# A hydrogel system based on a lactose-modified chitosan for viscosupplementation in osteoarthritis

F. Scognamiglio[a,*], A. Travan[b], I. Donati[c], M. Borgogna[b], E. Marsich[a]

[a] Department of Medical, Surgical and Health Sciences, University of Trieste, Piazza dell'Ospitale 1, I-34129, Trieste, Italy
[b] Biopolife S.r.l., Via Caboto 19/7, I-34147, Trieste, Italy
[c] Department of Life Sciences, University of Trieste, Via Licio Giorgieri 5, I-34127, Trieste, Italy

ARTICLE INFO

*Keywords:*
Lactose-modified chitosan
Polysaccharides
Hydrogels
Viscosupplementation
Osteoarthritis

ABSTRACT

Osteoarthritis (OA) is a chronic disease affecting joint functionality and often managed with hyaluronic acid (HA) administration. In this study, a hydrogel based on a lactose-modified chitosan (CTL) reticulated with boric acid has been developed as a viscosupplement for OA treatment. The rheological characterization allowed to identify a composition whose properties were in line with those of commercial products (in the order of tens of Pascal). The selected CTL-hydrogel showed biocompatibility and antioxidant activity in vitro, and it did not influence cytokines release by macrophages. Degradation studies carried out over 24 h pointed out its higher resistance to chemical degradation with respect to HA samples.

Overall, this study underlines the advantages of the CTL-hydrogel to address the treatment of OA and shed light on an innovative application of CTL polymer, which is one of the main component of the proposed hydrogel system and not used in mixture with other molecules.

## 1. Introduction

Osteoarthritis (OA) is a degenerative disease affecting synovial joints and characterized by cartilage degradation, chronic inflammation, pain, stiffness and reduced mobility (Berenbaum, 2013; Chen et al., 2017; Loeser, Goldring, Scanzello, & Goldring, 2012). Common therapies for OA often involve the use of analgesics, topical compounds, or the administration of solutions based on hyaluronic acid (HA) by infiltrative therapy (Fakhari & Berkland, 2013; Rani et al., 2016; Zheng et al., 2019). The main aim of intra-articular injections of HA is to restore the mechanical properties of synovial joints and to stimulate specific biological responses that can improve the pathological condition (Fakhari & Berkland, 2013; Gupta, Lall, Srivastava, & Sinha, 2019; Legré-Boyer, 2015). Although some beneficial effects in terms of reducing pain and improving joint functionality have been reported, at present the remission of the pathology remains a challenge. In spite of the common use of HA-based solutions for OA treatment, these products display several disadvantages such as the fast degradation of HA molecules when injected into the joints (Larsen, Dursema, Pollak, & Skrabut, 2012; Lindenhayn, Heilmann, Niederhausen, Walther, & Pohlenz, 1997; Lindqvist et al., 2002). The degradation of HA is mainly due to the action of hyaluronidases (Bowman, Awad, Hamrick, Hunter, & Fulzele, 2018; Piccirilli et al., 2016) and reactive oxygen species (ROS) (Mendoza et al., 2007; Stern, Kogan, Jedrzejas, & Soltes, 2007). This aspect limits the permanence of HA at the intra-articular level and accounts for the repeated number of injections that must be performed in a short time frame (Bowman et al., 2018; Piccirilli et al., 2016). Beside HA, the use of other natural polymers such as chitosan might provide several advantages mainly due to their similarity to the structure, composition and mechanical behavior of the components of the extracellular matrix (ECM) (Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015; Sultankulov, Berillo, Sultankulova, Tokay, & Saparov, 2019). In this perspective, a lactose-modified chitosan (namely "CTL") reticulated with boric acid has shown very peculiar and intriguing physical properties. Recently dynamic cross-linked polymer networks of CTL and boric acid were shown to possess a strain-hardening behavior under increasing mechanical stress resembling that already known for biopolymers of the natural ECM such as collagen and actin (Cok et al., 2018; Furlani et al., 2019). Moreover, the strong dependence in the scaling law between the concentration of CTL and the zero-shear viscosity suggested interesting applications of CTL–boric acid systems in viscosupplementation (Sacco et al., 2017). Remarkable biological features of CTL have been identified both in vitro and in vivo: Donati and coworkers reported that this polymer supports chondrocytes aggregation and proliferation stimulating the production of collagen and glycosaminoglycans (GAGs)

* Corresponding author.
*E-mail address:* fscognamiglio@units.it (F. Scognamiglio).

Accepted 16 July 2020

within an otherwise inert 3D architecture, in which lactose moieties play a fundamental role in inducing this biological response (Donati et al., 2005). The biological significance of this lactose-modified polymer has been traced back to the interaction with the ubiquitous Galectin1 (Marcon et al., 2005). In addition, the beneficial effect of intra-articular injections of a formulation based on CTL and HA was recently reported in an in vivo rat model of OA knee (Salamanna et al., 2019). The results pointed out that this formulation led to a reduction of cartilage degeneration and synovium inflammation with respect to the controls, a result that opens up for a potential use of this chitosan-derivative in the field of OA treatment. Other in vitro and in vivo studies reported that this polymer is biocompatible when applied on bone tissue (Marsich et al., 2013; Travan et al., 2012). Considering these premises, the present study aims at the development and characterization of a CTL-boric acid cross-linked hydrogel to be employed as a viscosupplement for OA treatment. The CTL-boric acid system can be of peculiar interest in the osteoarticular field, given the possibility to tune the rheological properties by modulating the polymer and cross-linker concentration. Previous studies pointed out that the strain-hardening behavior owing to the CTL-boric acid systems and resembling those of the ECM of tissues can play a fundamental role in a mechanotransduction process (Cok et al., 2018; Sacco et al., 2017). This can in turn stimulate peculiar biological responses that can improve the pathological condition in OA. The ability of CTL hydrogel to act as a ROS scavenger system and its increased resistance to degradation with respect to HA pointed out some of the other advantages of using such systems in case of OA occurrence. In this view, the properties of the CTL-boric acid hydrogels were investigated in light of the final medical application. This represents an innovative aspect of the proposed research study, since the polymer CTL is not used as a component in mixture with other molecules of known activities, but it is the CTL-boric acid hydrogel itself the key system for the development of the final medical device.

## 2. Materials and methods

### 2.1. Materials

Lactose-modified chitosan-hydrochloride form (CTL) with fractions of N-acetyl-glucosamine (GlcNAc; “acetylated”, A) ($F_A$) = 0.16; glucosamine ($GlcNH_2$; “deacetylated”, D) ($F_D$) = 0.21; lactitol-substituted D unit (N-alkylated GlcLac; “lactitol”, L) ($F_L$) = 0.63 was kindly provided by BiopoLife S.r.L. (Trieste, Italy).

Dulbecco Modified Eagle Medium (DMEM), RPMI 1640, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Euroclone S.p.A.

Phosphate-buffered saline (PBS), boric acid ($H_3BO_3$), sodium chloride (NaCl), lactate dehydrogenase-based TOX-7 kit, collagenase type II, hyaluronidase from bovine testes, lysozyme from chicken eggs white, phorbol-12-myristate-13-acetate (PMA), lipopolysaccharides (LPS), acetic acid, ethanol, Neutral Red (NR), Triton X-100, sodium hypochlorite (NaOCl) solution (6–14 % active chlorine, EMPLURA®), hydrogen peroxide ($H_2O_2$), iron(II) sulfate heptahydrate ($FeSO_4$-$7H_2O$), dichloro-dihydro-fluorescein diacetate (DCFH-DA), potassium hydroxide (KOH), methanol, 2,2′-Azobis-(2-amidinopropane) (ABAP), sodium hydroxide (NaOH) hyaluronic acid sodium salt from *Streptococcus equi* (53747; molecular weight: $\sim 1.5-1.8 \times 10^6$ Da) were purchased from Sigma Aldrich (USA). Human TNF-α ELISA kit and IL-10 Human ELISA kit were purchased from Novex® by Life Technologies™. For hydrogel preparation, sterile water for pharmaceutical use (Eurospital Spa) was used.

### 2.2. Preparation of CTL-boric acid hydrogels

CTL-based hydrogels reticulated with boric acid were prepared by solubilizing CTL in sterile water for pharmaceutical use, considering final concentrations in the range of 2 %–4 % w/v (Furlani et al., 2019). After solubilization, PBS 10X was added to the mixture at the final concentration of 1X and the pH was set at 7.4 by adding sodium hydroxide (NaOH) to the CTL-solutions. Boric acid (initial concentration 50 mM, pH 7.4 in PBS 1X) was added to the mixture under mechanical stirring to enable hydrogel formation. Hydrogels with different composition were prepared by varying the final concentration of CTL (in the range of 2% w/v - 4% w/v) and boric acid (in the range of 2 mM - 3 mM). The hydrogels were named C2-B2 (CTL 2% w/v – boric acid 2 mM), C2-B3 (CTL 2% w/v – boric acid 3 mM), C3-B2 (CTL 3% w/v – boric acid 2 mM), C4-B2 (CTL 4% w/v – boric acid 2 mM).

### 2.3. Sterilization of CTL-hydrogels by autoclave

CTL-hydrogels were sterilized by autoclave (Steristream 2 – Newmed S.r.l) to characterize them in their final form, for uses at the clinical level. The sterilization cycle comprises a pre-heating phase, followed by the sterilization phase (121 °C for 15 min, pressure in the range of 1.1–1.2 bars) (Rafael et al., 2019).

### 2.4. Frequency sweep (FS) and long stress sweep (LSS) measurements on CTL-boric acid hydrogels

Frequency sweep (FS) and long stress sweep (LSS) measurements on both autoclaved and non-autoclaved CTL-boric acid hydrogels were performed by means of a controlled stress rheometer Haake Rheo-Stress RS150 operating at 25 °C or 37 °C (Cok et al., 2018; Furlani et al., 2019; Sacco et al., 2017). FS and LSS analyses allowed to investigate the variation of the elastic (G′) and viscous (G″) moduli as a function of frequency and applied stress (τ), respectively. For FS tests, a roughened plate device was employed (HPP35 profiliert: Ø = 35 mm; gap: 1 mm; τ = 4 Pa; frequency range: 0.01 − 100 hertz - Hz). For each hydrogel composition, the samples were tested in duplicate. For LSS measurements, the C3-B2 AC hydrogels were tested by employing the same plate geometry (frequency: 2.5 Hz; gap = 0.5 mm). During each measurement, a solvent trap was used to prevent sample evaporation.

