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A hydrophobically-modified alginate gel system: utility in the repair of articular cartilage defects

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Abstract Alginate is a family of natural polysaccharides, widely used in industry and medicine for many applications, with its non toxic nature, gentle sol/gel transition procedure and low cost, alginate inferior biomechanical properties have limited its utility especially in tissue engineering. Additionally, ionically cross-linked alginate hydrogels generally lose most of their initial mechanical and swelling properties within a few hours in physiological solution. In order to overcome these limitations, the referenced alginate was treated by covalent fixation of octadecyl chains onto the polysaccharide backbone by esterification. In semi dilute solution, intermolecular hydrophobic interactions of long alkyl chains result in the formation of physical hydrogels, which can then be reinforced by the addition of calcium chloride. FTIR studies clearly showed the presence of ester bonds at 1612 and 1730 cm^{-1} indicating that the alkyl groups are incorporated in the backbone of resulting polymer. The endothermic peak and exothermic peak present in the DSC thermogram of Alg-C18 had shifted to lower temperatures comparing to native alginate (from 106 to 83°C and from 250 to 245°C, respectively) due to the esterification reaction that leads to high hydrophobic nature of the modified sample. From rheological experiments, it can be inferred that the combination of both calcium bridges and intermolecular hydrophobic interaction in the treated alginate leads to enhanced gel strength accompanied by more stable structure in physiological solution comparing to native sodium alginate hydrogel. Finally, the modified alginate tended to have no toxic effects on mesenchymal stem cell culture, rather it supported MSC chondrogenic differentiation.

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1 Introduction

Cartilage degeneration caused by congenital abnormalities or disease and trauma is of great clinical consequence, given the limited intrinsic healing potential of the tissue. Because of the lack of blood supply and subsequent wound-healing response, damage to cartilage alone, or chondral lesions, results in an incomplete attempt at repair by local chondrocytes [1]. Full-thickness articular cartilage damage, or osteochondral lesions, allow for the normal inflammatory response, but result in inferior fibrocartilage formation [2]. To prevent progressive joint degeneration in diseases such as osteoarthritis, surgical intervention is often the only option. In spite of the success of total joint replacement, treatments for repair of cartilage damage are often less than satisfactory, and rarely restore full function or return the tissue to its native normal state [3–6].

Innovative efforts to induce cartilage healing and regeneration are currently directed towards cell-based repair approaches and today autologous chondrocytes implantation (ACI), in which the expanded cells are delivered to the cartilage lesion in a liquid suspension, is the only cell-based therapy for cartilage repair approved by FDA [7]. A potential improvement to this technique involves the use of solid cell carriers in order to facilitate

the retention of transplanted cells at the implantation site and protects them during the healing process [8].

Therefore, tissue engineering approaches emerges as an alternative therapeutic process to treat severely injured patients; however, for designing a successful cell delivery system, several criteria must be met including biomechanical properties, biocompatibility and biodegradability of the constructs. The ideal material should, in addition, promote cell attachment and proliferation, thus leading to the reconstruction of the natural tissue [9–11].

A variety of natural and synthetic materials have been examined as potential carriers of cells or therapeutic agents for cartilage repair [12–16]. Of these, hydrogels are among the most promising alternatives since these materials can provide a temporary support structure during the repair process and possessing similar properties to native cartilage. Additionally, many hydrogel formulations can be cross-linked in situ and thus can be used in minimally invasive surgical procedures [17].

The seaweed-derived alginate, which is a family of unbranched copolymers, composed of alternating block of 1-4 linked α-L-guluronic acid (G-block) and β-D-mannurunic acid (M-block) residues (Fig. 1) is a member of the hydrogel family that has found some applications in cell encapsulation, drug delivery and macromolecule immobilization due to its wonderful properties [18–24]. The gelation of alginate is mainly achieved by the exchange of sodium ions with divalent cations predominantly Ca^{2+}.

It should be emphasized that Even though Ba^{2+} and Sr^{2+} may provide more strong gels, they may be slightly toxic to the cells and therefore their bioapplication are limited [25].

There is widespread agreement that the gel network, induced by a cooperative binding of Ca^{2+} by poly G chain segments, forms stable junctions consisting mainly of dimers (egg-box model) [20]. With its non toxic nature and gentle sol/gel transition procedure and the ease of its utility as an ionically cross linked polymer, its inferior biomechanical properties have limited its application especially in cartilage tissue engineering.

Additionally, Ca-alginate beads are sensitive toward chelating agents such as phosphate and citrate and non-gelling agents such as sodium and magnesium ions. In physiological solution, this ion replacement results in osmotic swelling of the beads inevitably leading to increased pore size and destabilization and rupture of the gel [26].

Traditionally, an outer polycation layer has been added to the capsules in order to decrease porosity and increase the long-term stability of alginate gel. Several studies have, however, indicated that the polycation provokes inflammatory reactions [27]. Hence, one of the main goals and challenges of the present work has been to make alginate gel with higher gel strength and long-term stability under physiological conditions over extended periods of time, without the use of polycations.

Lately, Alternative approaches focus on employing epimerized alginate [28] or exchanging the traditional Ca^{2+} ions with several divalent ions with higher affinity toward the alginate chain [27].

