Alginate/lactose-modified chitosan hydrogels: A bioactive biomaterial for chondrocyte encapsulation

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Abstract: A new bioactive scaffold was prepared from a binary polysaccharide mixture composed of a polyanion (alginate) and a polycation (a lactose-modified chitosan, chitlac). Its potential use for articular chondrocytes encapsulation and cartilage reconstructive surgery applications has been studied. The hydrogel combines the ability of alginate to act as a 3D supporting structure with the capability of the second component (chitlac) to provide interactions with porcine articular chondrocytes. Physico-chemical characterization of the scaffold was accomplished by gel kinetics and compression measurements and demonstrated that alginate-chitlac mixture (AC-mixture) hydrogels exhibit better mechanical properties when compared with sole alginate hydrogels. Furthermore, biochemical and biological studies showed that these 3D scaffolds are able to maintain chondrocyte phenotype and particularly to significantly stimulate and promote chondrocyte growth and proliferation. In conclusion, the present study can be considered as a first step towards an engineered, biologically active scaffold for chondrocyte *in vitro* cultivation, expansion, and cell delivery. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 84A: 364–376, 2008

Key words: alginate; chitosan; lactose; chitlac; chondrocytes; cell encapsulation; polyanion-polycation mixture

INTRODUCTION

Cartilage degeneration represents a serious health problem, causing progressive debilitation and marked decrease of quality of life. Its damages occur as a consequence of aging, congenital abnormalities, diseases and traumas, and are related to the limited intrinsic healing potential of this tissue. As a consequence of the lack of blood supply and subsequent insufficient inflammatory response, cartilage lesions result in an incomplete repairing attempt by local chondrocytes.[1,2] Recently, an approach based on the so-called A.C.I. (*A*utologous *C*hondrocyte *I*mplantation) has been presented as the ultimate technique in the field of cartilage regeneration. Delivery of autologous chondrocytes to the sites of wound requires cell isolation, *in vitro* cultivation, and expansion. However, chondrocytes undergo a process of phenotypic and functional de-differentiation when cultured in monolayer systems, due to the lack of the crucial influence of physiological cell–cell and cell–extracellular matrix (ECM) interactions. For this reason chondrocytes are generally seeded in biomimetic porous scaffolds that recreate the cellular microenvironment, providing a defined 3D structure that guides the tissue development.[2]

The tissue engineering approach (also known as regenerative strategy) has been regarded as an extremely promising and appealing technique for the potential to manipulate and combine cells and supportive biocompatible matrices aiming at restoring the original architecture and function of the tissue and hence providing cartilaginous constructs to be transplanted. Until now, a wide variety of biocompatible polymer-based materials (fibrin, hyaluronan

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(HA), collagen, and polyesters of α-hydroxyacids to name a few) have been proposed for 3D-scaffold design.[3–6]

Algal polysaccharide hydrogels represent very good candidates for 3D-scaffold design, having the advantage of being biocompatible and allowing a uniform distribution of seeded cells throughout the matrix.[7] Among these versatile biomaterials, alginate has been widely employed for cell immobilization procedures. Alginate is a family of linear copolymers (produced by brown algae and bacteria), containing 1-4-linked β-D-mannuronic acid (M) and α-L-gulur-onic acid (G) arranged in a blockwise pattern along the chain with homopolymeric regions of M (M blocks) and G (G blocks) residues interspersed with regions of alternating structure (MG blocks). Alginate has the ability to form stable gels in presence of millimolar concentrations of calcium or other divalent cations.[8] Cell encapsulation in calcium alginate beads represents a well established method for cell protection from host immune system,[9–11] but the biological inertness of alginate has largely hampered its use in all those applications where cell adhesion is mandatory for survival and proliferation.[12] As an example, although alginate beads have been used to encapsulate chondrocytes, allowing them to preserve their original phenotype,[13–17] a reduced cell proliferation was reported.[18]

Biopolymer engineering is an appealing approach to overcome the limit of nonbioadhesivity displayed by alginate.[18] Introduction of biologically active signals within the gel material might enable its interaction with the cells, hence enhancing their embedding.[19,20] We have recently reported on the biological properties of a lactose-modified chitosan (chitlac) in the aggregation of porcine articular chondrocytes and stimulation of chondro-specific glycosaminoglycans (GAGs) and collagen production.[21] It has been shown that the interaction between the engineered material and the cells is mediated by galectin-1 that specifically recognizes the galactose inserted as side chain into the chitosan backbone.[22] However, the biological significance of chitlac is counterbalanced by the complete lack of gel forming properties under cell-friendly conditions.

Prompted by these considerations, we decided to merge the properties displayed singularly by the two polysaccharides presented above, i.e. alginate and chitlac, thus designing a new bioactive biomaterial based on their soluble binary mixtures.[23,24] In such way, the final construct benefits both the characteristics of the two polysaccharides, i.e. good gel forming properties due to the presence of alginate and ability to interact with cells induced by the presence of chitlac. Along this line, the present contribution explores the developed system for chondrocyte *in vitro* cultivation, expansion, and cell delivery based on cell encapsulation in alginate/chitlac blended matrices. In particular, the mechanical properties of the calcium hydrogels produced with alginate/chitlac mixture have been characterized by gel kinetics and gel strength measurements. Moreover, the soluble mixture of the two polysaccharides was used to encapsulate porcine articular chondrocytes and the stimulation of chondro-specific markers expression and cellular growth has been evaluated.

MATERIALS AND METHODS

Materials

Sodium alginate samples isolated from *Laminaria hyperborea* stipe were purchased/ provided by FMC Biopolymer (Norway) ($M_w = 1.3{\cdot}10^5$, $F_G = 0.69$; $F_{GG} = 0.56$). Chitlac (lactose modified chitosan, $M_w \sim 1.5 \times 10^6$ CAS registry number 85941-43-1) sample was prepared according to the procedure reported elsewhere.[21,25] Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FCS), penicillin, streptomycin, trypsin/EDTA solutions, phosphate-buffered saline (PBS), and glutamine were purchased from Biochrom KG Seromed (Germany). Hyaluronidase, fluoresceine isothiocyanate (FITC), *N*-hydroxysuccinimide (NHS), 2-[*N*-morpholino]ethanesulfonic acid (MES), Tween 20, and Taq polymerase were from Sigma (USA). Moloney murine leukemia virus reverse transcriptase (MLV-RT) was from Life technologies (USA). Collagenase type II was from Worthington Biochemical Corp. (USA). Chitosan, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) and sodium cyanoborohydride were purchased from Aldrich Chemical Co. (USA). Live/dead® Reduced Biohazard viability/cytotoxicity kit and Rhodamine were from Molecular Probes (Leiden, NL). All other chemicals were of analytical grade.

Sodium alginate/chitlac binary mixture formation (AC-mixture)

The sodium alginate/chitlac binary mixture was obtained by dropwise addition of a chitlac solution (1% w/v, 0.15*M* NaCl, 0.01*M* Hepes, pH 7.4) to an equal volume of a sodium alginate solution (3% w/v, 0.15*M* NaCl, 0.01*M* Hepes, pH 7.4) under vigorous stirring. The final total polymer concentration was 2% with an alginate weight fraction of 0.75%.