### 2.5. Isolation of chondrocytes from porcine articular cartilage

Chondrocyte cells were obtained from knee-articular cartilage harvested from adult pigs kindly provided by a slaughterhouse, following a procedure previously described (Scognamiglio, Travan, Borgogna, Donati, & Marsich, 2020). Thin slices of cartilage tissue were cut and incubated with a solution containing hyaluronidase (270 U/mL), penicillin (500 U/mL) and streptomycin (500 U/mL). Enzymatic digestion was allowed for 1 h at 37 °C. A second enzymatic incubation was performed in collagenase type II (250 U/mL), penicillin (500 U/mL), streptomycin (500 U/mL) at 37 °C overnight under shaking. Tissue fragments were removed by filtration and isolated cells were cultured in DMEM supplemented with FBS 10 %, penicillin/streptomycin 0.25 %, at 37 °C and 5% $CO_2$.

### 2.6. In vitro biocompatibility (lactate dehydrogenase assay)

Lactate dehydrogenase (LDH) assay was performed on primary chondrocytes isolated from pig’s articular cartilage and on an osteoblast cell line (Osteosarcoma MG-63 cell line, ATCC number: CRL-1427) to evaluate in vitro the biocompatibility of C3-B2 hydrogels after autoclave (C3-B2 AC), following a protocol reported in the literature with slight modifications (Scognamiglio et al., 2020). The cells were cultured in cell medium (DMEM) supplemented with FBS 10 % and penicillin/streptomycin 0.25 %, at 37 °C and 5% $CO_2$. MG-63 cells were plated at the final density of 50,000 cell/well in a 12-well plate. The day after seeding, fresh DMEM was added to each well (1.5 mL) and the hydrogel (0.5 mL) was added to cells by using trans-well permeable supports (Costar® Transwell® Permeable Supports; diameter: 12 mm,

polycarbonate membrane with 0.4 μm pore size), which favor the diffusion of substances through a permeable membrane. Chondrocytes were seeded at the final density of 50,000 cell/well in 24-well plates. The day after seeding, 1.5 mL of fresh DMEM was added to the wells and a trans-well system (Costar® Transwell® Permeable Supports; diameter: 6.5 mm, polyester membrane with 3 μm pore size) was used for the delivery of the hydrogel to cells. Cells cultured in plain medium were employed as growth control, while cells treated with Triton X-100 (final concentration 0.01 % v/v) were considered as positive control of cell death. For LDH assay, cellular medium (45 μL) from each well was collected 24 and 72 h from treatment and incubated with the LDH mix solution (30 μL LDH assay substrate, 30 μL LDH cofactor, 30 μL dye solution) for 30 min in the dark. To stop the enzymatic reaction, 15 μL HCl 1 N were added to each well. Absorbance values were read at 490 nm and 690 nm with a spectrophotometer (Infinite 200PRO NanoQuant, Tecan). At each time point, the percentage of released LDH was calculated by normalizing the absorbance values of treated or untreated cells over the absorbance of the cellular lysis. For each series of samples, four replicates were considered. Statistical analysis of data was performed using Student's T-test.

### 2.7. In vitro tests with macrophages

Human U937 cell line from human lung (monocytic-like lymphoblast - ECACC 85011440) was cultured in RPMI-1640 supplemented with FBS 10 %, penicillin 100 U/mL, streptomycin 100 μg/mL and L-glutamine 2 mM, at 37 °C, 5% $CO_2$, following a protocol of culturing conditions reported in the literature with slight modifications (Baek et al., 2009; Garrelds et al., 1999). Undifferentiated U937 cells were seeded on 12-well plate (150,000 cell/well) and differentiation in macrophage-like cells was induced by the addition of phorbol-12-myristate-13-acetate (PMA; final concentration: 15 ng/mL). After 3 days incubation, cellular medium was discarded and replaced with RPMI-1640 (1 mL) to allow restoration of cells for 1 day. Fresh medium (1.5 mL) was then added and the cells were treated with C3-B2 AC hydrogels ("C3-B2") and C3-B2 AC hydrogels in the presence of lipopolysaccharides (LPS) – ("C3-B2-LPS"; final concentration of LPS: 1 μg/mL). C3-B2 AC hydrogel (0.5 mL) was delivered to cells by using a trans-well system (Costar® Transwell® Permeable Supports; diameter: 12 mm, polyester membrane with 0.4 μm pore size). In the case of "C3-B2-LPS" samples, LPS was added to the wells 1 h after the incubation with the hydrogel. In the case of cells treated with C3-B2 hydrogels autoclaved samples were employed. Cells cultured in RPMI-1640 (untreated cells) and cells cultured in the presence of LPS (LPS; final concentration: 1 μg/mL) were considered as negative and positive controls, respectively.

### 2.8. Quantification of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) release by U937 cells

Enzyme-Linked Immunosorbent Assay (ELISA) for the quantification of Tumor Necrosis Factor-α (TNF-α; Human TNF-α ELISA kit) and Interleukin-10 (IL-10; IL-10 Human ELISA kit) were used according to the manufacturer's protocols. The aim of these tests was to investigate the effect of CTL-hydrogel treatment in stimulating macrophages to synthesize cytokines that might be involved in the inflammatory process in case of OA occurrence. Cellular supernatants from differentiated U937 macrophages were collected 6 h (for the analyses of TNF-α) and 24 h (for the analyses of IL-10) after treatment. The absorbance was read at 450 nm (FLUOStar® Omega – BMG Labtech spectrophotometer) and the concentration of cytokines in each sample was calculated by using a calibration curve. The values of cytokine concentration (pg/mL) were normalized over the viability of macrophages, established by Neutral Red assay (see section "*2.9 Neutral red (NR) assay on macrophages*"). Three replicates were considered for each series of samples. For statistical analyses of data one-way ANOVA test was used, and p-value $< 0.05$ was considered as statistically significant.

### 2.9. Neutral red (NR) assay on macrophages

The viability of adherent U937 macrophages was evaluated by the neutral red (NR) assay following a protocol described in the literature with slight modifications (Cordier, Gulumian, Cromarty, & Steenkamp, 2013). This test allowed to normalize the signals corresponding to cytokines synthesis (see section "*2.8 Quantification of Tumor Necrosis Factor-α (TNF-α) and Interleukin-10 (IL-10) release by U937 cells*") over cellular viability. Neutral red (NR) powder was prepared by dissolution in water (5 mg/mL) and centrifuged at 16,000*g* for 10 min to remove any insoluble residue. A diluted solution of NR (final concentration: 100 μg/mL in PBS 1X) was added to each well (400 μL) and incubation was allowed at 37 °C for 20–30 min. After incubation, the wells were washed with PBS 1X and a solution containing ethanol (50 % v/v), acetic acid (1% v/v) in water was added to each well (400 μL). The solution (150 μL) was transferred into a 96 multiwell plate and the absorbance was measured at 540 nm using a plate reader spectrophotometer (Infinite 200Pro NanoQuant, Tecan). For each series of sample, three replicates were considered.

### 2.10. Peroxyl-radical scavenging capacity (PSC) assay

The antioxidant capacity of CTL-hydrogels and solutions containing CTL, boric acid and hyaluronic acid (HA) as single components was evaluated by the peroxyl-radical scavenging capacity (PSC) assay (Adom & Liu, 2005). This test enabled to evaluate the ability of compounds to scavenge peroxyl-radicals. The polymeric hydrogel (CTL 3% - boric acid 2 mM) was prepared as described in section "*2.2 Preparation of CTL-boric acid hydrogels*". Solutions containing CTL and HA were prepared at the concentration of 3% w/v in PBS 1X, pH 7.4. All samples were sterilized by autoclave at the conditions reported in section "*2.3 Sterilization of CTL-hydrogels by autoclave*", diluted in water and tested at three final concentrations, after the addition of the reagents required for the test. The final concentration of samples tested for PSC assay is reported in Table 1.

For the assay, non-fluorescent dichloro-dihydro-fluorescein diacetate (DCFH-DA) was dissolved in methanol (final concentration: 2.5 mM) and 80 μL of solution were added to 900 μL of potassium hydroxide (KOH) solution (final concentration: 1 mM) to hydrolyze diacetate (DA) groups. The reaction was carried out for 5 min at room temperature (RT), and the solution was then diluted by the addition PBS 1X to reach the final volume of 6 mL (DCFH solution). The samples (100 μL) were transferred on a 96-well plate and an equal volume of DCFH solution was added. A solution containing 2,2′-Azobis-(2-amidinopropane) (ABAP) at the concentration of 20 mM in PBS 1X was prepared, and 50 μL of this solution were added to each well to trigger the oxidation of DCFH to fluorescent DCF. As control reaction, samples containing only DCFH and ABAP were employed. Quercetin (final

**Table 1**
Final composition of samples for PSC assay.

| Sample name | Final composition |
|---|---|
| CTL-B (1) | CTL 0.4 % - boric acid 0.27 mM |
| CTL-B (2) | CTL 0.2 % - boric acid 0.13 mM |
| CTL-B (3) | CTL 0.1 % - boric acid 0.07 mM |
| CTL (1) | CTL 0.4 % |
| CTL (2) | CTL 0.2 % |
| CTL (3) | CTL 0.1 % |
| B (1) | boric acid 0.27 mM |
| B (2) | boric acid 0.13 mM |
| B (3) | boric acid 0.07 mM |
| HA (1) | HA 0.4 % |
| HA (2) | HA 0.2 % |
| HA (3) | HA 0.1 % |