Another method recently exploited by many groups is the covalent modification of alginate, whereby different organic compounds are incorporated into alginate chains [29].

The presence of anchored side chains on water-soluble polysaccharides capable of forming noncovalent interactions (mainly hydrophobic interactions) result in the formation of physical hydrogels, which can then be reinforced by ionic interaction as a consequence of the addition divalent cations.

The alkyl groups are among the most promising compounds which can be grafted to the backbone of alginate chains via ester functions [30]. However, in the esterification method employed several criteria must be met including the fact that grafting of very long alkyl chains to the backbone of referenced alginate make the entire polymer insoluble. Further more hydrophobic chains substitution ratios should be kept in a narrow range so as to avoid polymer precipitation in physiological condition.

In the present study the synthesis and properties of the Alg-C_{18}, a Hydrophobically-associating alginate gels, as

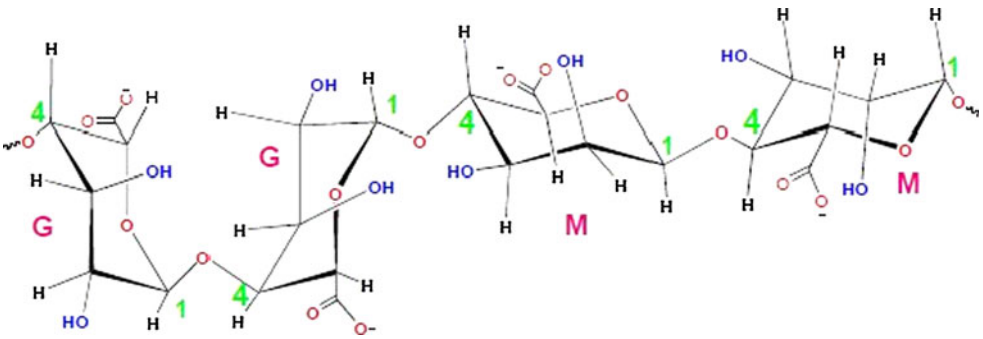

Fig. 1 Structural of alginate chains. The length of M-block and G-block and sequential distribution along the polymer chain varies depending on the source of the alginate

mesenchymal stem cells carriers was investigated. Furthermore, the capability of the prepared bioartificial matrixes in the in vitro cell culture of mesenchymal stem cells for cartilage tissue engineering applications was evaluated.

2 Materials and methods

2.1 Materials

All materials were obtained from Merck chemical Co (Ger) unless stated otherwise. Medium viscosity Sodium alginate was obtained from Sigma-Aldrich (Germany). Dulbecco's Modified Eagle Medium (DMEM), HEPES buffer, Fetal Bovine Serum (FBS), Phosphate buffer saline (PBS) without $CaCl_2$ and $MgCl_2$ were obtained from Gibco (Germany), Trans forming growth factor-β(TGF-β), bone morphogenetic Protein-6(BMP-6), insulin/transferrin/selenium (ITS) and bovine serum albumin were obtained from Sigma-Aldrich Co (USA), dimethylsulfoxid was obtained from Sigma-Aldrich Co (DMSO, Germany).

2.2 Preparation of Alg-C_{18}

The synthesis of Alg-C_{18} is derived from the procedure previously described by Della Valle and Romeo [31]. Briefly, Na-alginate was first transformed into acidic form (EtOH/0.6, HCL, 4C, 30 min, over ice bath). The TBA salts of alginate acid were then prepared by neutralization of the alginic acid with TBAOH. The TBA-alginate solution was then lyophilized in order to remove all water molecules from their matrix. The TBA-alginate salt (1gr) was dissolved in 100 ml DMSO and stirred overnight to allow its complete dissolution. Then the alkyl bromide (C_{18}) was introduced at adequate stoichiometry and the mixture was left to react for 24 h under stirring. A solution of NaCl (96 ml, 2.5 M) in H_2O was added to the TBA-alginate solution after 24 h. The purpose was to replace the existing TBA^+ ions with Na^+. The product was in a strong gel state and was not mixed with Na^+ solution, so the mixture was remounted to stir for 2 h. Afterwards, 30 ml of EtOH (70%) was added to the solution and agitated for 15 min. The prepared alginate-C_{18} was then washed in two separate runs with 250 ml EtOH (70%), 50 ml acetone, and dried in a drying oven at 25°C (Fig. 2). The mixture was filtrated and washed three times with EtOH/Acetone (3:1) solution [30]. Substitution ratios X in AAxC_{18} (x = number of alkyl chains per 100 uronic acid units) were determined by gas chromatography (Shimadzu GC 17 AAF, column SE 30 Chromosorb W-HP, length 2 m; injection temperature 285°C, column temperature 250°C; Nitrogen flow 25 ml/min) on aliquots (120 mg) first subjected to

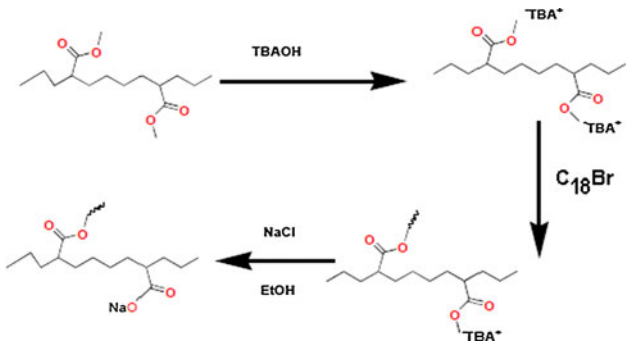

Fig. 2 Schematic of preparation method

alkaline hydrolysis (0.1 M NaOH for 4 h at room temperature), followed by toluene extraction of the resulting octadecyl alcohol. As expected the substitution ratio should be kept in the narrow range approximately 3–7%. At higher substitution ratios, the polymer was no longer soluble in water as exhibited by viscosity values determined at low shear rate, in the first Newtonian plateau.