Gelling kinetics and rheological characterization

Gelling kinetics and dynamic viscoelastic characterization were carried out on a Stress-Tech general-purpose rheometer (Reologica instruments AB, 22363 Lund, Sweden). Briefly, to an AC-mixture sample (final concentrations: 1.5% w/v alginate, 0.5% w/v chitlac, 0.15*M* NaCl, 0.01*M* Hepes, pH 7.4), $CaCO_3$ (20 m*M*) and GDL (40 m*M*) were added and the mixture was stirred for 30 s prior to the measurements. A *L. hyperborea* solution (1.5% w/v

alginate, 0.15*M* NaCl, 0.01*M* Hepes, pH 7.4) was used as a control. A serrated plate–plate (d =40 mm) measuring geometry with $T = 25^{\circ}C$ and gap = 1.00 mm was used. The kinetics of gelation was followed by repeated determination of G' and G'' ($\omega = 6.28$ rad s^{-1}) at intervals of 3 min for ~18 h. The dynamic viscoelastic characterization was carried out 24 h after induction of gelation by determining the frequency dependence of the storage (G') and loss moduli (G''). Frequency sweeps were performed at a constant strain (0.001) in the frequency range 0.01–50 Hz. The samples were sealed with a low-density silicon oil to avoid adverse effects associated with evaporation of the solvent throughout the gelation experiments.

Preparation of gels cylinders

Two approaches for the preparation of gel cylinders were followed. In the first case ("*in situ* calcium release" cylinders), the AC-mixture was blended with an inactivated form of Ca^{2+} (20 m*M* $CaCO_3$) followed by the addition of the slowly hydrolyzing D-glucono-δ-lactone (GDL) (GDL/Ca^{2+} = 2). Aliquots of this gelling solution were cured in 24-well tissue culture plates (h = 18 mm, Ø = 16 mm, Costar, Cambridge, MA) for 24 h prior to measurement. In the second case ("calcium excess" cylinders), the AC-mixture was dialyzed against a solution containing $CaCl_2$ (50 m*M*) and NaCl (0.2*M*) for 48 h at 4°C prior to analysis.[26] In both cases, a 1.5% alginate solution in the same buffer solutions was used as control.

The Young's modulus (E) was calculated from the initial slope of the force/deformation curve[27] as measured with a Stable Micro Systems TA-XT2 texture analyzer at 20°C. For all gels exhibiting syneresis, the final polymer concentration was determined and E was corrected by adaptation of $E \propto c^2$.[28]

Measurement of the creep compliance response

The creep compliance of the AC-mixture and from the 1.5% alginate solution was measured in uniaxial compression on gel cylinders (prepared following the above reported "*in situ* calcium release" procedure) using a Stable Micro System TA-XT2 Texture analyzer at 22°C. For each specimen, a stress (σ) was applied in order to obtain an "instantaneous" strain (ε) equal to 0.08. The developing strain was monitored for 2000 s. The compliance at a given instant t, $J(t)$, was calculated from the time dependent strain ($J(t) = \varepsilon(t)/\sigma$).

Bead formation

Calcium beads from AC-mixture (final concentrations: 1.5% w/v alginate, 0.5%w/v chitlac, 0.15*M* NaCl, 0.01*M* Hepes, pH 7.4) were obtained by dripping the polymer blend into the gelling solution (0.05*M* $CaCl_2$, 0.15*M* mannitol, 0.01*M* Hepes, pH 7.4). The droplet size was controlled by use of a high-voltage electrostatic bead generator[29] (5 kV, 10 mL/h, steel needle with 0.4 mm outer diameter, 1.7 cm distance from the needle to the gelling solution). The gel beads obtained were stirred for 30 min in the gelling solution prior to use.

^{1}H NMR spectroscopy

The ^{1}H NMR spectra were recorded in D_2O at 90°C with Bruker WM 300. The chemical shifts are expressed in parts per million (ppm) downfield from the signal for 3-(trimethylsilyl)propanesulfonate. Beads from AC-mixture were treated with HCl (0.1*M*) to remove the calcium ions prior to analysis.

Bead stability in saline solution (0.9% NaCl)

The dimensional stability of calcium beads obtained from AC-mixture was measured with an inverted light microscope (Zeiss) adding 0.5 mL of gel beads to 3 mL of saline solution (0.9% NaCl). The sample was stirred for 1 h. The saline solution was replaced several times and the diameter of the beads was determined (n = 15) before each change.

Visualization of the beads by confocal laser scan microscopy

Beads from labeled AC-mixture, obtained by coupling alginate and chitlac with rhodamine 123 and FITC respectively,[23] have been prepared and visualized in the CLSM. Gelling solution composition: 0.05*M* $CaCl_2$, 0.003*M* $BaCl_2$, 0.15*M* mannitol, 0.01*M* Hepes (pH 7.4), and 0.1% (v/v) Tween 20. High-voltage electrostatic bead generator parameters: 5 kV, 10 mL/h, steel needle with 0.7 mm outer diameter, 2.5 cm distance from the needle to the gelling solution.

The capsules were examined in a Zeiss LSM 510 Confocal Microscope with a C-Apochromat 10×/45 W objective and by software LSM 510, release 2.02 (Carl Zeiss, Oberkochen, Germany). All the settings for the confocal microscope and the imaging of the beads were computer-controlled. The settings used for imaging of the AC beads were as follows: for visualization of alginates (Rhodamin 123 labeled) a 543 nm HeNe-laser was used and the fluorescence was detected with a 560 nm long pass filter; for visualization of chitlac (FITC labeled) a 488 nm Argon-laser was used and the fluorescence was detected with a 505–530 nm bypass filter. To obtain an image of the capsules' core centre, all beads were examined by scanning through an equatorial slice of the capsules. Eight scans were performed and the mean used to reduce the noise.

Isolation and encapsulation of porcine articular chondrocytes

Thin slices of articular cartilage were aseptically removed from the humeral proximal head of mature pigs within 2 h from the sacrifice. Cells were then isolated by enzymatic digestion of the tissue as described.[21] Sterile

polymer solutions (AC-mixture and control alginate solution) were poured on chondrocyte pellets previously washed with PBS to prepare a mixture containing 5×10^5 cells/mL. Chondrocytes have been encapsulated in gel beads of different size, by extruding the cell suspension through a 23-gauche needle into a gelling solution (0.05*M* $CaCl_2$, 0.15*M* mannitol, 0.01*M* Hepes, pH 7.4; resulting bead diameter = 2 ± 0.5 mm) or using the high-voltage electrostatic bead generator (bead diameter = 0.5 ± 0.1 mm; bead generator parameters: 5 kV, 10 mL/h, steel needle with 0.7 mm outer diameter, 2.5 cm distance from the needle to the gelling solution; gelling solution composition: 0.05*M* $CaCl_2$, 0.003*M* $BaCl_2$, 0.15*M* mannitol, 0.01*M* Hepes, pH 7.4). The gel beads obtained were stirred for 10 min in the gelling solution and then rinsed with saline solution (0.9% NaCl) and DMEM medium prior to use.

Viability assays

Viability was evaluated by means of a Live/dead assay kit (Molecular Probes). Cells encapsulated using AC-mixture and sodium alginate as control were recovered from beads by chelating the Ca^{2+} ions with citrate (50 m*M* citrate, 100 m*M* NaCl, 10 mm glucose, pH 7.4), washed twice with PBS, and stained with a mixture of two fluorescent probes according manufacturer's protocol. This staining procedure allowed for a clear distinction between live cells (in green) and dead cells (in red).