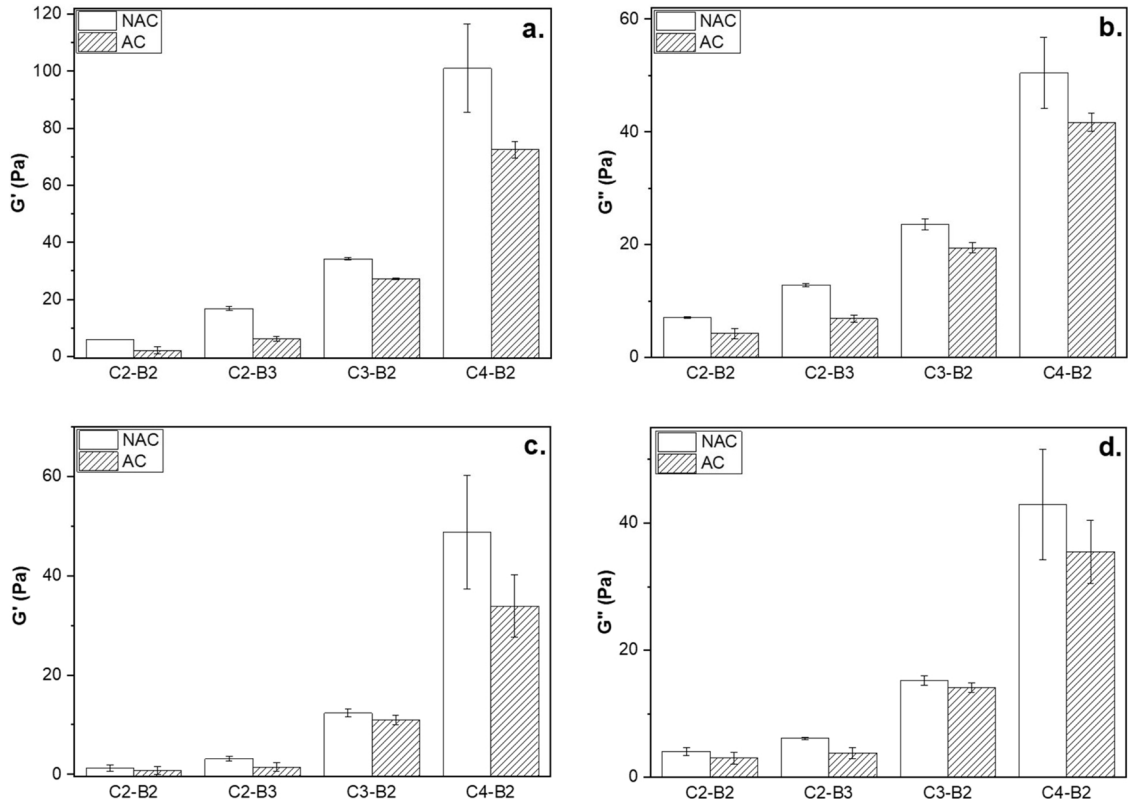


**Fig. 1.** a, b) elastic - G′ (a) and viscous - G″ (b) moduli of non-autoclaved (NAC; white bars) and autoclaved (AC; patterned bars) CTL-boric acid hydrogels at 25 °C. c, d) elastic - G′ (c) and viscous - G″ (d) moduli of non-autoclaved (NAC; white bars) and autoclaved (AC; patterned bars) CTL-boric acid hydrogels at 37 °C. The G′ and G″ values were considered at the frequency of running - 2.5 Hz (n = 2).

concentration: 8 μM) was used as positive control of scavenger activity. The kinetic of the reaction was carried out at room temperature for 40 min. Fluorescence was monitored at 485 nm (excitation) and 540 nm (emission) with a fluorescent spectrophotometer (FLUOStar® Omega – BMG Labtech spectrophotometer). For all samples, the areas under curve (AUC) for the fluorescent reaction were integrated and data employed for calculating the scavenger activity, according to Eq. (1)

$$\text{Scavenger activity (\%)} = (1 - (\text{S.A.}/\text{C.A.})) * 100 \quad (1)$$

Where S.A. = AUC for samples; C.A. = AUC for control reaction.

Data were reported as mean values ± standard deviation of three independent experiments. Statistical analysis of data was performed using Student's T-test.

### *2.11. Degradation of CTL hydrogels by sodium hypochlorite (NaOCl) solution and Fenton's reagent*

The degradation of CTL hydrogels and HA solutions by means of sodium hypochlorite (NaOCl) and Fenton's reagent was monitored over time by FS measurements on autoclaved samples. Degradation studies performed by chemicals or enzymes (see section "*2.12 Enzymatic degradation of CTL hydrogels by lysozyme*") were performed (CTL-hydrogels and HA solutions) to investigate the degradation kinetics of hydrogels under conditions that simulate or emphasize the in vivo conditions and which might contribute to CTL-hydrogels degradation in case of OA occurrence.

In the case of degradation by NaOCl solutions, autoclaved CTL hydrogels (final concentration: CTL 3% w/v – boric acid 2 mM) and HA solutions (final concentration: 1.5 % w/v) were treated by adding NaOCl solutions at the final concentration of active chlorine 0.015 %, 0.037 %, 0.074 % and 0.150 %.

For Fenton reaction, $H_2O_2$ and $FeSO_4$-$7H_2O$ solutions (150 μL) were added to the autoclaved samples (1.5 mL) at the final concentration of 20 mM and 1.6 mM, respectively. This solution was named "$H_2O_2$-$Fe^{2+}$ (A)". The effect of a second solution named "$H_2O_2$-$Fe^{2+}$ (B)" was also investigated at the final concentration of $H_2O_2$ 10 mM and $FeSO_4$-$7H_2O$ 0.8 mM, by adding the solution (150 μL) to the autoclaved samples (1.5 mL).

FS measurements were performed after 6 h and 24 h incubation with the chemicals by means of a controlled stress rheometer Haake Rheo-Stress RS150 operating at 25 °C and equipped with a smooth plate device. At each time point, the hydrogels were loaded onto the plate of rheometer (CC60/1°-99018; Ø = 60 mm; gap = 51 μm; τ = 4 Pa) for the measurement. For each condition and time point, the values of G′ at 2.5 Hz were considered. The results were reported assuming the G′ values of untreated C3-B2 AC hydrogels and autoclaved HA solutions (G′ at time 0) as 100 %. For each condition, three replicates were performed.

### *2.12. Enzymatic degradation of CTL hydrogels by lysozyme*

The effect of lysozyme on CTL-hydrogels was evaluated by time oscillatory measurements. For the enzymatic reaction, CTL hydrogels were autoclaved and lysozyme from chicken eggs white (final concentration: 12,000 U and 37,500 U) were added to the hydrogel (final concentration: CTL 3% w/v – boric acid 2 mM AC) and mixed for 1 min before rheological measurements. Time oscillatory measurements were performed by means of a controlled stress rheometer Haake Rheo-Stress RS150 operating at 25 °C and equipped with a smooth cone-plate device (geometry: CC60/1°-99018; Ø = 60 mm; gap: 1 mm; τ: 4 Pa; frequency: 2.5 Hz).

## 3. Results

### 3.1. Preparation of CTL-boric acid hydrogels and rheological characterization

CTL macromolecules are known to reticulate with the inorganic agent boric acid, leading to the formation of dynamic cross-linked polymeric networks (Cok et al., 2018; Furlani et al., 2019). CTL-hydrogels with different compositions were prepared by varying parameters such as the final concentration of the polymer (in the range of 2% w/v - 4% w/v) and boric acid (in the range of 2 mM–3 mM) in the mixture. The hydrogels underwent sterilization by autoclave and the rheological properties of both autoclaved (AC) and non-autoclaved (NAC) hydrogels were investigated by frequency sweep (FS) measurements. For each formulation type, the values of elastic (G′) and viscous (G″) moduli at the frequency of 2.5 Hz (frequency of running) were considered. These measurements were performed at the temperature of 25 °C and 37 °C (Fig. 1 and Table S1, Supporting information). The values of G′ and G″ moduli at the frequency of 0.5 Hz (frequency of walking) were also reported (Table S2, Supporting information).

The values of G′ and G″ at the frequency of 2.5 Hz were in the range from few Pascal to tens of Pascal, depending on hydrogel composition. At this frequency, only a negligible effect of autoclave treatment on the complex viscosity of the hydrogels was observed (data not shown). Among the formulations tested, C3-B2 AC hydrogels display viscous and elastic moduli in the range typically encompassed by commercial products for viscosupplementation (Nicholls, Manjoo, Shaw, Niazi, & Rosen, 2018) and close to those of healthy young synovial fluid, which displays values of 23 Pa and 7 Pa for G′ and G″ respectively, at the frequency of 2.5 Hz (Fakhari & Berkland, 2013; Fam, Bryant, & Kontopoulou, 2007). For these reasons, this specific formulation was employed for further characterization. The FS spectra of C3-B2 AC hydrogels at the temperature of 25 °C and 37 °C are reported in Fig. 2(a and b).

At both temperatures, the hydrogel composed by CTL and boric acid displays a viscoelastic behavior with a viscous modulus (G") higher than the elastic modulus (G') at low frequencies. The frequency sweep performed at 25 °C shows a cross-over point between elastic and viscous response at around 1.5 Hz.

LSS measurements were performed on C3-B2 AC hydrogels to evaluate the stress-strain relationship after autoclave, both at 25 °C and 37 °C (Fig. 3).

The spectra show the presence of a linear response to applied stress that extends up to approximately 100 Pa and point out a minor influence of temperature. For larger values of the applied stress, a strain-hardening behavior emerges at both 25 °C and 37 °C although to a different extent. Indeed, G″ shows a six-fold and a three-fold increase at 25 °C and at 37 °C, respectively.

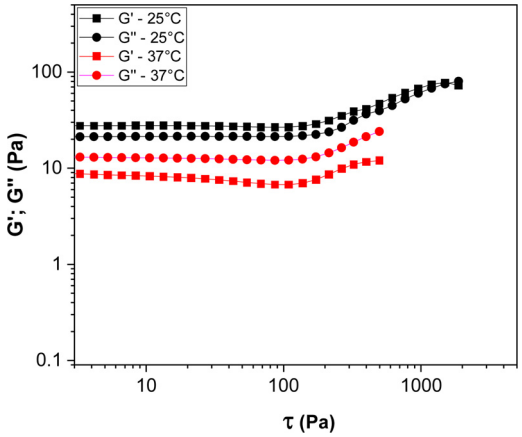


**Fig. 3.** Long stress sweep spectrum of C3-B2 AC hydrogels at 25 °C (black) and 37 °C (red). The elastic - G′ (squares) and viscous - G″ (rounds) moduli are reported as function of stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.2. In vitro biocompatibility (LDH assay) on cells treated with C3-B2 AC hydrogels

Given the potential use of this material in direct contact with osteoarticular tissues, the hydrogel C3-B2 AC was assayed for in vitro biocompatibility on both osteoblast-like and chondrocyte cells (Fig. 4).

The data pointed out that, for both cell types, the treatment with the CTL-hydrogel did not affect cellular viability with respect to untreated cells. At variance, in the case of cells treated with the positive control (Triton X-100) the percentage of released LDH was significantly enhanced, indicating a cytotoxic response. Overall, these results showed the absence of cytotoxic reactions caused by the treatment with the hydrogel for both cell types.