2.3 Fourier transforms infrared spectroscopy (FTIR)

The FTIR spectra of the native alginate and Alg-C_{18} samples were obtained using a Shimadzu 8400S FTIR spectrometer. A total of 2% (w/w) of sample was mixed with dry potassium bromide (KBr), the mixture was grounded into a fine powder, before being compressed to form KBr disc. Each KBr disc was scanned at 4 mm/s at a resolution of 4 cm^{-1} between 400 and 4000 cm^{-1}.

2.4 Differential scanning calorimetry (DSC)

Thermal analysis by DSC of the native alginate and Alg-C_{18} were undertaken using a Mettler-Toledo DSC821 (Mettler-Toledo, Leicester, UK) incorporating a nitrogen cooling system. The system was calibrated with indium (melting point 156.6°C) before use. Individual samples (approximately 17 mg) were placed in 40 µl aluminum pans, sealed and weighed before being placed in the thermal chamber. Measurements were performed over the temperature range of 0–400°C at a heating rate of 10°C/min.

2.5 Preparation of the samples

Sodium alginate hydrogel is derived from the procedure previously described by Langer et al. [32]. Briefly, Sodium alginate (1.5% w/v) were made up in 30 mM HEPES containing 150 mM NaCl and 10 mM KCl and left to stand overnight to ensure complete dissolution. As a crosslinking agent the equal volume of a solution including 100 mM

$CaCl_2$ in 10 mM HEPES containing 150 mM NaCl and 10 mM KCl were added to polymer solutions.

AA-3C_{18} (1.5% w/v) were made up in 30 mM HEPES containing 150 mM NaCl and 10 mM KCl and left to stand overnight to ensure complete dissolution. As a crosslinking agent the equal volume of a solution including 100 mM $CaCl_2$ in 10 mM HEPES containing 150 mM NaCl and 10 mM KCl were added to polymer solutions.

In each instance, gelation was induced in the presence of non-gelling ions as previous studies have demonstrated that this leads to increased homogeneity of the formed gel [32].

2.6 Injection apparatus

Injection devices were fabricated using a sterile Y-shape mixer, to which separate syringes containing alginate/Alg-C_{18} or crosslinking solutions were attached and directly connected to a 22-gauge needle.

2.7 Rheological experiments

In this study, native Alginate/AA-3C_{18} samples were prepared as cylindrical disks, 15 mm diameter and 8 mm in thickness, in a custom-made cylindrical Derlin mold by employing the diffusional setting method for 12 h.

All rheometrical determinations were performed at 37°C using a dynamic stress rheometer (MCR 300, physical anton paar, GER) Equipped with a 25 mm parallel plate coated with photocopy paper disks. This helped to minimize samples slippage by letting the small amount of water extruded to be absorbed by the paper disks themselves.

The new zero position of the upper plate was determined in accordance with Morresi et al. [33] and 5 min delay was given to samples to let all shear history be relaxed.

In study A, a series of Dynamic frequency sweep tests were carried out within the linear viscoelasticity region, where the storage (G′) and loss (G″) moduli are independent of the frequency.

In study B, the stability of the alginate/alginate derivative gels in physiological solution was investigated by exposing the gels to NaCl–$CaCl_2$ for different periods of time. Briefly, after gelation, the samples were removed from the molds and placed in 1.8 mM $CaCl_2$ with 0.15 M NaCl for at 4°C for either 15 h or 7 days. The concentrations of $CaCl_2$ and NaCl were chosen to match their respective concentrations in Dulbecco's modified Eagle's medium, which is commonly used for many cell types, including fibroblasts [34] and chondrocytes [35].

Again, by performing a series of Dynamic frequency sweep tests within the linear viscoelasticity region, the storage (G′) and loss (G″) moduli were studied for different periods of time.

All the tests were replicated at least 5 times and the obtained results are given as the mean ± SD of 5 measurements.