DNA content determination

Samples were assayed for DNA content using the Fluorescence Assay DNA Quantization Kit (Sigma).

Cell proliferation assay

Cell proliferation of chondrocytes grown in calcium beads produced using AC-mixture was assessed by examining the incorporation of labeled thymidine into nucleic acids. A defined number of beads have been incubated with 1 μCi/mL [^{3}H]-thymidine (specific activity 25 Ci/mmol) in tubes containing complete DMEM at 37°C for 24 h. Gel beads have been dissolved using a sodium citrate solution (0.05*M* sodium citrate, 0.1*M* NaCl, 0.01*M* glucose, pH 7.4). The tubes were centrifuged to separate cells from the polymers. Cell pellets were washed twice with ice-cold PBS, once with 5% trichloroacetic acid and disrupted by adding 0.5 mL 0.5*N* NaOH/0.5% SDS. The suspensions were transferred into tubes containing 4 mL of scintillation cocktail and radioactivity incorporated in DNA was measured in a scintillation counter (Betamatic V, Kontron Instruments). Chondrocytes grown in calcium alginate beads have been used as control. The values obtained have been normalized on the total DNA content.

GAGs content and collagen synthesis

Sulphated GAGs content was measured using a modified dimethylmetylene blue (DMMB) assay as previously described.[30] Briefly, AC-mixture or alginate beads were dissolved and digested for 24 h at 65°C using a sodium citrate-papaine solution (0.055*M* sodium citrate, 0.005*M* EDTA, 0.005*M* cysteine hydrochloride, 0.5 U/mL papain, pH 7.4). To avoid any electrostatic interference with the colorimetric assay, alginate has been removed from the sample by means of acid precipitation, adjusting pH to 1.5 by adding HCl (6*M*). Forty microliters of papain-digested sample was then added to 250 μL of DMMB solution (pH 1.5) and absorbance measured at 525 nm. Chondrotin sulphate has been used to construct a standard curve and GAGs content was normalized on the DNA content.

Collagen synthesis by encapsulated chondrocytes was determined by pulse-labeling using [^{3}H] proline. Alginate beads were incubated for 12 h in culture medium supplemented with 1.5 μCi/mL [^{3}H] proline and washed in PBS containing 0.001*M* $CaCl_2$. Beads were then digested for 12 h at 60°C in an EDTA-papain buffered solution (PBS supplemented with 0.01*M* EDTA and 2 U/mL papain), each digested sample added to 4 mL of scintillation cocktail and radioactivity was measured with a scintillation counter (Betamatic V, Kontron Instruments). Chondrocytes grown in calcium alginate beads were used as control. Aliquots of each sample were mixed with PBS and used to determine the DNA content.

RNA extraction and RT-PCR analysis

Total RNA from encapsulated chondrocytes was obtained, after gel beads dissolution, with the TRIZOL® based Gibco RNA isolation system as indicated by the manufacturer. For cDNA synthesis, 1 μg total RNA was denatured at 70°C for 5 min and quickly chilled on ice, added to the reverse transcriptase (RT) mixture containing 1× M-MLV-RT buffer, 30 U Rnase out, 10 m*M* DTT, 25 pmol random hexanucleotides, 0.5 m*M* dNTPs, and 200 U of M-MLV-RT to a total volume of 50 μL, and incubated for 1 h at 37°C. Reaction was blocked by heating mixture at 95°C for 5 min. Polymerase chain reaction (PCR) was carried out in 100 μL reaction volume using 5 μL of the cDNA reaction product (corresponding to 100 ng of RNA equivalent) as template mixed with PCR mix (1× Taq buffer, 50 pmol of each primer, 0.1 m*M* dNTPs, 0.5 U Taq polymerase, 2% DMSO, 0.0015*M* $MgCl_2$) using the following conditions for each couple of primers: 94°C for 30 s, 55°C for 1 min, 74°C for 1 min for 35 PCR cycles. PCR primers sequences have been reported in Ref. 19. TaqMan GAPDH Control Reagents (Applied Biosystems) were used to evaluate the transcription of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) housekeeping gene as an endogenous reference.

Optical microscopy

The beads were incubated at room temperature for 30 min in a buffer 10 m*M* HEPES pH 7.4 containing 10 m*M* $CaCl_2$, 100 m*M* NaCl, 5 m*M* glucose, and 5% BSA. They were then fixated with 10% neutral formalin for 4 h at 4°C and dehydrated in acetone. A final embedding in Immuno-bed (Electron Microscopy Sciences, WA) was

done according to the manufacturer instructions. Toluidine blue and silver impregnation staining for GAGs and collagen identification was performed on 5 μm thick sections.

Statistical analysis

The significance was determined using the two-tailed Student's test. Differences are accepted as significant at $p < 0.05$.

RESULTS

Gel formation and properties

The presence of 1.5% alginate in the binary polymer mixture allowed obtaining, upon treatment with calcium ions, hydrogels from the AC-solutions. Hence, the overall mechanical properties of the calcium gels obtained from the system composed of the two oppositely charged polysaccharides were assessed. Since the gel formation process is due to the chelation of calcium ions by the alginate G-residues,[31] a 1.5% alginate solution has been used for comparison. An "internal gelation" process, that comprises the use of inactivated form of calcium ions ($CaCO_3$), and the slow hydrolyzing lactone GDL, was used to evaluate the kinetics of gel formation. The slow hydrolysis of GDL releases protons that convert the insoluble $CaCO_3$ into HCO_3^-, thus providing the free calcium ions required for the gel formation. The delay between the mixing of the lactone and the gel formation allows investigating the formation and curing of the hydrogel in the rheometer. Figure 1(a) reports the variation of the storage (G') and loss (G'') moduli for the AC-mixture and for the 1.5% alginate solution in the first 1000 s of gel formation. In both cases, a rapid increase of the storage modulus is detected, leading to the conclusion that the presence of the polycation (chitlac) in the binary polymer mixture, which has no active role in calcium binding, does not hinder the formation of network junctions by interaction between the G residues on alginate and the cross-linking divalent cation. On the contrary, the comparison of the phase angle (δ)-time profile displayed by the two samples above reported (Fig. 1, inset) seems to suggest a slightly faster sol–gel transition for the AC-mixture.

The curing of the gel obtained by internal gelation was continued for $\sim 7 \times 10^4$ s for both the AC-mixture and the 1.5% alginate solution reaching, in both cases, a plateau value of the storage modulus (G'). After the complete formation of the gel, mechanical spectra of the hydrogels obtained from the two samples, i.e. AC-mixture and 1.5%-alginate solution, were measured [Fig. 1(b)]. Both samples showed a typical gel behavior, with the storage modulus (G') always higher than the loss modulus (G'') and constant over the entire range of frequency explored. However, the hydrogel obtained from the binary mixture displayed a 1.7-fold higher storage modulus that the 1.5% alginate solution pointing at the benefit of the presence of the second polymeric component on the overall mechanical properties of the construct.