### 3.3. Release of cytokines by macrophages treated with C3-B2 AC hydrogels

CTL-boric acid hydrogels were employed to evaluate the ability to influence cytokine expression in both activated and non-activated macrophages in vitro. For this test, macrophages were incubated with C3-B2 AC hydrogels, in the presence and in the absence of lipopolysaccharides (LPS), and the release of Tumor Necrosis Factor-α (TNF-α) and Interleukin-10 (IL-10) was quantified by ELISA assay. As control, cells grown in cellular medium (untreated) and cells treated with LPS only were considered. At selected time-points, the cellular medium was

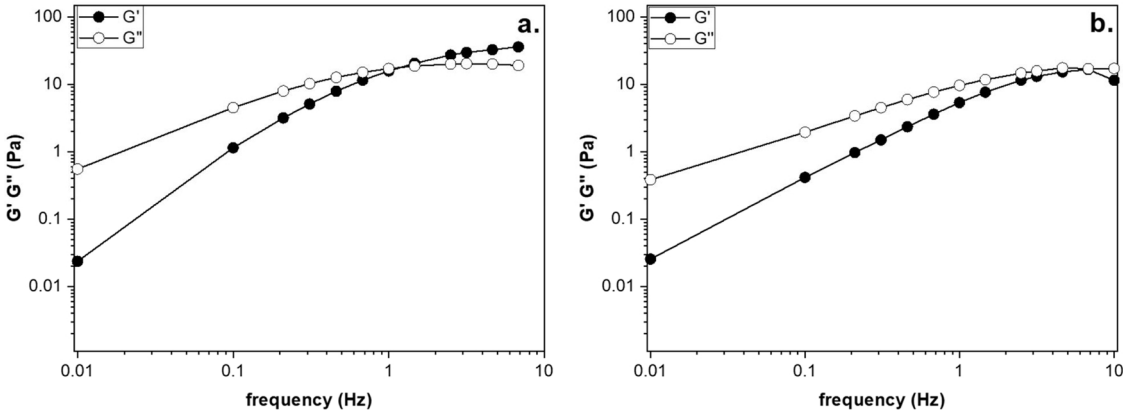


**Fig. 2.** Frequency sweep spectra of C3-B2 AC hydrogels at 25 °C (a) and 37 °C (b). The variation of G′ (full symbols) and G″ (open symbols) moduli is reported as function of frequency.

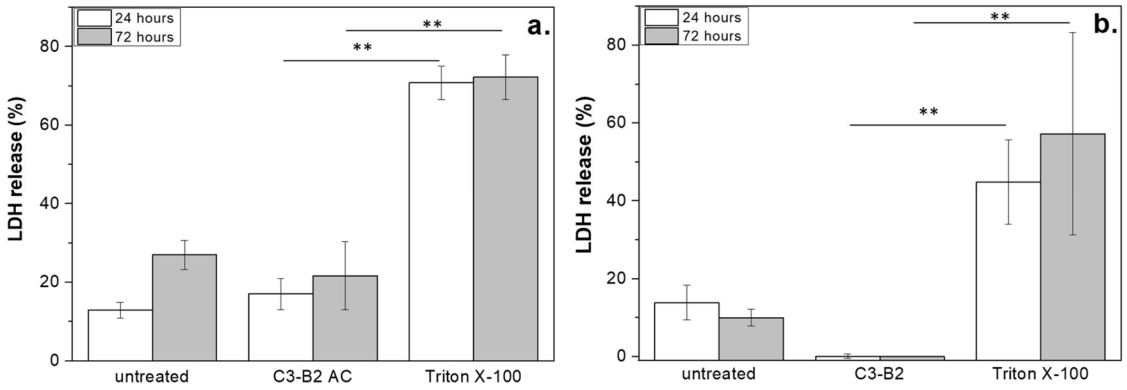


**Fig. 4.** Lactate dehydrogenase assay on osteoblasts (a) and primary chondrocytes (b) at 24 h (white bars) and 72 h (grey bars) treated with C3-B2 AC hydrogels and Triton X-100 (positive control of cell death). As negative control, cells grown in plain medium (untreated cells) were considered (n = 4; **: p-value < 0.01).

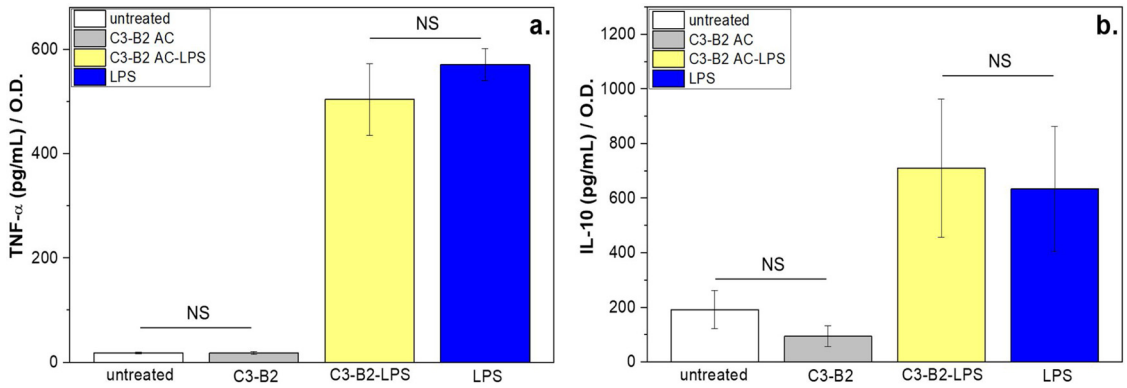


**Fig. 5.** Tumor Necrosis Factor-α (a) and Interleukin-10 (b) released by macrophages after the treatment with C3-B2 AC hydrogels ("C3-B2″; grey bar), C3-B2 hydrogels in the presence of lipopolysaccharide ("C3-B2-LPS"; yellow bars) and lipopolysaccharide ("LPS"; blue bars). As control, cells cultured in cellular medium only ("untreated"; white bars) were considered (n = 3; NS: not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

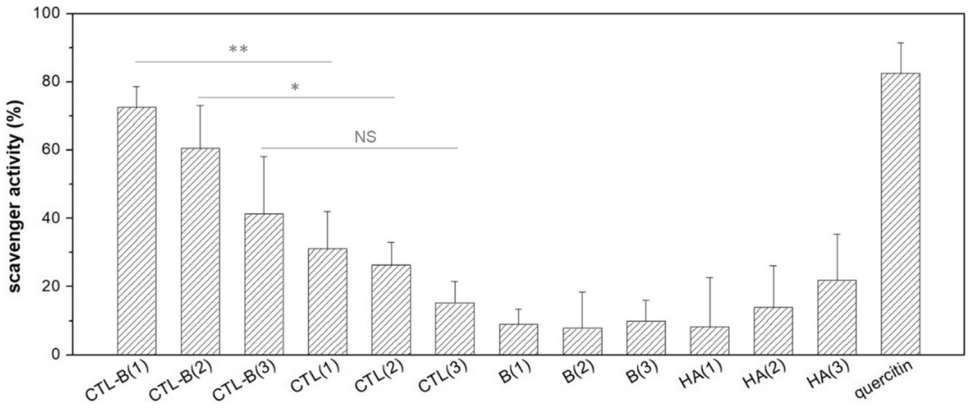


**Fig. 6.** Scavenger activity of CTL-hydrogels and solutions containing CTL, boric acid and hyaluronic acid at three concentrations (high: (1); medium: (2); low: (3)). Quercitin represents the positive control of scavenger activity (n = 3; *: p-value < 0.05; **: p-value < 0.01; NS: not significant).

collected and the concentration of the two cytokines quantified. The results were normalized over cellular viability as determined by neutral red assay. The results are reported in Fig. 5.

The production of TNF-α was evaluated 6 h after treatment of cells, since this cytokine is known to be produced during the early phase of the inflammatory response (Parameswaran & Patial, 2010). The results pointed out that the treatment of cells with the hydrogel C3-B2 AC did not stimulate the synthesis of TNF-α, since no significant differences were observed with respect to untreated cells. At variance, the addition of LPS to cell culture medium stimulates the release of TNF-α by macrophages. Interestingly, in the presence of both the C3-B2 hydrogel and LPS, the synthesis of TNF-α did not significantly differ with respect to the treatment with LPS only. IL-10 release by macrophages led to the same conclusions: no significant increase in IL-10 titer was noticed in the presence of CTL-boric acid hydrogel with respect to untreated cells; conversely, for the samples "LPS" and "C3-B2-LPS" an increased release of IL-10 occurred. No significant variations in IL-10 expression occurred between LPS-treated samples. Overall, these results indicate that the hydrogel *per se* did not stimulate macrophages and that it did not enhance the inflammatory response triggered by a pro-inflammatory stimulus (LPS).

### 3.4. Scavenger properties of CTL-boric acid hydrogels

The peroxyl-radical scavenging capacity (PSC) of CTL-boric acid hydrogels was evaluated in vitro. This test was performed by employing autoclaved CTL-boric acid hydrogels at three concentrations. As reference samples, solutions of CTL, of boric acid and of a high molecular weight hyaluronic acid (HA) were employed. As positive control of scavenger capacity, a compound known for its antioxidant ability (quercetin) was considered (Fig. 6).

The results pointed out that the CTL-boric acid hydrogels display the highest scavenger capacity among the compounds considered. CTL solutions tested at the same concentration display a lower scavenger capacity with respect to corresponding cross-linked hydrogels. This is more evident at the highest concentrations (CTL-B (1) and CTL-B (2)), for which significant differences in the ability to scavenge ROS were noticed, while at the lowest concentration (CTL-B (3) and CTL (3)) no significant differences occurred.

In the case of boric acid and HA solutions only, a minimal scavenger activity was detected (Fig. 6). For both CTL-hydrogels and CTL solutions, a concentration-dependent scavenger activity occurred.

### 3.5. Degradation of C3-B2 AC hydrogels by Fenton reaction

The resistance of C3-B2 AC hydrogels and HA solutions to degradation by Fenton reaction was evaluated by frequency sweep measurements at 6 and 24 h after incubation, using $H_2O_2$ and an iron containing solution at two concentrations, reported as "$H_2O_2$-$Fe^{2+}$(A)" and "$H_2O_2$-$Fe^{2+}$(B)". The concentration of both components in the former case (A) was twice as much as the one in the latter case (B). The results are reported in Fig. 7.