2.8 Bone marrow cell culture

In this study, 10 New Zealand 2–3 month-old white rabbits were used. Prior to the investigation, the approval for the use of animals in the experiments was obtained from the ethic committee of Royan Institute. The animals were anesthetized by intramuscular injection of 1.5 ml ketamin (100 mg/ml) and 0.5 ml xylezine (20 mg/ml). A 19-gauge needle was inserted into the tibial medullary canal, about 0.5 ml bone marrow was aspirated, mixed with 5 ml DMEM (Dulbecco's Modified Eagle's Medium; Gibco; Germany) containing 100 IU/ml penicillin, 100 IU/ml streptomycin and 15% FBS (Fetal Bovine Serum) and centrifuged at 300 g for 5 min. The cell pellet was suspended in 15 ml DMEM medium, plated in 75-cm^2 culture flasks and incubated at 37°C and 5% CO_2. The cultures were kept until confluency with the medium replacement of twice a week. Passaged-3 cells were used for below experimentation with modified alginate. To evaluate the osteogenic potential of the isolated cells, the medium of the passaged-3 culture was replaced by osteogenic DMEM medium containing 50 μg/ml ascorbic acid 2-phosphate (Sigma; USA), 10 nM dexamethasone (Sigma; USA) and 10 mM ß-glycerol phosphate (Sigma; USA) for 21 days at the end of which the cells were fixed with 10% formalin for 10 min and stained with alizarin red (Sigma; USA) for 15 min. For adipogenic differentiation, confluent passaged-3 cells cultivated in 6-well culture plates were provided with adipogenic DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA). The cultures were then incubated for 21 days at the end of which the culture was fixed with 4% formalin at room temperature, washed by 70% ethanol and then stained by oil red solution in 99% isopropanol for 15 min. After the dye solution was removed the cultures were washed with 70% ethanol and observed by light microscopy.

2.9 Mesenchymal stem cells (MSC) culture in ALg-C_{18}

About 1×10^6 passaged-3 MSCs were uniformly suspended in 1 ml ALg-C_{18} gel and the mixture was loaded into 2 ml sterile syringe having a 22-gauge needle. ALg-C_{18} beads (approximately 2 mm) were then made by dropping the modified alginate cell suspension through the injection needle into 100 mM $CaCl_2$-suspension [36]. (Each beads contained an average of 12,000 cells). The beads were cultured either in DMEM medium supplemented with 15%

FBS and antibiotic to examine the cell proliferation or in DMEM medium containing chondrogenic substances including 10 ng TGF-ß (Transforming growth factor, Sigma; USA), BMP-6 (bone morphogenetic protein Sigma; USA), 50 mg ITS (insulin/transferrin/selenium, Sigma; USA) + premix, 5.35 mg linoleic acid and 1.25 mg BSA (bovine serum albumin Sigma; USA) and 1% FBS in order to investigate the cell differentiation within the ALg-C_{18} gel. The cultures maintained for 21 days during which cell viability, proliferation and differentiation were evaluated. All experiments were replicated 3 times.

2.10 Histological observation

To observe the cell distribution in modified alginate scaffold, some beads were processed for light microscopic observation. For this purpose, the beads were fixed in 4% paraformadehyde for 24 h followed by dehydration using ascending concentrations of ethanol. The beads were then cleared by xylene and embedded in paraffin. Six micrometer-thick sections were then made and stained with toluidine blue at room temperature.

2.11 Propidium iodide (PI) staining

To evaluate the effect of ALg-C_{18} on cell viability, MSCs were released from ALg-C_{18} by sodium citrate and suspended in PBS, stained with 10 µl PI (100 µg/ml) solution and evaluated by flowcytometry (BD FACScalibur, USA). PI is membrane impermeable and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population.

2.12 MTT assay for cell proliferation

This assay was performed in several time points including day 3, 7, 10, 14 and 20. ALg-C_{18} beads were dissolved in sodium citrate to release the cells from the gel. To perform MTT [3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide] assay, 1:5 diluted MTT solution in fresh DMEM medium was added to the cells. The cells were then incubated at 37°C for 2 h that resulted in the formation of formazan crystal due to the action of succinate-tetrazolium reductase belonging to mitochondrial respiratory chain on MTT. Supernatant was then removed and 200 µl dimethyl sulfoxide (DMSO, Sigma, Germany) was added to crystals. This caused the crystals to be dissolved creating a purple color. The absorbance of the color was recorded by Eliza-reader at 540 nm in a dark room. Using the standard curve plotted for the known number of cells, the number of the cells in alginate was determined.

2.13 RT-PCR analysis

To examine the cell differentiation, RT-PCR analysis for collagen II, aggrecan and sox9 were performed for the cell cultivated in modified alginate scaffold on the days 14 and 21. For this purpose, total RNA was collected from the cells, using nucleospine RNAII kit. Before reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove contaminating genomic DNA. Standard reverse-transcription reaction was performed with 5 µg total RNA using Oligo (dT) 18 as a primer and RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR was as follows: 2.5 µl cDNA, 1× PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were utilized to detect the differentiations.

3 Result and discussion

3.1 FTIR analysis

The FTIR spectra of the native and the modified alginate samples are shown in Fig. 3. In the FTIR spectra of the native alginate, the presence of two bands at 1425 and 1610 cm^{-1} are ascribed to the C=O component in

Table 1 The primers were utilized to detect differentiations

Gene name	Direction	Sequence	Ta	Product size
Collagen type II	Forward	F: 5′ aat ccc aga acc cat cag g 3′	54	325
	Reverse	R: 5′ cca gta gtc acc gct ctt cc 3′		
Sox9	Forward	F: 5′ aag atg acc gac gag cag 3′	58	284
	Reverse	R: 5′ ggc ttg ttc ttg ctg gag 3′		
Aggrecan	Forward	F: 5′ gga ggt ggt ggt gaa agg tg 3′	54	255
	Reverse	R: 5′ ctc acc ctc cat ctc ctc tg 3′		
GAPDH (housekeeping gene)	Forward	F: 5′ cac cca ctc ctc tac ctt cg 3′	60	276
	Reverse	R: 5′ ggt ctg gga tgg aaa ctg tg 3′		