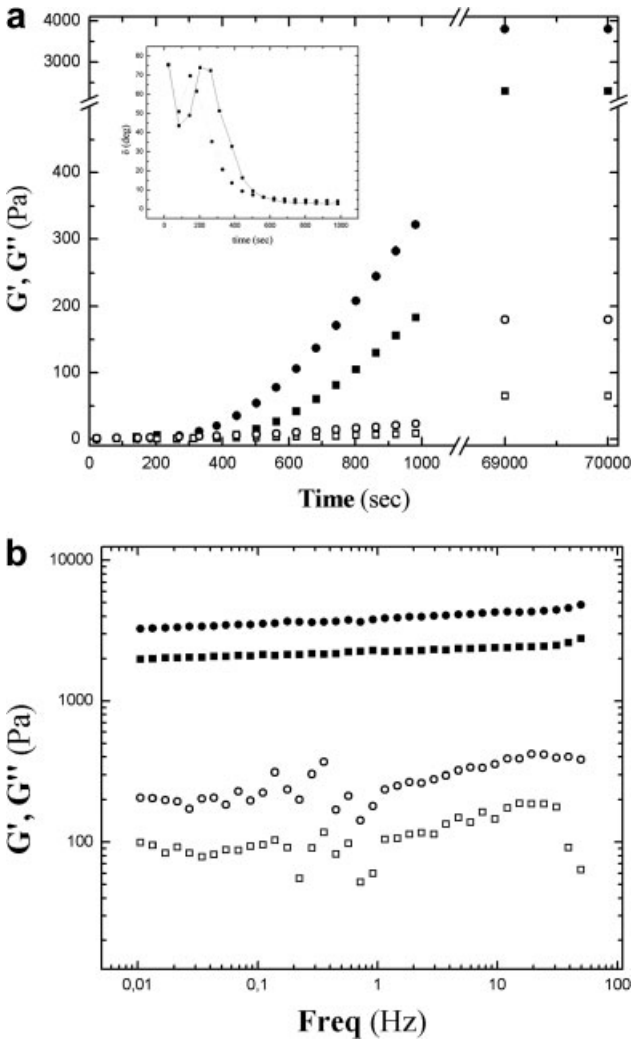

Figure 1. (a) Variation of G' (solid symbols) and G'' (open symbols) in the first 1000 s for gels obtained from an AC-mixture (circles) and from *L. hyperborea* (L.h.) alginate alone (squares). INSET: variation of δ for AC-mixture (circles) and alginate (L.h.) (squares). (b) Storage G' (solid symbols) and loss G'' (open symbols) moduli for hydrogels obtained from AC-mixture (circles) and alginate (L.h.) (squares). Gels were obtained by adding at 25°C $CaCO_3$ (20 m*M*) and GDL (40 m*M*) to the polymer solutions.

To confirm the results reported in Figure 1(b), it was decided to perform a further characterization of the rigidity of the constructs, both from a static and a dynamic standpoint. In the former case, this has been carried out by measuring the Young's modulus (E) of gel cylinders prepared according to the two

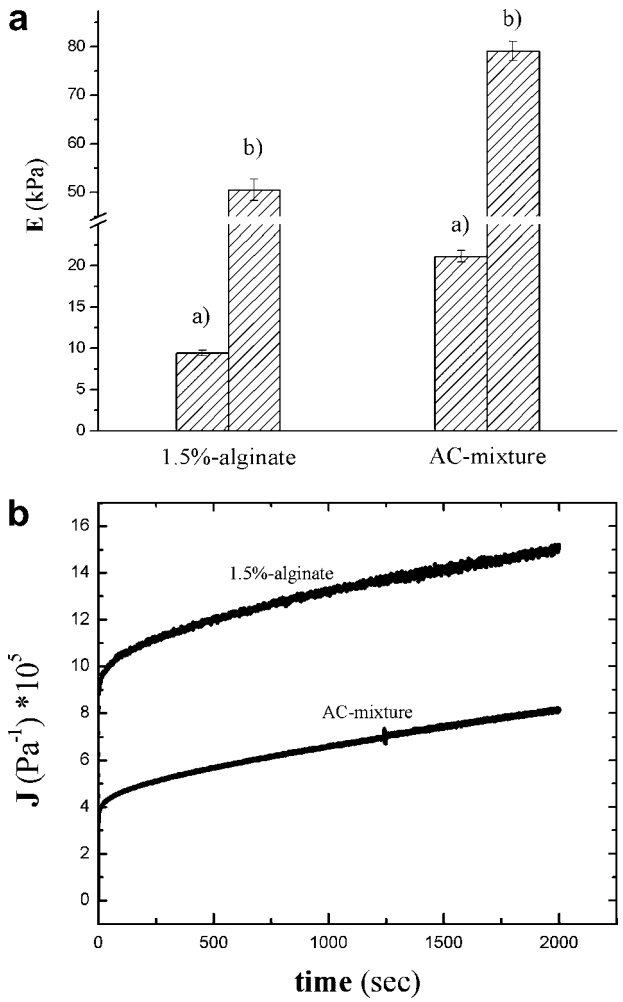

Figure 2. (**a**) Young's modulus (E) of gel cylinders from AC-mixture and *L. hyperborea*, obtained: (a) from internal gelation (20 m*M* $CaCO_3$ and 40 m*M* GDL); (b) from dialysis. Values are reported as mean ± SD ($n = 8$). (**b**) Creep compliance curves for hydrogels from AC-mixture and 1.5%-alginate solution. Gels were obtained by adding $CaCO_3$ (20 m*M*) and GDL (40 m*M*) to the polymer solutions at 25°C.

different gelation procedures (reported in Materials and Methods section) [Fig. 2(a)]. It should be noted that the different gelation procedure brings about a notable effect on the Young's modulus (E) displayed by the two samples. In particular, for both the AC mixture and the alginate solution, the dialysis procedure led to a higher compressive modulus than the internal gelation procedure. This is easily explained considering that in the latter method a limited amount of calcium is present to the gelling system thus a non complete filling of all available calcium binding sites is accomplished. On the contrary, in the dialysis procedure a large reservoir of calcium is provided and all the binding sites of the polyuronate (alginate) are saturated by the cross-linking ion. However in both cases a higher Young's modulus is detected for hydrogels prepared from the AC-mixture when compared to those obtained from the 1.5%-alginate solution (2.3- and 1.6-fold in the case of internal gelation and dialysis, respectively). This observation strengthens the results obtained by oscillatory measurements and points at a nonnegligible contribution of the polycation (chitlac) to the overall mechanical properties. The dynamic aspect of the rigidity of the calcium-alginate hydrogels was further studied with time-domain rheological experiments. In particular, creep compliance for gel cylinders obtained, via internal gelling, from the AC-mixture and from the 1.5%-alginate solution was evaluated by applying an "instantaneous" stress (σ) and measuring the time dependent increase of strain (ε) [Fig. 2(b)]. The creep compliance J ($= \varepsilon/\sigma$) was recorded for 2000 s before removing the load. The creep compliance was analyzed by means of a model composed of a Maxwell element in series with one Voigt element [Eq. (1)], which accurately fitted the experimental data[32]:

$$J(t) = J_0 + J_1\left(1 - e^{-\frac{t}{\tau}}\right) + \frac{t}{\eta_N}$$

where $J(t)$ is the measured compliance, J_0 and J_1 are the compliances of the Maxwell and Voigt springs respectively, τ is the retardation time associated with the Voigt element, and η_N is the so-called Newtonian viscosity of the Maxwell dashpot. Figure 2(b) shows that, as predictable, the instantaneous spring component ($E_0 = 1/J_0$) is higher for the hydrogel obtained from the AC-mixture than that of the 1.5%-alginte solution, thus paralleling the trend exhibited by the Young's modulus of the two samples. Moreover, the values of η_N obtained from the fitting of the experimental data with Eq. (1) are 61.5 and 54 kPa s for the AC-mixture and for the sole alginate solution, respectively, thus pointing to a (slightly) higher resistance of the former network toward slipping.