The experimental data indicate that the two polymer systems show a different degradation kinetics as well as a different sensitivity of the elastic modulus to the oxidative degradation process. Overall, the C3-B2 hydrogel after 24 h of treatment shows a 51.1 % ± 12.1 % reduction of the elastic modulus at the highest concentration of Fenton reagent used, against 81.5 % ± 7.9 % of reduction shown by HA at the same time point. Interestingly, the degradation rate of the C3-B2 hydrogel decreases over time at both reagent concentrations, and reaches a value close to zero at around 24 h. The behavior of the HA solutions appears opposite, with a more pronounced increase in the rate of degradation after 6 h of treatment.

### 3.6. Degradation of C3-B2 AC hydrogels by sodium hypochlorite (NaOCl) solutions

The resistance to oxidative degradation exerted by sodium hypochlorite (NaOCl) solutions was evaluated over time on C3-B2 AC hydrogels and HA solutions after autoclave. At each time point, FS measurements were performed and the values of G′ at 2.5 Hz recorded (Fig. 8).

The results point out that C3-B2 AC hydrogels and HA solutions display a different behavior after the treatment with NaOCl solutions. In the case of C3-B2 hydrogels, a concentration-dependent increase of the elastic modulus was observed up to the final concentration of 0.074 % active chlorine. This effect can be ascribed to the increase of pH due to the addition of NaOCl solutions. Indeed, the bind of CTL molecules to boric acid increases by increasing the pH (Pezron, Leibler, Ricard, Lafuma, & Audebert, 1989), and this process might result in the enhancement of the rheological properties of the CTL-hydrogels. In this case, it can be hypothesized that a double effect occurs: when added to the CTL-hydrogels, NaOCl triggers the degradation of CTL polymer, while in the meantime the increase of pH determines an enhancement of the cross-linking degree among the CTL-molecules; indeed the binding of such cross-linkers to diols occurs in a pH-dependent manner (Pezron et al., 1989). Overall, these simultaneous events result in the increase of G′ values for CTL-hydrogels. When the final concentration of 0.150 % active chlorine is used, a concentration-dependent reduction of the elastic modulus is observed over time. In this case, the effect of degradation on the cross-linked polymeric matrix overcomes the additional cross-linking of boric acid so causing the progressive reduction of the elastic modulus over time. In the case of HA solutions, the decrease of the elastic modulus is almost completed during the first six hours incubation. Moreover, under these experimental conditions, there is no dependence of degradation from active chlorine concentration; indeed, concentrations equal to and higher than 0.037 % of active chlorine determine a complete degradation of HA molecules after few hours of incubation.

### 3.7. Enzymatic degradation of C3-B2 AC hydrogels

Resistance to enzymatic degradation was assessed upon the treatment of C3-B2 AC hydrogels with lysozyme, which can cleave glycoside bonds in chitosan and peptidoglycans of bacterial walls (Tomihata & Ikada, 1997; Torsteinsdóttir et al., 1999). Lysozyme enzyme is expressed in different tissues and variations in the level of its expression were found in the different biological fluids (Hankiewicz & Swierczek, 1974). In the present case, lysozyme was employed at concentrations 7- and 20-times higher than that previously found in serum (Hankiewicz & Swierczek, 1974), i.e. 12,000 U and 37,500 U, respectively (Fig. 9).

The results show that when lysozyme is used at a concentration 7-fold higher than that of serum, only a slight reduction in G′ and G″ moduli occurs over time, indicating that the enzyme exerts a negligible

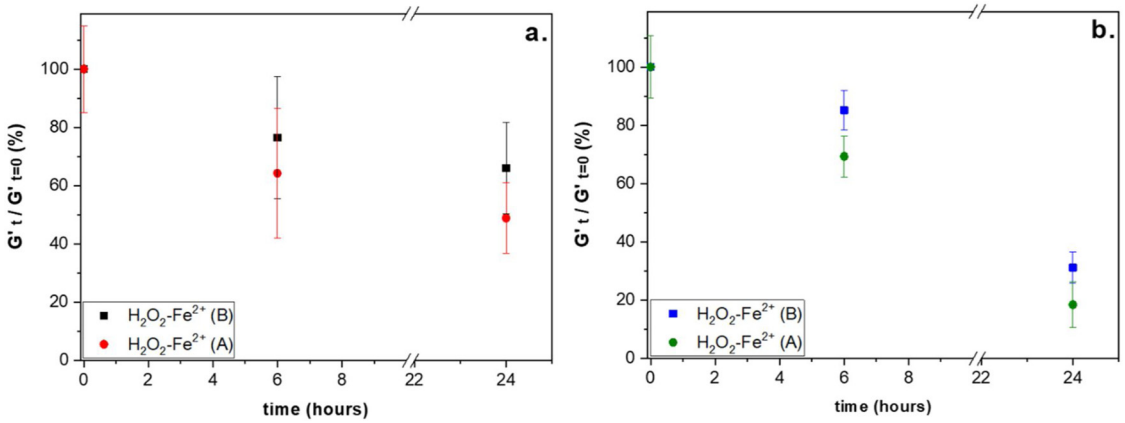


**Fig. 7.** (a) Relative variation of G′ modulus with time for C3-B2 AC hydrogels undergoing Fenton reaction with $H_2O_2$-$Fe^{2+}$ in conditions (A) (red) and (B) (black). (b) Relative variation of G′ modulus with time for hyaluronic acid solutions undergoing Fenton reaction with $H_2O_2$-$Fe^{2+}$ in conditions (A) (green) and (B) (blue) (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

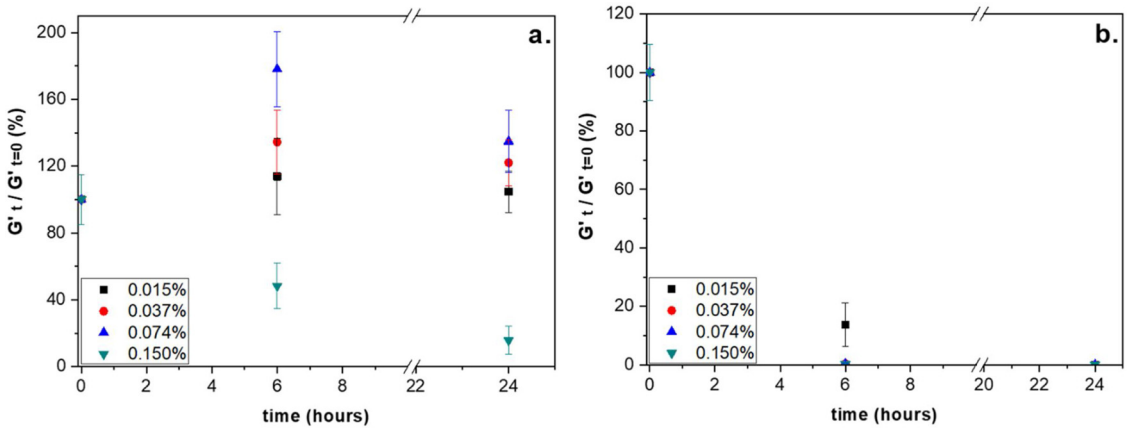


**Fig. 8.** Relative variation of G′ modulus from incubation time for C3-B2 AC hydrogels (a) and hyaluronic acid solutions (b) treated with sodium hypochlorite solutions containing active chlorine at the final concentration of 0.015 % (black line), 0.037 % (red line), 0.074 % (blue line) and 0.150 % (green line) (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect on CTL-hydrogels. As expected, increasing the enzyme concentration up to 20-fold higher than that of serum leads to a considerable variation of elastic and viscous moduli over time.

## 4. Discussion

The management at clinical level of osteoarthritis (OA) has long been carried out by the administration of products based on hyaluronic acid (HA), although their short life-time in OA joints due to chemical and enzymatic degradation highlighted the need for more effective therapeutic solutions. In order to overcome the main limitations associated to the use of HA-based products, the addition of protective components such as mannitol was explored to prevent HA degradation and extend its permanence in situ (Conrozier, Mathieu, & Rinaudo, 2014; Conrozier, 2018). Other polymers such as chitosan and chitosan-derivatives have been recently employed in combination with HA to improve the efficacy of viscosupplements and confer additional functional properties (Kaderli et al., 2015; Salamanna et al., 2019). Among chitosan derivatives, CTL, a lactose-modified chitosan, is of particular interest for its increased biological and physical-chemical properties. Indeed, CTL-boric acid hydrogels are characterized by the presence of transient cross-links in the polymeric network that account for the non-linear rheological behavior of the constructs. In this perspective, the presence of lactose flanking groups is the key feature of the CTL polymer that allows the formation of transient crosslinks through the binding of boric acid (Cok et al., 2018; Sacco et al., 2017). This rheological response resembles that of proteins composing ECM of tissues, and it represents a signal that regulates mechanisms such as cells fate, spreading and activity in a complex process known as mechanotransduction (Chaudhuri et al., 2015, 2016). As an additional advantage, the addition of boric acid to the CTL samples allows the formation of 3D reticulated structures without using covalent crosslinks that can cause cell toxicity. In view of an application as viscosupplement, the possibility of CTL-hydrogels to mediate the cellular response through a mechanotransduction process provides an additional advantage of these systems for OA applications and cartilage regeneration. A deeper investigation on the correlation between the strain-hardening behavior of the hydrogels and their bioactivity will be carried out in future work.

In this research study, several formulations of CTL-hydrogels were prepared by varying the final concentration of polymer (CTL) and cross-linker (boric acid). The hydrogels were sterilized by autoclave and characterized from a rheological and biological point of view.