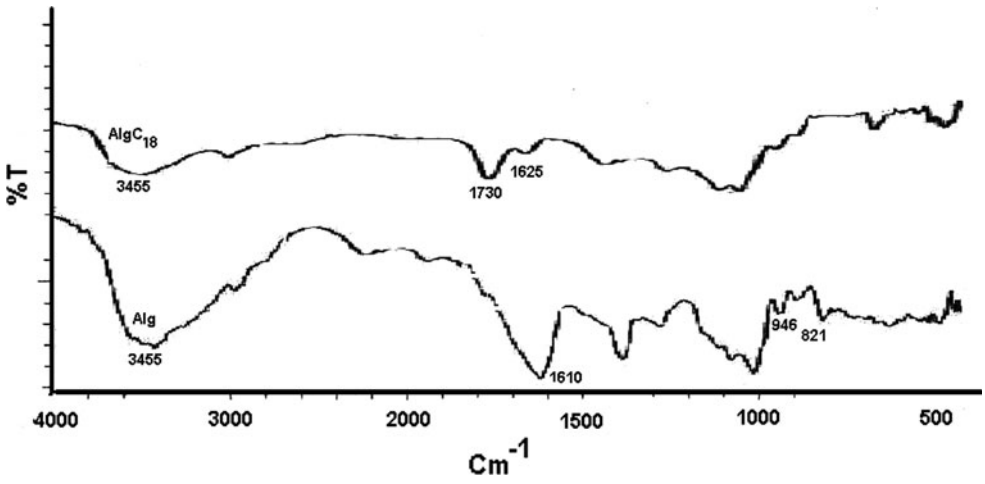

Fig. 3 FTIR spectra of the native alginate and Alg-C_{18}

carboxylic acid groups [37]. These peaks are also present in the Alg-C_{18} spectra, however, the reduction in the peaks size, accompanied by appearance of new bands at 1612 and 1730 cm^{-1} suggesting that the estrification reaction took place on some of the carboxylic acid groups.

Additionally, the peaks at 821 and 946 Cm^{-1} in native alginate FTIR spectra represent the Gluronic (G) and Manuronic (M) acid functional group respectively [38]. These peaks are also occurred in modified alginate; but the magnitude of the peak for Manuronic acid reduced, suggesting that esterification of carboxylic group is merely performed on the M blocks.

Moreover, the magnitude of the peak at 3455 cm^{-1}, which can be attributed to the hydrogen-bonds of the hydroxyl groups [39], has been decreased in modified sample comparing to the native alginate. This observation can be attributed to the hydrophobic nature of the modified sample, which is in consistency with the esterified M blocks in treated samples.

3.2 Differential scanning calorimetry

Due to its simple and interpretable spectra comparing to many other characterization methods, DSC method has been largely developed for characterization of polymer materials [40]. Figure 4 represents the DSC spectra of the native alginate and the modified sample. As is observed in the DSC spectrum of the native alginate the endothermic peak at 106°C is attributed to the water absorption, which is related to the hydrophilic nature of the functional groups of native alginate [41, 42]. On the other hand, In the DSC spectrum of the treated sample, the endothermic peak of the Alg-C_{18} sample is shifted to 83°C suggesting a lower affinity for water absorption in Alg-C_{18} comparing to native alginate. This observation can be interpreted as a result of esterification reaction that leads to higher hydrophobic nature of the modified functional groups.

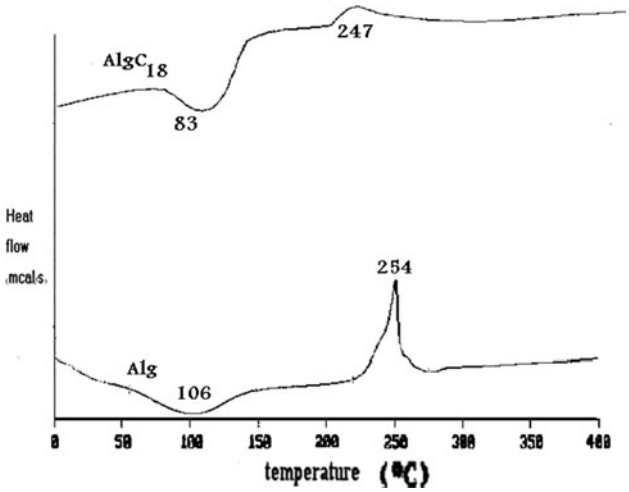

Fig. 4 DSC spectra of the native alginate and Alg-C_{18}

Additionally, the DSC spectra of the native alginate represent the exothermic peak at 254°C which is described by decomposition of the carboxylic group releasing in the form of CO_2 [37]. Similarly, this peak is also occurred in the thermogram of modified alginate but has shifted to 247°C. This is related to the fact that ester functional group can decompose to CO_2 more easily than the carboxylic group.