Bead formation

Calcium gel beads have been prepared from AC-mixture by means of an electrostatic bead generator using the standard procedure developed for calcium alginate gel beads (see Materials and Methods section). Since gel beads formation occurs as soon as the alginate gets in contact with a solution containing one or more gelling ions, it has been possible to obtain gel beads from the AC-mixture, where the chitlac chains result to be entrapped within the network created by alginate. This was demonstrated by ^{1}H NMR: samples of AC-mixture analyzed by means of ^{1}H NMR prior and after the bead formation displayed nondetectable differences as to

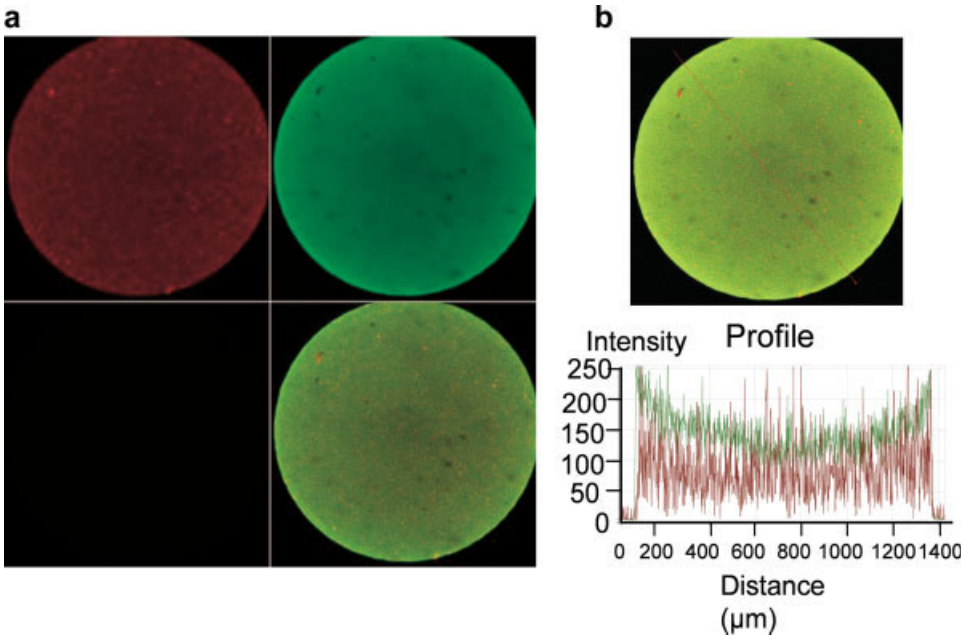

Figure 3. (a) CLSM image of the AC-bead obtained from Rhodamine-labeled alginate (red, left image) Fluorescein-labeled chitlac (green, right image). (b) The image represents the two images in (a) overlaid, and the distribution profile of the two polysaccharides in the AC-bead. The images represent a slice along the bead equator. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the polymer–mixture composition and relative amount. In both spectra it is possible to detect the doublet centered at around 4.55 ppm assigned to the anomeric proton of galactose moiety present in side-chain in chitlac[21] (data not reported). This conclusion was confirmed by the CLSM microphotographs of a bead produced using a mixture of Rhodamine-labeled alginate (red color in the picture) and Fluorescein-labeled chitlac (green color in the picture)[24] (Fig. 3). In this case the presence of each polysaccharide throughout the bead is clearly evident [Fig. 3(a)]. It is important to note the absence of a pattern characterized by higher fluorescent emission typical of phase separated systems; hence this analysis excludes the occurrence of a large-scale aggregation upon gelation. A preliminary analysis of the equatorial plane of the bead revealed a homogeneous distribution of alginate in the capsule. This is basically in accordance with previous findings.[33] On the contrary, chitlac, the free diffusion of which is not halted by the gelation process, exhibited a slightly inhomogeneous distribution, with higher polymer content on the outer shell of the bead [Fig. 3(b)]. Far from being conclusive, this seems to imply some mutual influence of the two oppositely charged polysaccharides on their spatial distribution, likely due to electrostatic interactions.[24] However, additional measurements are now in progress to confirm and further characterize this peculiar distribution of the chitlac component and its implications. In particular, a higher concentration of the polymer mixture on the outer border of the capsule is likely to alter the pore size of the hydrogel network and thus its permeability.

The stability of the beads obtained from AC-mixture was tested by measuring the average diameter variation upon treatment with saline solution (0.9% NaCl), using calcium alginate beads from *L. hyperborea* (1.5%) as control (Fig. 4). The comparison between the two different samples allows concluding

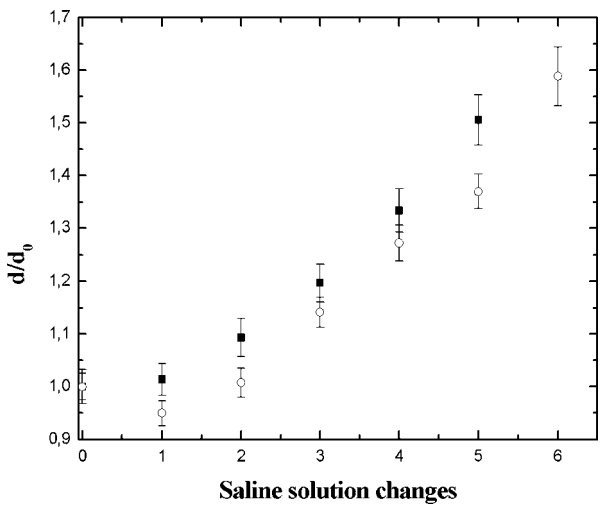

Figure 4. Stability of calcium beads from AC-mixture (open circles) and from *L. hyperborea* alginate (full squares) upon saline solution replacements expressed as the ratio of the bead diameter with respect to the initial diameter (d_0) ("relative diameter"). Values are reported as mean ± SD (n = 15).