The rheological properties of CTL-boric acid hydrogels were investigated by frequency sweep (FS) and long stress sweep (LSS) analyses. FS analyses pointed out the impact of sterilization on the rheological properties of the hydrogel formulation and showed that G′ and G″ moduli are influenced by the temperature and by the polymer-cross-linker ratio. The mechanical spectra of C3-B2 AC hydrogels underline their viscoelastic features and the presence of a cross-over point between the elastic and viscous moduli (Fig. 2). This behavior nicely mirrors the one displayed by the synovial fluid (Madkhali, Chernos,

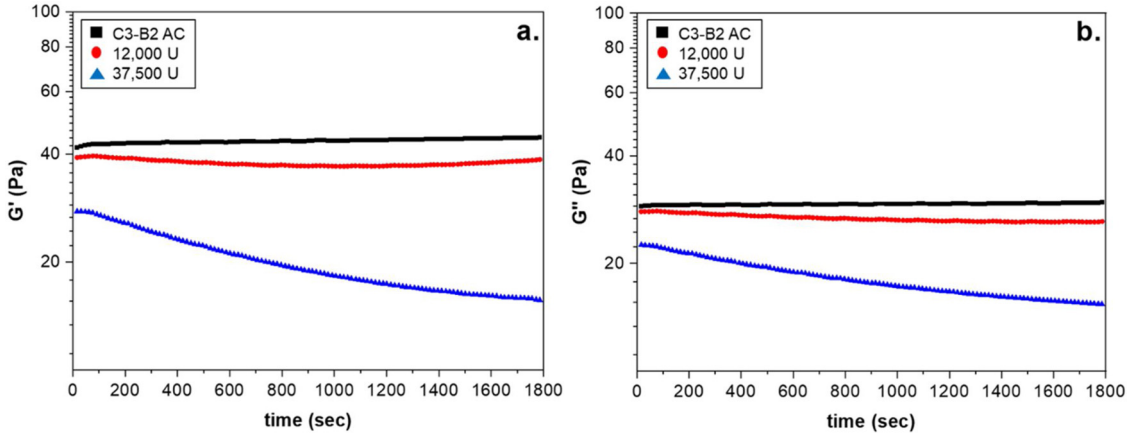


**Fig. 9.** Time oscillatory measurements on C3-B2 AC hydrogels incubated with lysozyme 12,000 U (red line) and 37,500 U (blue line). Untreated C3-B2 AC hydrogel (black line) is also reported. The variation of G′ (a) and G″ moduli (b) over time are reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Grecov, & Kwok, 2016) and that shown by a high molecular weight autoclaved hyaluronic acid at a concentration of 1.5 % (Fig. S1, Supporting information). FS results highlight the variation of G′ and G″ moduli among the different CTL-hydrogel formulations (Fig. 1). To model the in vivo behavior of the formulations, the frequency of 2.5 Hz was selected as resembling the knee-joint under solicitation from running (Nicholls et al., 2018). For all formulations both G′ and G″ moduli at 37 °C were lower than those at 25 °C as the increase in temperature weakens the boric acid binding thus lowering the transient entanglements in the polymeric network. The rheological properties of CTL hydrogels can be modulated by varying the ratio between polymer and cross-linker, a feature that can be exploited when a medical device with tunable mechanical properties is sought. Rheological measurements performed on commercial products for infiltrative therapy indicate that viscosupplements can appear either as dilute solutions or as materials with elastic-solid characteristics (Nicholls et al., 2018) with a difference in G′ and G″ spanning over approximately two orders of magnitude. It should be noted that the formulation C3-B2 AC displays viscous and elastic moduli in the range of those of commercial products (Nicholls et al., 2018) and close to those of healthy synovial fluid (Fakhari & Berkland, 2013; Fam et al., 2007), and this oriented the selection of C3-B2 hydrogel formulation for further characterization. The sterilization of the hydrogels is of paramount importance for any potential use in the field of biomaterials. The use of the autoclave resulted an efficient and validated method for sterilization of hydrogels with limited effect on their mechanical performance regardless the composition. Indeed, focusing on the C3-B2 hydrogel, the autoclave sterilization caused a reduction in G′ and G″ moduli of about 11 % and 7%, respectively. LSS measurements enabled to investigate the variation of the rheological properties on applied stress for the formulation C3-B2 and pointed out its strain-hardening behavior, which might play a relevant role in a mechanotransduction process. In the case of CTL-boric acid systems, LSS spectra are characterized by an extended linear response to the stress applied, followed by strain-hardening for a value of the stress which exceeds 172 Pa and 196 Pa at 25 °C and 37 °C, respectively (Fig. 3). In general terms, the extent of this response depends on the hydrogel composition and on the conditions employed for the analysis. The strain-hardening behavior of CTL-boric acid hydrogels has already been described in the literature on non-autoclaved hydrogels (Cok et al., 2018; Furlani et al., 2019). In this study, this peculiar mechanical response was investigated on sterilized hydrogels (C3-B2 AC) thus in their potential final form for application in viscosupplementation. The LSS spectra pointed out that, at the frequency of 2.5 Hz, the autoclaved C3-B2 hydrogels retain their strain-hardening behavior both at 25 °C and at 37 °C, suggesting that this property might be maintained when the hydrogel is employed in the human body. This mechanical response to applied stress is of particular interest because it mimics the behavior of the ECM of tissues (Cok et al., 2018; Furlani et al., 2019).

The treatment of macrophages with C3-B2 AC hydrogels did not affect the expression of TNF-α and IL-10. With reference to the specific field of OA, recent studies highlighted that OA is a multifactorial disorder caused by events such as mechanical stress, inflammation, biochemical processes and metabolic factors (Ayhan, Kesmezacar, & Akgun, 2014). The contribution of inflammation in OA has long been investigated, and it is still debated whether inflammatory reactions play a major role in the onset of OA or in OA progression (Ayhan et al., 2014). In OA joints, inflammation mainly involves the innate immune response (Mora, Przkora, & Cruz-Almeida, 2018) and it is characterized by the production of pro-inflammatory and anti-inflammatory cytokines, which can alter the metabolic activity of tissues and lead to a progressive degeneration of articular cartilage (Goldring & Goldring, 2004; Mueller & Tuan, 2011; Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). In particular, the role of macrophages in OA progression has been described: these cells can differentiate into pro-inflammatory or anti-inflammatory cells (Murray & Wynn, 2011) in response to various stimuli and environmental changes (Porcheray et al., 2005; Stout et al., 2005). The activity of macrophages can also be stimulated by endogenous HA fragments originated from degraded ECM components (Jiang, Liang, & Noble, 2011). Considering that external stimuli can promote the activation of macrophages, in vitro tests were performed to investigate whether CTL-hydrogels influence cytokine production. The results pointed out that the treatment of non-activated U937 cells with C3-B2 hydrogels did not lead to any significant increase of TNF-α and IL-10 basal production with respect to untreated cells (Fig. 5). At variance, a significant increase of released TNF-α and IL-10 occurred in the presence of a pro-inflammatory stimulus (LPS). C3-B2 AC hydrogels were also added to cells in combination with LPS, to simulate a condition in which a viscosupplement is administered in an inflammatory environment (OA joints). Also in this case, the hydrogel does not alter the expression of the cytokines with respect to the cells treated with only LPS. Taken together, these results point out that C3-B2 hydrogels do not stimulate non-activated macrophages and do not affect the activity of macrophage cells in term of cytokines synthesis and release.

The peroxyl scavenger activity of CTL-hydrogels was higher than that of CTL and HA solutions. Inflammatory processes are often associated to an increased oxidative stress. The role of reactive oxygen species (ROS) in OA pathogenesis has been widely reported in the literature (Duan, Zhang, Zhang, & Cai, 2018; Lepetsos & Papavassiliou, 2016; Paździor et al., 2019; Pinto, Rao, & Rao, 2008). In OA, the increased amount of ROS leads to events such as synovial inflammation, cartilage degradation, cellular damages and apoptosis (Henrotin, Kurz, & Aigner, 2005; Lepetsos & Papavassiliou, 2016). In this perspective, scavengers for free radicals can be beneficial in case of OA occurrence. The effect of compounds with antioxidant and scavenger activity has long been investigated in the onset and prevention of OA progression. For instance, N-acetylcysteine internalized by cells can scavenge ROS through increasing the production of glutathione (Schreck, Rieber, & Baeuerle, 1991). Other anti-inflammatory compounds employed for OA applications have the ability to inactivate ROS and inhibit cellular apoptosis (Hu et al., 2019). The CTL-boric acid hydrogels developed in this research display the ability to inactivate peroxyl radicals in vitro, in a dose-dependent manner (Fig. 6). The concentration-dependent effect observed for CTL-hydrogels serving as scavenger has been described also for other polysaccharide-based materials and compounds (Luan et al., 2018; Mu et al., 2012; Olasehinde, Mabinya, Olaniran, & Okoh, 2019). Although to a lower extent, the same trend of CTL hydrogels and the dose-dependent scavenger activity was observed for CTL solutions. Interestingly, at the highest concentrations, the scavenger capacity of CTL in solution was lower than that shown by the corresponding hydrogel, which suggests the central role held by the cross-linked polymeric network for ROS inactivation. Indeed, it can be hypothesized that the presence of crosslinking points in CTL hydrogels enhances the exposure of the CTL molecules to ROS, which increases the number of inactivated peroxyl radicals, while in the case of CTL in solution a minor accessibility of the polysaccharidic chains results in a lower scavenger capacity. In this view, the CTL hydrogels cross-linked with boric acid can be considered as high-capacity scavenger systems. In the latest years, biomaterials employed for OA treatment have been supplemented with polyols such as mannitol and sorbitol that display scavenging activity (Conrozier et al., 2014; Conrozier, 2018; Migliore et al., 2014). The use of these molecules in combination with HA viscosupplements limits polysaccharide degradation by ROS and improves its permanence in situ (Conrozier et al., 2014; Conrozier, 2018).

CTL hydrogels display a higher resistance to degradation induced by Fenton reaction and NaOCl solutions than HA samples. In the case of CTL-hydrogels, the resistance to radical degradation was assessed by the Fenton reaction. Two concentrations of $H_2O_2$-iron ions in solution were employed leading to the formation of two different amounts of hydroxyl radicals. The data (Fig. 7) show that in the case of HA samples the degradation process occurs gradually over time, leading to an almost complete degradation after 24 h (Fig. 7). In the case of C3-B2 sample, the degradation process that takes place during the first 6 h

incubation is more pronounced than that observed for HA solutions (Fig. 7a). In the case of C3-B2 hydrogel, it can be hypothesized that hydroxyl radicals affect the structure of the polysaccharidic cross-linked matrix in a first instance; after an initial degradation phase of the polysaccharide molecules, the matrix re-arrange and new transient bonds form within the hydrogel due to its dynamic feature (Cok et al., 2018; Sacco et al., 2017).

The rearrangement of the polysaccharide matrix prevents a more extensive reduction of the rheological properties for prolonged incubation. Indeed, a limited reduction in G′ modulus is further observed after 6 h of incubation and up to 24 h, indicating that hydroxyl radicals have only a negligible effect on the hydrogel structure. In the case of HA, the absence of covalent bonds among HA molecules turns out in a more pronounced reduction of the rheological properties that occurs gradually over time (Fig. 7b). The transient features of the molecular interactions in C3-B2 hydrogels might account for the higher variability of G′ values with respect to HA samples.

Further studies will clarify the molecular mechanism by which ROS are scavenged by CTL-hydrogels.