3.3 Rheometrical tests

In study A, By performing a series of frequency sweep tests at an amplitude γ 0 of 0.005 and an angular frequency ω, from 0.01 to 100 rad/se, all the samples showed apparently elastic dominant behavior across the accessed frequency range ($G' > G''$) with the complex shear modulus of the Alg-C18 about 5 order of magnitude greater than the complex shear modulus of parent alginate at all frequency decades (Fig. 5).

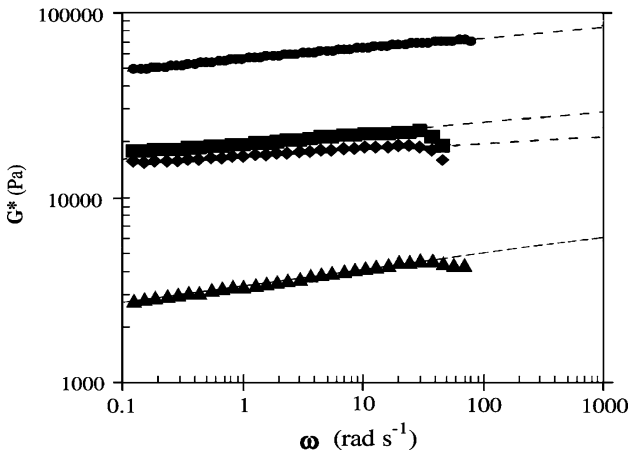

Fig. 5 Complex shear modulus of parent alginate at all frequency

It can be pointed out that the combination of both calcium bridges and intermolecular hydrophobic interaction in hydrogels prepared from Alg-C_{18} leads to an increase in elastic and viscose moduli of Alg-C18 comparing to native sodium alginate hydrogel.

In study B, The effect of NaCl and $CaCl_2$ at physiological concentration was determined in dynamic testing configurations. From Fig. 5 it can be inferred that the physiologic buffer decreased the complex dynamic shear moduli of both alginate and Alg-C_{18} due to some loss of crosslinking from competition between the relatively scarce Ca^{2+} and the abundant Na^{+}. However, the presence of hydrophobic interaction between the alkyl chains in Alg-C_{18} lead to more stable structure as exhibited by higher dynamic shear moduli as compared to non treated alginate. Additionally, a longer period of storage in NaCl (10 days) was not associated with significant changes in the material properties as compared to a 24-h exposure time, reflecting the stability of the alginate gel over this time period. These findings are in agreement with Morresi et al. [43].

3.4 Cell culture

Fibroblastic cell colonies appeared in rabbit bone marrow primary culture and were grown into confluency in 2 weeks after culture initiation. Confluent culture consists of uniform fibroblastic mesenchymal stem cells. Sufficient MSCs were obtained for further experiment after two rounds of cell passages. MSCs have two properties that could be used for their isolation. The first one is adhesion to the tissue culture substrate and the second one is capability of differentiation to bone, chondrocyte and adipocyte cell lineage [44]. So we evaluated bone and adipo differentiation of isolated cells. The osteogenic cultures stained positively with alizarin red indicating that the culture undergoes mineralization following osteogenic differentiation. In the adipogenic cultures, lipid droplets were observed in differentiating cells several days after culture initiation. These were identified using oil red staining (Fig. 6).

3.5 Histological observation

According to the sections that were stained with toluidine blue, MSCs observed as round cells distributed almost uniformly in the lacuna like cavity within the modified alginate gel (Fig. 7) indicating that the used alginate was compatible with MSC culture. Such morphology has been reported in some investigations using alginate cultures [45, 46].

3.6 Cell viability

PI staining results demonstrated that about 87% of cultivated cells in modified alginate kept their viability. These results indicated that the modified alginate was compatible with the 3D culture of MSCs. About 13% of cultivated cells which appeared to be died in our 3D culture in modified alginate would be expectable. It has long been known that the supply of oxygen and soluble nutrients becomes critically limiting for in vitro culture of 3D tissues as well as 3D cell culture in scaffolds under static conditions(Fig. 8) [47, 48]. In other words about 87% viable cells indicate that the modified alginate possesses an adequate porosity through which nutrients and O_2 can penetrate toward cultivated cells.

3.7 Proliferation and differentiation

According to the MTT results, there was no proliferation in alginate culture (Fig. 9). There are multiple reports on alginate culture of chondrogenic cells including chondrocytes and MSCs. In general reports on proliferative effects of alginate on cells are different. While regarding chondrocyte cultures some investigations have indicated that alginate can promote the cell proliferation [49–51] another study using MSCs in alginate culture have demonstrated no proliferative effects of alginate on the cell. Weber et al. [36] reported no proliferation of MSCs in the culture of a novel class of alginate matrix. In contrast to these results Wang et al. [52] have reported MSCs can proliferate in alginate depending on the structural characteristics of the used alginate. According to our findings modified alginate cannot stimulate the MSC proliferation. This would be the results from addition of alkyl groups to the alginate structure. It should be mentioned that according to some investigation cell encapsulation increase the G0–G1 and decrease the S phase frequency rate in culture. Since proliferation and differentiation are contrasting process it can