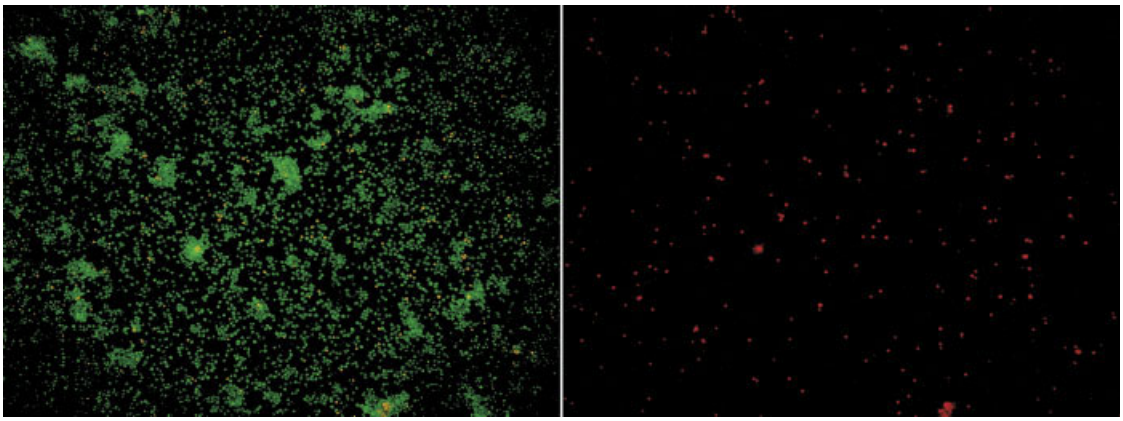

Figure 5. Fluorescence micrographs of chondrocytes from AC-mixture beads, stained with two different fluorescent probes to evidence cell viability after 20 culture days. Dead cells appear red, live cells appear green. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that the swelling behavior of AC-mixture beads closely parallels the trend of those composed exclusively of alginate, showing in addition a slight increase of stability (less than 1.4-fold diameter increase after five saline solution replacements) when compared to alginate alone (more than 1.5-fold increase). It is remarkable that after six saline solution replacements, it is no more possible to visualize and measure intact alginate beads, while there are still intact and measurable AC-mixture beads, presenting a 1.6-fold mean diameter increase. Since the swelling of the beads is likely to be accompanied by an increase in the diameter of the pores of the gel, the presence of the polycation (chitlac) after the six-saline shifts was assessed. Samples of AC-mixture beads were collected prior and after the swelling stability tests and treated with HCl to remove calcium ions and thus dissolve the beads. The ^{1}H NMR spectra of the solution proves the presence of a considerable amount of chitlac at the end of the test. Indeed, by comparing the peaks corresponding to the alginate component and that of the galactose

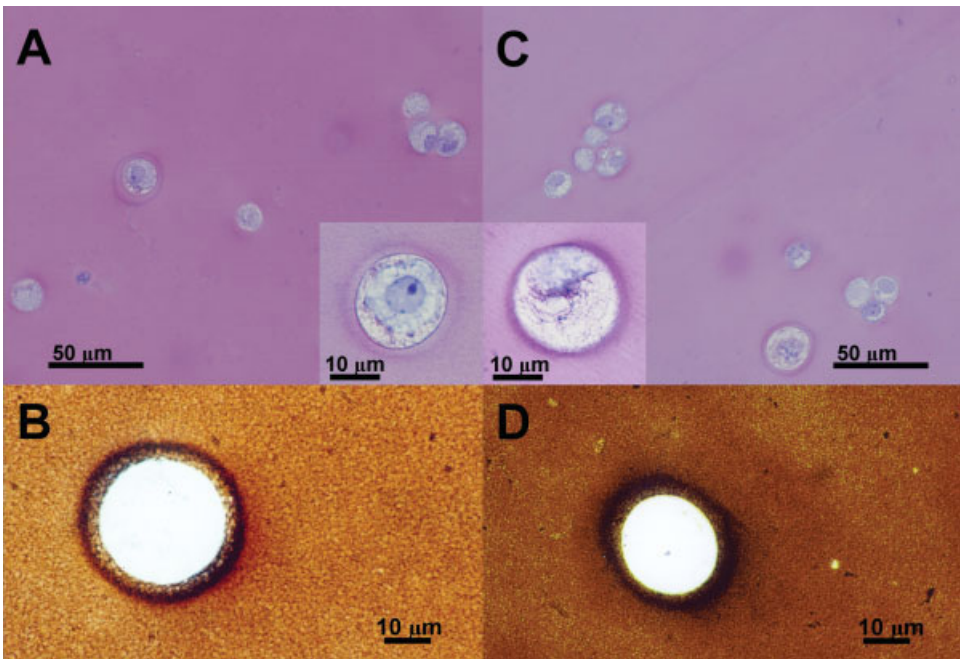

Figure 6. Optical microscopy of bio-constructs after 20 days of culture in alginate (a, b) and AC-mixture (c, d) hydrogels. Toluidine blue (a, c) and silver impregnation staining (b, d) were performed for evidencing GAGs and collagen: Chondrocytes entrapped appear isolated or in groups of two or more elements. GAGs and collagen are visualized as a purple and a brown ring around the cells or group of cells on a clearer background, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

group in chitlac, there seem to be an increase of the chitlac/alginate ratio after six saline shifts for AC-mixture. This is actually due to the leakage of alginate chains from the bead structure, as a consequence of the calcium displacement and the corresponding gel weakening. However, the network created by the alginate chains still remains dense enough to trap the chitlac chains, not allowing their diffusion from the gel structure after six saline shifts.

Biological properties

The influence of bead composition on chondrocytes biology and metabolism was evaluated by means of biochemical analysis: cell viability, cell proliferation, GAGs and collagen synthesis at different times. Cell viability in AC-mixture scaffold was assessed using a live/dead kit. The basis of this viability test is the differential permeability of live and dead cells to a pair of fluorescent stains (SYTO® 10 and DEAD Red™), respectively. SYTO 10 is a green fluorescent nucleic acid stain, highly membrane-permeable, which labels all cells, including those with intact plasma membranes. DEAD Red is a cell-impermeable red fluorescent nucleic acid stain which labels only cells with compromised membranes. Live cells appear fluorescent green and dead cells fluorescent red. As reported in Figure 5, it is evident that after 20 culture days more than 95% of chondrocytes from AC-mixture beads are fully viable as well as chondrocytes grown in alginate scaffolds.

It was important to verify if chondrocytes in beads from binary polymer mixture were active in the synthesis of extracellular matrix components. To this end, histological analysis was applied to detect the production of GAGs and collagen: toluidine blue and silver impregnation staining clearly visualized GAGs and collagen, respectively, on alginate and AC-mixture beads sections after 20 days of culture (Fig. 6). The synthesis of extracellular matrix components, GAGs and collagen, was then quantified in AC-mixture in comparison to alginate scaffolds (as control). The amount of GAGs was evaluated by colorimetric DMMB assay[30] and the collagen synthesis by [^{3}H]-proline incorporation. The results are reported in Figure 7. For GAGs content quantification, beads were collected after 5, 10, 15, 19, and 24 days of culture; optical density after DMMB reaction was comparatively measured as a function of time. Although the content of GAGs was lower in AC-mixture scaffolds than in alginate alone until day 24, the former interestingly showed a higher amount of GAGs with respect to the latter after day 24 ($p < 0.05$) [Fig. 7(a)]. In another set of experiments, the use of [^{3}H]-proline labeling allowed to test the collagen synthetic activity of embedded chondrocytes [Fig. 7(b)]. It should be noted that the amount of synthesized collagen was not affected by the composition of the hydrogel bead, i.e. the presence of the positively charged polysaccharide. Additionally, regardless of scaffold composition, collagen synthesis rates decreased significantly with time just after the fifth day of culture ($p < 0.01$).