The degradation of C3-B2 hydrogel and HA solutions by NaOCl solutions has also been investigated in this research study (Fig. 8). Hypochlorite is one of the ROS involved in OA pathogenesis (Lepetsos & Papavassiliou, 2016). The CTL-hydrogel and HA were treated with NaOCl solutions at different final concentration of active chlorine. As expected, the presence of NaOCl even at the lowest tested concentration of active chlorine (0.015 %) has a detrimental effect on hyaluronan in 24 h (Fig. 8b), leading to the cleavage of the polysaccharidic chains (Stern et al., 2007). At variance, in the case of C3-B2 AC hydrogels, the effect of degradation was evident over time only when the samples were treated with 0.150 % active chlorine, while with lower concentrations an increase of G′ modulus was observed after 6 h incubation. The different behavior of these two polysaccharide systems might be related to their different structure. As to the case of degradation induced by ROS formed by Fenton reaction, it can be hypothesized that also for NaOCl solutions the cross-linked matrix of CTL-hydrogels has a protective role in preventing degradation of the system, which suggests a higher resistance of CTL-hydrogels when undergoing to increased oxidative stress conditions. Along this line, it is interesting to note that the use of concentrations of active chlorine up to 0.074 % determines an increase in G′ in the first 6 h. This could be likely traced back to the increase of pH, which causes a higher binding of boric acid. At variance, 0.150 % of active chlorine degrades the CTL chains to a higher extent which is likely non-compensated by extra crosslinking due to pH increase and results in the overall loss of the elastic modulus over time (Fig. 8a). As for samples treated with Fenton's reagent, also in this case the higher variability of data for C3-B2 hydrogels can be ascribed to the transient features of the chemical interactions between CTL and boric acid.

The treatment of CTL hydrogels with lysozyme at concentrations higher that those found in the human body showed a concentration-dependent effect on CTL-hydrogels degradation; considerable effects were noticed only for the higher of the two tested conditions. When injected in synovial space the lifetime of HA viscosupplements can be threatened also by hyaluronidases that lead to chain cleavage. In this perspective, the use of CTL-hydrogels provides the advantage of not being sensitive to hyaluronidases, an aspect that might account for an increased hydrogel stability in situ. The stability of CTL-hydrogels can instead be affected by lysozyme, which is found in human body fluids and tissues at different concentrations (Hankiewicz & Swierczek, 1974). This aspect was investigated in vitro by measuring the rheological properties of the hydrogel after enzyme treatment (Fig. 9). The lysozyme concentrations employed were 7-folds and 20-folds higher than those found in serum (Hankiewicz & Swierczek, 1974), in order to boost chain cleavage in vitro with respect to the in vivo environment. After the addition of lysozyme to the samples, the rheological properties of C3-B2 AC hydrogel were investigated by time oscillatory measurements. The enzyme catalysis was allowed for the duration of the time oscillatory test (30 min) and variation of the viscous and elastic moduli were monitored over time. As expected, a concentration-dependent effect on hydrogel degradation was noticed, although only the higher of the two concentrations tested could exert a detrimental effect on the CTL-hydrogel structure. The lower concentration (7-fold higher than that of serum) had only a minimal effect on the hydrogel structure, suggesting that the C3-B2 hydrogel is likely to withstand enzymatic degradation at the in vivo conditions.

## 5. Conclusions

This study describes the development and characterization of a hydrogel based on the polysaccharide CTL reticulated with boric acid for viscosupplementation in OA treatment. Rheological analyses on different CTL-hydrogel formulations pointed out the effect of autoclave and enabled to identify a formulation whose mechanical properties were in the range of those of commercial products for viscosupplementation. The CTL-hydrogel formulation selected in this study display a strain-hardening behavior in response to applied stress and the results highlight the influence of the temperature in determining the extent of such behavior, which might account for a mechanotransduction process. C3-B2 AC hydrogels showed a good biocompatibility in vitro and the ability of not stimulating the activity of macrophages in terms of cytokines release. The beneficial effect of the CTL-boric acid hydrogel was related also to its ability of acting as a scavenger system for ROS, which are involved in the OA pathology. These studies pointed out that the CTL-hydrogel displays a higher ROS scavenger capacity than the corresponding CTL samples in solution when tested at the highest concentration, suggesting a central role held by the cross-linked polymeric network in ROS inactivation. The hydrogel degradation was investigated by Fenton's reaction, NaOCl and lysozyme treatment. When treated with Fenton's reagents, C3-B2 AC hydrogels display a higher resistance to degradation and a different degradation kinetics than HA samples, suggesting the occurrence of a matrix rearrangement and the formation of new transient bonds inside the hydrogel network that prevent further degradation. Similarly, low concentrations of NaOCl exert a detrimental effect on HA samples, while determining an enhancement of the elastic modulus of CTL-hydrogels owing to the increase of pH, which leads to a higher binding of boric acid and to the strengthening of the hydrogel structures. At concentration 0.150 % of active chlorine, the effect of hydrogel degradation was more pronounced as it was not compensated by extra-binding of boric acid due to the increase of pH. The increased resistance to chemical degradation with respect to HA points out that an additional advantage in using these reticulated hydrogels might be related to their increased stability and half-life after injection in OA joints. Further studies will be performed to evaluate the efficacy of the proposed system in vivo. Enzymatic degradation studies pointed out a very limited effect of lysozyme on hydrogel degradation. Overall, these results support the use of this hydrogel biomaterial in a pathological condition involving inflammation and ROS production and provide some evidences for its potential beneficial effects in case of OA occurrence.

## CRediT authorship contribution statement

**F. Scognamiglio:** Conceptualization, Investigation, Writing - review & editing. **A. Travan:** Conceptualization, Investigation, Writing - review & editing. **I. Donati:** Conceptualization, Writing - review & editing. **M. Borgogna:** Writing - review & editing. **E. Marsich:** Conceptualization, Writing - review & editing, Supervision.

## Acknowledgments

The authors acknowledge Mr. Gennaro Sessa for technical support in the experimental part. This study was supported by Regione

Autonoma Friuli Venezia Giulia - Progetto HEaD "Higher Education and Development" Units Operazione 2 - (Codice FP1619892003, canale di finanziamento 1420AFPLO2) - Fondo Sociale Europeo - Investimenti in favore della crescita e dell'occupazione - POR 2014-2020 and by Interreg V-A Italia-Slovenia 2014-2020 bando 1/2016 asse 1 – project BioApp 1472551605.

## References


Adom, K. K., & Liu, R. H. (2005). Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. *Journal of Agricultural and Food Chemistry, 53*, 6572–6580.

Ayhan, E., Kesmezacar, H., & Akgun, I. (2014). Intraarticular injections (corticosteroid, hyaluronic acid, platelet rich plasma) for the knee osteoarthritis. *World Journal of Orthopedics, 5*, 351–361.

Baek, Y. S., Haas, S., Hackstein, H., Bein, G., Hernandez-Santana, M., Lehrach, H., et al. (2009). Identification of novel transcriptional regulators involved in macrophage differentiation and activation in U937 cells. *BMC Immunology, 10*, 18.

Berenbaum, F. (2013). Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis Cartilage, 21*, 16–21.

Bowman, S., Awad, M. E., Hamrick, M. W., Hunter, M., & Fulzele, S. (2018). Recent advances in hyaluronic acid based therapy for osteoarthritis. *Clinical and Translational Medicine, 7*, 6.

Chaudhuri, O., Gu, L., Darnell, M., Klumpers, D., Bencherif, S. A., Weaver, J. C., et al. (2015). Substrate stress relaxation regulates cell spreading. *Nature Communications, 6*, 6364.

Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S. A., Weaver, J. C., et al. (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nature Materials, 15*, 326–334.

Chen, D., Shen, J., Zhao, W., Wang, T., Han, L., Hamilton, J. L., et al. (2017). Osteoarthritis: Toward a comprehensive understanding of pathological mechanism. *Bone Research, 5*, 16044.

Cok, M., Sacco, P., Porrelli, D., Travan, A., Borgogna, M., Marsich, E., et al. (2018). Mimicking mechanical response of natural tissues. Strain hardening induced by transient reticulation in lactose-modified chitosan (chitlac). *International Journal of Biological Macromolecules, 106*, 656–660.

Conrozier, T. (2018). Is the addition of a polyol to hyaluronic acid a significant advance in the treatment of osteoarthritis? *Current Rheumatology Reviews, 14*, 226–230.

Conrozier, T., Mathieu, P., & Rinaudo, M. (2014). Mannitol preserves the viscoelastic properties of hyaluronic acid in an in vitro model of oxidative stress. *Rheumatology and Therapy, 1*, 45–54.

Cordier, W., Gulumian, M., Cromarty, A. D., & Steenkamp, V. (2013). Attenuation of oxidative stress in U937 cells by polyphenolic-rich bark fractions of Burkea africana and Syzygium cordatum. *BMC Complementary and Alternative Medicine, 13*, 116.

Donati, I., Stredanska, S., Silvestrini, G., Vetere, A., Marcon, P., Marsich, E., et al. (2005). The aggregation of pig articular chondrocyte and synthesis of extracellular matrix by a lactose-modified chitosan. *Biomaterials, 26*, 987–998.

Duan, L., Zhang, W., Zhang, F., & Cai, H. (2018). Myrtol improves post-traumatic knee osteoarthritis by regulation of reactive oxygen species, transforming growth factor beta1 and apoptosis in a mouse model. *Experimental and Therapeutic Medicine, 15*, 393–399.

Fakhari, A., & Berkland, C. (2013). Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. *Acta Biomaterialia, 9*, 7081–7092.

Fam, H., Bryant, J. T., & Kontopoulou, M. (2007). Rheological properties of synovial fluids. *Biorheology, 44*, 59–74.

Furlani, F., Sacco, P., Scognamiglio, F., Asaro, F., Travan, A., Borgogna, M., et al. (2019). Nucleation, reorganization and disassembly of an active network from lactose-modified chitosan mimicking biological matrices. *Carbohydrate Polymers, 208*, 451–456.

Garrelds, I. M., van Hal, P. T., Haakmat, R. C., Hoogsteden, H. C., Saxena, P. R., & Zijlstra, F. J. (1999). Time dependent production of cytokines and eicosanoids by human monocytic leukaemia U937 cells; effects of glucocorticosteroids. *Mediators of Inflammation, 8*, 229–235.

Goldring, S. R., & Goldring, M. B. (2004). The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clinical Orthopaedics and Related Research,* S27–S36.