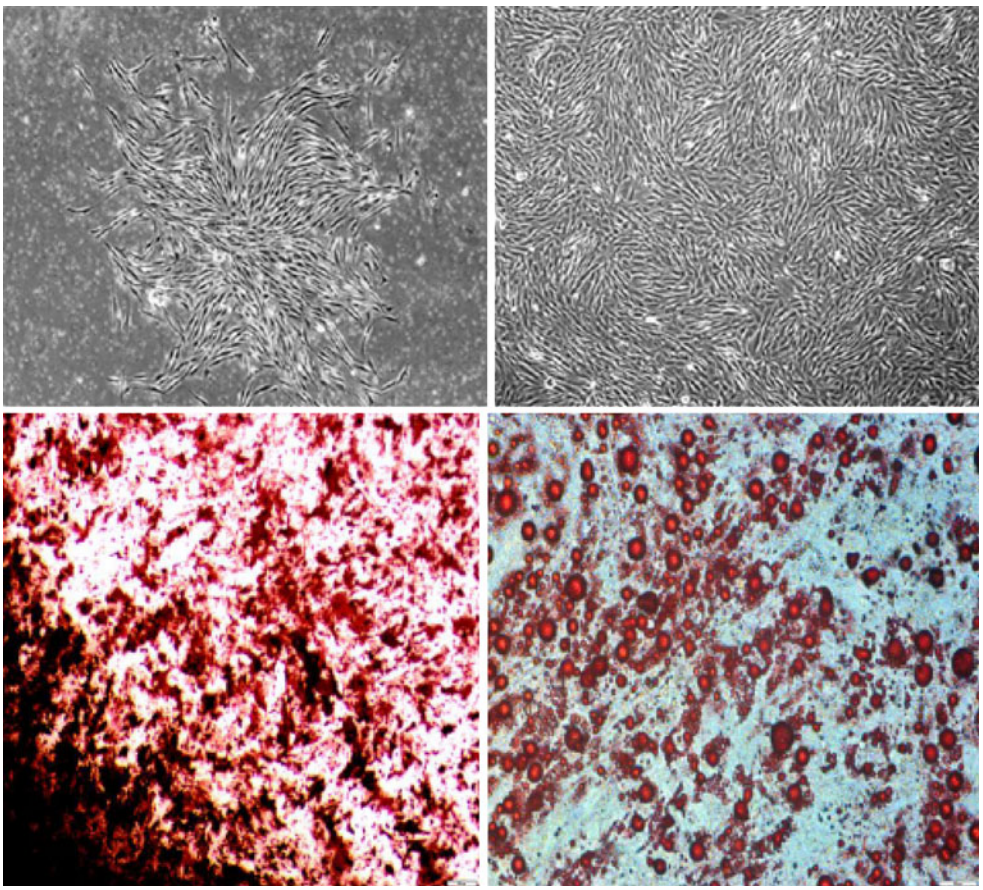

Fig. 6 Cell culture and differentiation: **a** A fibroblastic cell colony in bone marrow primary culture. **b** confluent culture of MSCs in passage-2. Phase contrast invert microscopy; magnification 40×. **c**, **d** alizarin red and oil red staining of passaged-3 mesenchymal stem cells., Phase contrast invert microscopy; magnification 100×

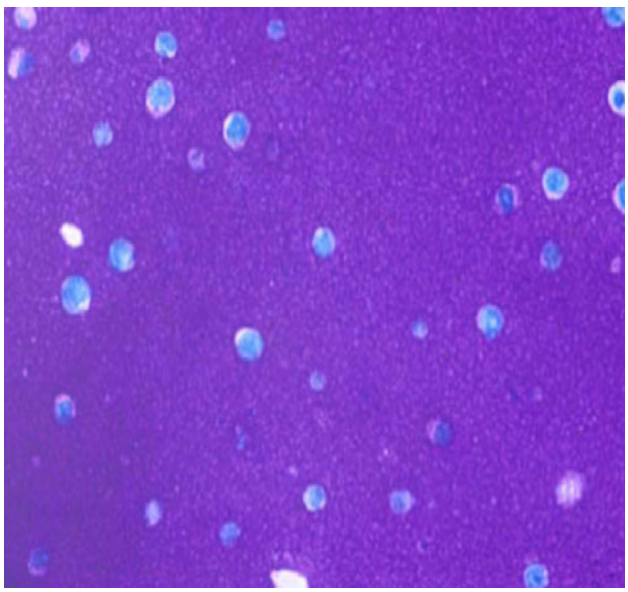

Fig. 7 Toluidine blue staining of MSCs in alginate indicating the cell distribution in lacuna-like cavity of alginate. Light microscopy; magnification 400×

be speculated that in such a condition differentiating cell rate would be increased [53, 54].

RT-PCR analyses showed MSCs cultivated in modified alginate expressed cartilage specific genes including collagen II, aggrecan and sox9 genes on either day 14 or 21. Sox9 is a transcription factor expressed in chondritic progenitors as well as chondritic cells. This factor induces cartilage-specific gene expression such as aggrecan and collagen II by binding to their enhancer region [55]. Since the cells succeeded to differentiate towards chondrogenic lineage it can be speculated that the modified alginate had no negative effects on the cell differentiation capacity (Fig. 10).