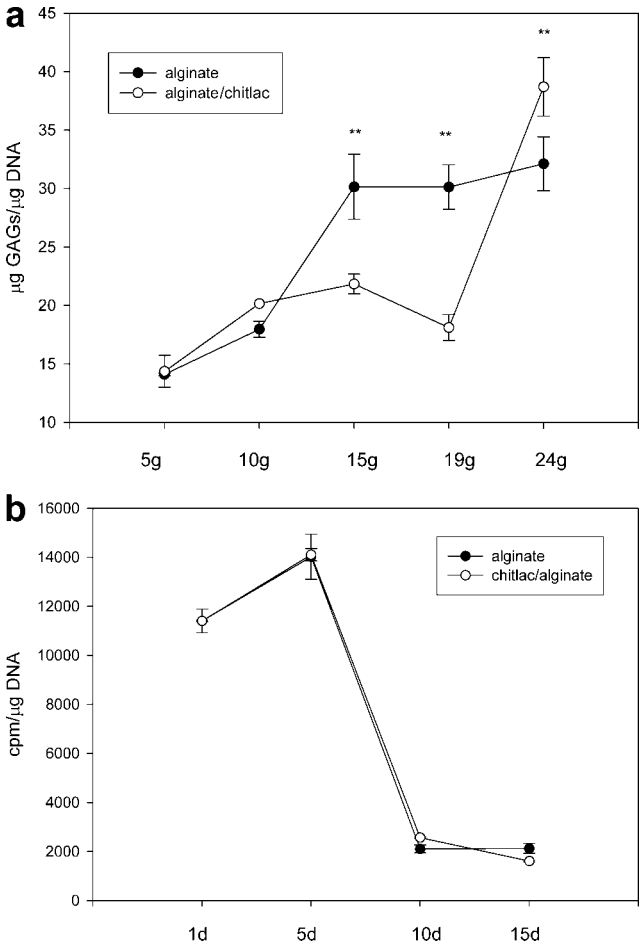

Figure 7. (a) Amount of synthesized GAGs and (b) collagen biosynthesis rates in alginate and AC-mixture chondrocyte beads. For GAGs quantization assay, forty microliters of papain-digested sample was added to 250 μL of DMMB solution (pH 1.5) and absorbance measured at 525 nm. Chondrotin sulphate has been used to construct a standard curve. Collagen synthesis by chondrocytes was determined by pulse-labeling using [^{3}H] proline. Beads were digested in 600 μL of EDTA-papain buffered solution and digested samples added to 4 mL of scintillation cocktail for radioactivity measure. Data of four samples from a representative out of three independent experiments are reported. All data were evaluated as mean ± SD ($n = 4$) normalized to relative DNA quantity and presented as cpm on μg of DNA for proline incorporation assays and as μg GAGs on μg of DNA in DMMB assay (*$p < 0.05$; **$p < 0.01$).

The extracellular matrix components produced by chondrocytes in articular cartilage consist of tissue-specific macromolecules, the biosynthetic program of which is determined by the expression of a set of

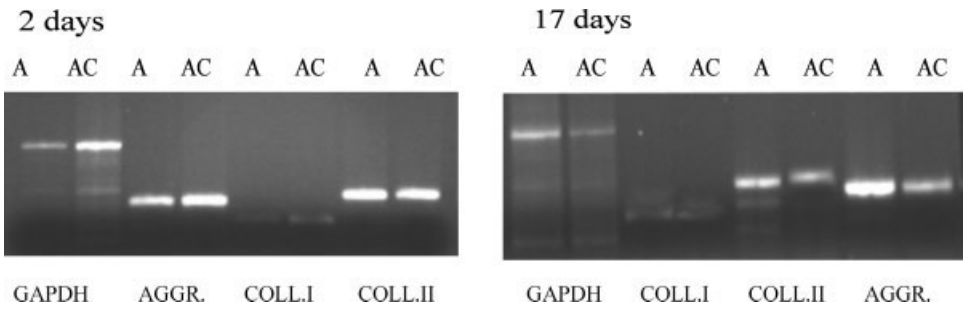

Figure 8. mRNA expression in porcine chondrocytes of GAPDH, Collagen II (COLL.II), Collagen I (COLL.I), and aggrecan (AGGR.) genes as detected by RT-PCR. RT-PCR analysis was performed on alginate beads cultured chondrocytes and AC-mixture beads chondrocytes after 2 and 17 days from encapsulation. Results are visualized on a 1% agarose gel stained with ethidium bromide (A, alginate beads; AC, alginate/chitlac mixture beads).

cartilage-specific genes such as collagen (of the types II, IX, and XI) and the proteoglycan aggrecan. The proline incorporation assay cannot discriminate between the different collagen types I and II. Therefore, in order to evaluate the effect of the culture system on the chondrocyte phenotype, the expression of some specific gene patterns by RT-PCR was qualitatively analyzed. Primers were used that specifically amplify porcine COL2A1, COLI, aggrecan transcripts and GAPDH housekeeping gene and total RNA was extracted at day 2 and day 17 postencapsulation from cells maintained in alginate and AC-mixture beads. As shown in Figure 8, chondrocytes in both culture systems express aggrecan and type II collagen, markers of cartilage matrix, whereas collagen I, marker of the undifferentiated phenotype, was almost undetectable throughout the time of culture.

Cell proliferation was measured in alginate and AC-mixture matrices by counting [^{3}H]-thymidine incorporation in DNA during cell division in individual beads. The number of cpm measured per scaffold was normalized by the μg of DNA per bead. After normalization with respect to DNA content, chondrocytes in the AC-mixture scaffolds showed at any culture time a significantly increased [^{3}H]-thymidine uptake when compared to scaffold with sole alginate, with a maximum rate of cell proliferation at day 5, which decreases afterwards (Fig. 9). After culture day 15, encapsulated cells seem to reach a constant state of proliferation that is higher in AC-mixture beads chondrocytes than in alginate ones ($p < 0.01$). Further analysis is now in progress to establish the phase of the cycle of the encapsulated cells.

DISCUSSION

A binary polysaccharide mixture composed of a polyanion (alginate) and a polycation (chitlac) has been studied as a potential bioactive biomaterial for chondrocyte encapsulation. In this respect, the new smart scaffold developed in the present work combines the ability of one of the components to provide the supporting structure (e.g. alginate hydrogel) with the capability of the second component (chitlac) to directly interact with porcine articular chondrocytes.[21,22] The study of the main characteristics of these binary polymer solutions has been tackled[23,24] and revealed that the presence of highly hydrophilic saccharidic moieties on chitosan grants a very good solubility to the engineered polycation thus allowing a complete miscibility with alginate under physiological conditions. Nevertheless, because of the

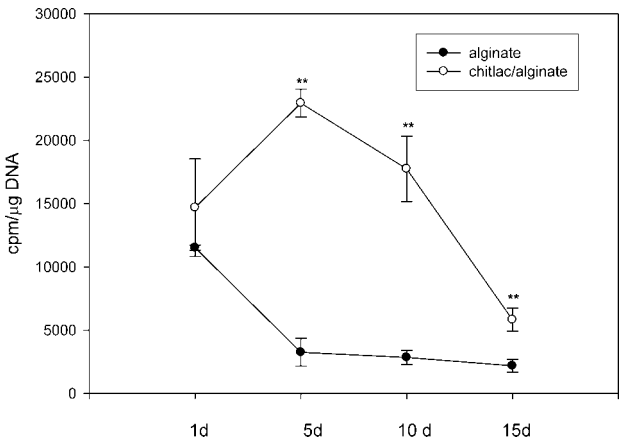

Figure 9. Proliferation of chondrocytes cultured in alginate and AC-mixture beads. [^{3}H]-thymidine uptake into nucleic acids after various culture times was measured in a scintillation counter. Gel beads have been dissolved in a sodium citrate disrupted by adding 0.5 mL 0.5*N* NaOH/0.5% SDS. The suspensions were transferred into tubes containing 4 mL of scintillation cocktail and radioactivity incorporated in DNA was measured in a scintillation counter. Six independent experiments were performed in quadruplicates and one as representative data was reported. Data were evaluated as mean ± SD ($n = 4$) normalized to relative DNA quantity and presented as cpm on μg of DNA. (**$p < 0.01$).