Gupta, R. C., Lall, R., Srivastava, A., & Sinha, A. (2019). Hyaluronic acid: Molecular mechanisms and therapeutic trajectory. *Frontiers in Veterinary Science, 6*, 192.

Hankiewicz, J., & Swierczek, E. (1974). Lysozyme in human body fluids. *Clinica Chimica Acta, 57*, 205–209.

Henrotin, Y., Kurz, B., & Aigner, T. (2005). Oxygen and reactive oxygen species in cartilage degradation: Friends or foes? *Osteoarthritis Cartilage, 13*, 643–654.

Hu, Y., Gui, Z., Zhou, Y., Xia, L., Lin, K., & Xu, Y. (2019). Quercetin alleviates rat osteoarthritis by inhibiting inflammation and apoptosis of chondrocytes, modulating synovial macrophages polarization to M2 macrophages. *Free Radical Biology & Medicine, 145*, 146–160.

Jiang, D., Liang, J., & Noble, P. W. (2011). Hyaluronan as an immune regulator in human diseases. *Physiological Reviews, 91*, 221–264.

Kaderli, S., Boulocher, C., Pillet, E., Watrelot-Virieux, D., Rougemont, A. L., Roger, T., et al. (2015). A novel biocompatible hyaluronic acid – chitosan hybrid hydrogel for osteoarthrosis therapy. *International Journal of Pharmaceutics, 483*, 158–168.

Larsen, N. E., Dursema, H. D., Pollak, C. T., & Skrabut, E. M. (2012). Clearance kinetics of a hylan-based viscosupplement after intra-articular and intravenous administration in animal models. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 100B*, 457–462.

Legré-Boyer, V. (2015). Viscosupplementation: Techniques, indications, results. *Orthopaedics & Traumatology: Surgery & Research, 101*, S101–S108.

Lepetsos, P., & Papavassiliou, A. G. (2016). ROS/oxidative stress signaling in osteoarthritis. *Biochimica et Biophysica Acta - Molecular Basis of Disease, 1862*, 576–591.

Lindenhayn, K., Heilmann, H. H., Niederhausen, T., Walther, H. U., & Pohlenz, K. (1997). Elimination of tritium-labelled hyaluronic acid from normal and osteoarthritic rabbit knee joints. *European Journal of Clinical Chemistry and Clinical Biochemistry, 35*, 355–363.

Lindqvist, U., Tolmachev, V., Kairemo, K., Aström, G., Jonsson, E., & Lundqvist, H. (2002). Elimination of stabilised hyaluronan from the knee joint in healthy men. *Clinical Pharmacokinetics, 41*, 603–613.

Loeser, R. F., Goldring, S. R., Scanzello, C. R., & Goldring, M. B. (2012). Osteoarthritis: A disease of the joint as an organ. *Arthritis and Rheumatism, 64*, 1697–1707.

Luan, F., Wei, L., Zhang, J., Mi, Y., Dong, F., Li, Q., et al. (2018). Antioxidant activity and antifungal activity of chitosan derivatives with propane sulfonate groups. *Polymers, 10*, 395.

Madkhali, A., Chernos, M., Grecov, D., & Kwok, E. (2016). Osteoarthritic synovial fluid rheology and correlations with protein concentration. *Biorheology, 53*, 111–122.

Marcon, P., Marsich, E., Vetere, A., Mozetic, P., Campa, C., Donati, I., et al. (2005). The role of galectin-1 in the interaction between chondrocytes and a lactose-modified chitosan. *Biomaterials, 26*, 4975–4984.

Marsich, E., Travan, A., Donati, I., Turco, G., Kulkova, J., Moritz, N., et al. (2013). Biological responses of silver-coated thermosets: An in vitro and in vivo study. *Acta Biomaterialia, 9*, 5088–5099.

Mendoza, G., Alvarez, A. I., Pulido, M. M., Molina, A. J., Merino, G., Real, R., et al. (2007). Inhibitory effects of different antioxidants on hyaluronan depolymerization. *Carbohydrate Research, 342*, 96–102.

Migliore, A., Massafra, U., Bizzi, E., Tormenta, S., Cassol, M., & Granata, M. (2014). Duration of symptom relief after intra-articular injection of hyaluronic acid combined with sorbitol (anti-OX-VS) in symptomatic HIP osteoarthritis. *International Journal of Immunopathology and Pharmacology, 27*, 245–252.

Mora, J. C., Przkora, R., & Cruz-Almeida, Y. (2018). Knee osteoarthritis: Pathophysiology and current treatment modalities. *Journal of Pain Research, 11*, 2189–2196.

Mu, H., Zhang, A., Zhang, W., Cui, G., Wang, S., & Duan, J. (2012). Antioxidative properties of crude polysaccharides from Inonotus obliquus. *International Journal of Molecular Sciences, 13*, 9194–9206.

Mueller, M. B., & Tuan, R. S. (2011). Anabolic/catabolic balance in pathogenesis of osteoarthritis: Identifying molecular targets. *PM&R, 3*, S3–S11.

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology, 11*, 723–737.

Nicholls, M., Manjoo, A., Shaw, P., Niazi, F., & Rosen, J. (2018). Rheological properties of commercially available hyaluronic acid products in the United States for the treatment of osteoarthritis knee pain. *Clinical Medicine Insights Arthritis and Musculoskeletal Disorders, 11* 1179544117751622.

Olasehinde, T. A., Mabinya, L. V., Olaniran, A. O., & Okoh, A. I. (2019). Chemical characterization of sulfated polysaccharides from Gracilaria gracilis and Ulva lactuca and their radical scavenging, metal chelating, and cholinesterase inhibitory activities. *International Journal of Food Properties, 22*, 100–110.

Parameswaran, N., & Patial, S. (2010). Tumor necrosis factor-a signaling in macrophages. *Critical Reviews in Eukaryotic Gene Expression, 20*, 87–103.

Paździor, M., Kiełczykowska, M., Kurzepa, J., Luchowska-Kocot, D., Kocot, J., & Musik, I. (2019). The oxidative stress in knee osteoarthritis patients. An attempt of evaluation of possible compensatory effects occurring in the disease development. *Medicina (Kaunas, Lithuania), 55*, 150.

Pezron, E., Leibler, L., Ricard, A., Lafuma, F., & Audebert, R. (1989). Complex formation in polymer-ion solution. 1. Polymer concentration effects. *Macromolecules, 22*, 1169–1174.

Piccirilli, E., Oliva, F., Murè, M. A., Mahmoud, A., Foti, C., Tarantino, U., et al. (2016). Viscosupplementation with intra-articular hyaluronic acid for hip disorders. A systematic review and meta-analysis. *Muscles, Ligaments and Tendons Journal, 6*, 293–299.

Pinto, S., Rao, A. V., & Rao, A. (2008). Lipid peroxidation, erythrocyte antioxidants and plasma antioxidants in osteoarthritis before and after homeopathic treatment. *Homeopathy, 97*, 185–189.

Porcheray, F., Viaud, S., Rimaniol, A. C., Léone, C., Samah, B., Dereuddre-Bosquet, N., et al. (2005). Macrophage activation switching: An asset for the resolution of inflammation. *Clinical and Experimental Immunology, 142*, 481–489.

Rafael, D., Andrade, F., Martinez-Trucharte, F., Basas, J., Seras-Franzoso, J., Palau, M., et al. (2019). Sterilization procedure for temperature-sensitive hydrogels loaded with silver nanoparticles for clinical applications. *Nanomaterials (Basel), 9*.

Rani, N., Sabbioni, G., Mazzotta, A., Rocchi, M., Stagni, C., Filanti, M., et al. (2016). Infiltrative therapy as conservative treatment in HIP osteoarthritis: A literature review. *HIP International, 26*, S8–S13.

Rodríguez-Vázquez, M., Vega-Ruiz, B., Ramos-Zúñiga, R., Saldaña-Koppel, D. A., & Quiñones-Olvera, L. F. (2015). Chitosan and its potential use as a scaffold for tissue engineering in regenerative medicine. *BioMed Research International, 2015*, 821279.

Sacco, P., Furlani, F., Cok, M., Travan, A., Borgogna, M., Marsich, E., et al. (2017). Boric acid induced transient cross-links in lactose-modified chitosan (Chitlac).

*Biomacromolecules, 18*, 4206–4213.
Salamanna, F., Giavaresi, G., Parrilli, A., Martini, L., Nicoli Aldini, N., Abatangelo, G., et al. (2019). Effects of intra-articular hyaluronic acid associated to Chitlac (arty-duo-R) in a rat knee osteoarthritis model. *Journal of Orthopaedic Research, 37*, 867–876.
Schreck, R., Rieber, P., & Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *The EMBO Journal, 10*, 2247–2258.
Scognamiglio, F., Travan, A., Borgogna, M., Donati, I., & Marsich, E. (2020). Development of biodegradable membranes for the delivery of a bioactive chitosan-derivative on cartilage defects: A preliminary investigation. *Journal of Biomedical Materials Research Part A, 108*, 1534–1545.
Stern, R., Kogan, G., Jedrzejas, M. J., & Soltes, L. (2007). The many ways to cleave hyaluronan. *Biotechnology Advances, 25*, 537–557.
Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., & Suttles, J. (2005). Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *Journal of Immunology, 175*, 342.
Sultankulov, B., Berillo, D., Sultankulova, K., Tokay, T., & Saparov, A. (2019). Progress in the development of chitosan-based biomaterials for tissue engineering and regenerative medicine. *Biomolecules, 9*.
Tomihata, K., & Ikada, Y. (1997). In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. *Biomaterials, 18*, 567–575.
Torsteinsdóttir, I., Håkansson, L., Hällgren, R., Gudbjörnsson, B., Arvidson, N. G., & Venge, P. (1999). Serum lysozyme: A potential marker of monocyte/macrophage activity in rheumatoid arthritis. *Rheumatology, 38*, 1249–1254.
Travan, A., Marsich, E., Donati, I., Foulc, M. P., Moritz, N., Aro, H. T., et al. (2012). Polysaccharide-coated thermosets for orthopedic applications: From material characterization to in vivo tests. *Biomacromolecules, 13*, 1564–1572.
Wojdasiewicz, P., Poniatowski, Ł. A., & Szukiewicz, D. (2014). The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators of Inflammation, 2014*, 561459.
Zheng, Y., Yang, J., Liang, J., Xu, X., Cui, W., Deng, L., et al. (2019). Bioinspired hyaluronic acid/phosphorylcholine polymer with enhanced lubrication and anti-inflammation. *Biomacromolecules, 20*, 4135–4142.