4 Conclusions

Although non toxic nature and gentle sol/gel transition procedure of native alginate make it a common material for cell encapsulation and macromolecule immobilization, its inferior biomechanical properties have limited its utility

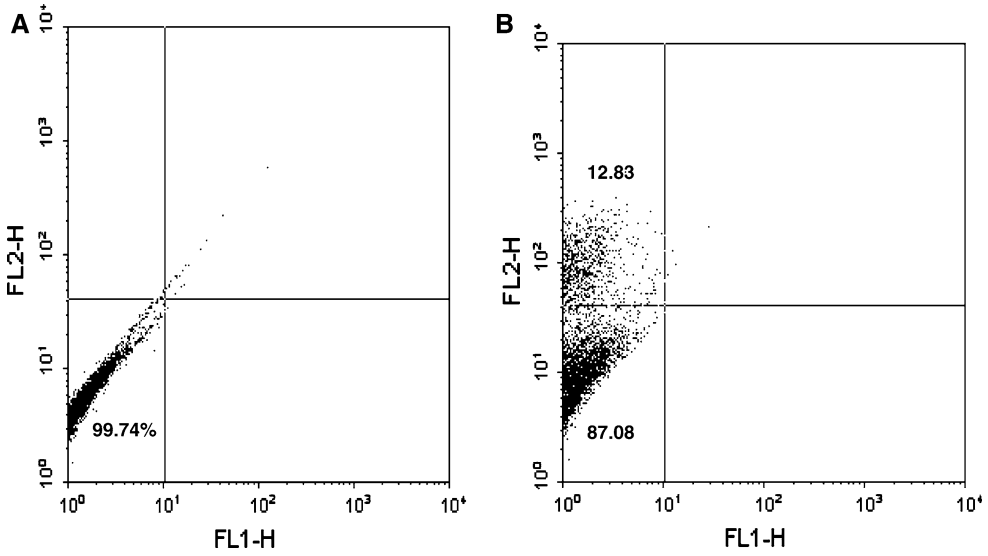

Fig. 8 Flow cytometry analysis for determining the quantity of viable MSCs in alginate culture. **a** Flow cytometry before PI staining, **b** Flow cytometry after PI staining, Upper Left (*UL*): dead cells. Lower Left (*LL*): viable cells

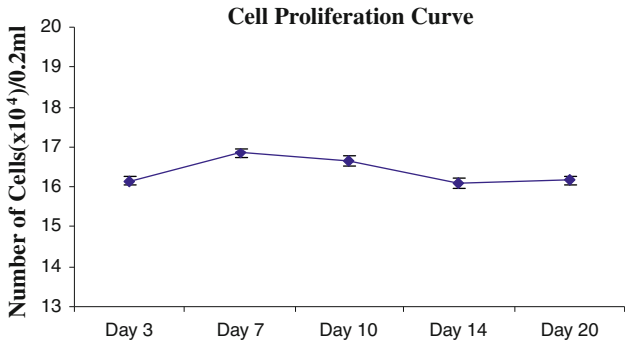

Fig. 9 Graph indicating the MSCs proliferation in alginate gel on day 3, 7, 10, 14 and 20 of culture period

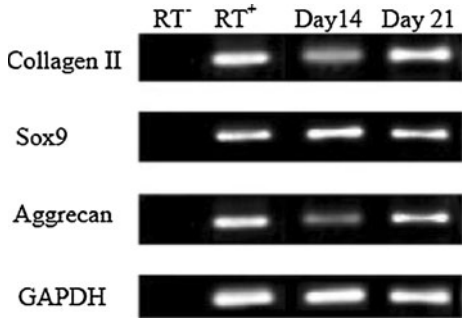

Fig. 10 RT-PCR analysis of cartilage specific gene expression in MSCs cultivated in alginate gel. RT^{-}: Specimen without reverse-transcriptase and RT^{+}: cartilage tissue from rabbit knee joint

especially in tissue engineering. Additionally, ionically cross-linked alginate hydrogels generally lose most of their initial mechanical and swelling properties within a few hours in physiological solution.

In this work an innovative approach to overcome these limitations was successfully developed, increasing the referenced alginate hydrophobic nature by the addition of octadecyl groups to the backbone of the native alginate. In semi dilute solution, intermolecular hydrophobic interactions of long alkyl chains result in the formation of physical hydrogels, which can then be reinforced by the addition of calcium chloride.

FTIR studies clearly show the presence of ester bonds at 1617 and 1736 cm^{-1} suggesting that the esterification reaction took place on some of the carboxylic acid groups.

The endothermic peak and exothermic peak present in the DSC thermogram of Alg-C_{18} had shifted to lower temperatures comparing to native alginate due to the esterification reaction that leads to high hydrophobic nature of the modified sample.

From the rheological experiments, it can be inferred that the combination of both calcium bridges and intermolecular hydrophobic interaction in the treated alginate leads to enhanced gel strength accompanied by more stable structure in physiological solution comparing to native sodium alginate hydrogel.

Finally, the modified alginate tended to have no toxic effects on mesenchymal stem cell culture; rather it supported MSC chondrogenic differentiation.

The findings of this study show the production of novel material capable of encapsulating MSCs for cartilage tissue engineering. Crucially, the all-important gelling and non-toxic properties of native alginate have been retained.

In forthcoming articles the result of the implantation of this material in rabbit knees will be described.

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