simultaneous presence of positive charges on chitlac and negative charges on alginate, electrostatic interactions occur between the two polymers resulting in the formation of soluble complexes. The overall effect of the latter in dilute and semi-dilute solution is the increase of the viscosity of the solution and the possibility of the formation of a temporary network based on inter-polyelectrolyte interactions of electrostatic origin. The presence of the latter and their consequences are readily detectable also in the present contribution. In fact, hydrogels obtained from the AC-mixture are characterized by a (slightly) faster gel kinetics (Fig. 1) and by a higher compressive and storage modulus than those obtained from a 1.5%-alginate solution (Fig. 2). This effect can be explained considering the presence of "electrostatic cross-links" between the oppositely charged polysaccharides. They can contribute in two ways to the stability of the network, besides representing the very first nucleus of formation: (i) they increase the "effective" local concentration of alginate molecules, with a positive contribution to both the kinetics and the thermodynamics of the calcium/alginate junctions; (ii) once the latter ones are formed, they additionally contribute to interpolymer networking by increase the total number of electrostatic "noncovalent" contacts. Thus, the hydrogels formed from the AC-mixtures benefit from an additional cross-linking contribution of electrostatic origin which does not interfere with the ionic bonding of Ca^{2+}, since it is not suppressed even in the presence of an overwhelming amount of Ca^{2+} ions, i.e. in the hydrogels formed from the dialysis procedure. The time-domain rheological experiments [Fig. 2(b)] revealed just slightly larger values of the so-called Newtonian viscosity, η_N, for the hydrogels from the AC-mixtures and from the 1.5%-alginate solution. Bearing in mind that the value of η_N is influenced by both the cross-linking density and the concentration of the polymer in the gel, one would expect a much higher Newtonian viscosity in the case of the AC-mixture hydrogels. This result might be explained by considering that the electrostatic cross-links do not show a remarkable stability toward the application of a persistent stress; thus the latter one likely leads to a rupture of the charge-induced polymer contacts enhancing the slipping of the polysaccharide chains in the hydrogels network. From this point of view, it can be stated that the presence of the positively charged polycation enhances the viscoelastic properties of the calcium-based alginate hydrogel.

The dimensional stability of the ionic gel beads is determined by the equilibrium between a positive osmotic pressure (swelling) and a negative pressure, related to the number of cross-links in the gel and therefore to the network elasticity. During the saline solution treatments, Na^+ counterions compete with and displace the calcium ions, reducing number and length of the alginate G-rich junctions and leading to an overall diameter increase. Therefore, the higher the dimensional variation for a given number of saline shifts, the lower the stability of the capsule. When the AC-mixture is considered, the presence of polyanion-polycation interactions enhances the bead stability by increasing the total number of effective cross-links that counterbalance the osmotic pressure; the overall effect is lower (relative) dimensional variation in the case of the mixed beads (Fig. 4).

The simultaneous presence of a good gel forming component and of a biologically active polysaccharide in the AC-mixture was exploited for the encapsulation of a primary line of porcine articular chondrocytes. Chondrocytes undergo a process of phenotypic de-differentiation when cultured in monolayer systems because of the lack of the crucial influence of physiological cell–cell and cell–extracellular matrix interaction.[34] To prevent de-differentiation, chondrocytes need being embedded within three-dimensional scaffolds to maintain their phenotype.[35–38] The effectiveness of any scaffold for cell culture in general, and for chondrocytes growth in particular, is highly dependent on the biological and physicochemical properties of molecules present on the scaffold surface. Molecular recognition between matrix and cell receptors should mimic the specific signals and physiological stimuli involving the cell membrane and the ECM components. Analysis reveals that when the AC-mixture beads are used to encapsulate chondrocytes, the presence of chitlac (i) does not impair the production of collagen and GAGs by chondrocytes (Figs. 6 and 7); (ii) stimulates, the synthesis of GAGs over long times. Moreover, the data reported in the present work clearly show that the presence of chitlac in the binary polymer mixture represents a cell signal that significantly enhances cell proliferation at any time of culture, with respect to the growth of chondrocytes in alginate alone (Fig. 9). The slow duplicating rate of chondrocytes in alginate 3D-scaffolds had been recently described[39] and natural ECM polymers such as HA[40–43] were used in combination with alginate to improve chondrocytes growth and metabolic activity. In some studies, alginate/HA association seemed to influence positively cells proliferation,[44] but more recent studies demonstrated that HA did not have a significant impact on chondrocytes replication when compared to alginate scaffolds.[45,46] In the present case, the replication stimulatory effect of chitlac might be explained by physico-chemical processes influencing the polymer interaction with the cell and the cellular activity (e.g. by the slightly positive charge) or, more probably, by a specific biological effect on chondrocytes induced by a chemical signal, as we have already

described.[21,22] In fact, this chitosan-derived glycopolymer exhibits the ability to induce chondrocyte aggregation and to stimulate cartilage matrix synthesis.[21] The specificity of the effect of chitlac stems from the presence of the galactose moiety; the bridging role between cellular membrane and polymer is carried out by a protein, a member of the S-type galactoside-binding animal lectins, Galectin-1.[22] Therefore, it can be very reasonably suggested that such a Galectin-mediated specific interaction between the galactose-engineered chitosan and chondrocyte might be responsible for the stimulation of the cell proliferation. Further work is being carried out to investigate the molecular mechanisms underlining the chondrocytes–glycopolymer specific interactions.

CONCLUSIONS

In this study we report experimental results about preparation of biocompatible three dimensional hydrogels for tissue engineering applications, using ternary aqueous solutions of alginate and a cationic chitosan derivate, chitlac (AC-mixture). The system combines the gelling properties of alginate with the demonstrated bioactive properties of chitlac.

The experimental data show that AC-mixture hydrogels exhibit better physical and mechanical properties when compared to sole alginate hydrogels, as can be evinced from the physico-chemical characterization. This can be explained considering the formation of soluble complexes between the two oppositely charged polysaccharides, which represents an ionic extra-contribution to the hydrogel formation and results in a structural stabilization. This phenomenon leads to the possibility to produce AC-mixture calcium beads characterized by a slightly higher stability than standard alginate beads. Moreover, these 3D-scaffolds represent a supportive matrix bearing, additionally, bioactive molecules able to maintain chondrocyte phenotype and particularly to stimulate and promote cell growth and proliferation. In view of the above reported properties, the AC-mixture beads could be successfully employed as an effective system for the culture of chondrocytes and as efficient support for their delivery in tissue regeneration.

In conclusion, the reported data represent the starting point for the development of a new multicomponent scaffold based on natural polymers, with improved stability, mechanical, and biological properties than sole alginate hydrogels.

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