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In-bioreactor ultrasonic monitoring of 3D culture human engineered cartilage

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ABSTRACT

Engineered cartilage tissue is one of the most promising treatments for articular cartilage defects. In this study, a bioreactor was designed to implement a non-invasive real-time monitoring of the neo-cartilage tissue formation processes through ultrasonic signal analysis. Polylactic acid (PLA) scaffolds were printed and seeded with human chondrocytes. Then, they were cultured in an ultrasound (US)-integrated bioreactor. The readings from the ultrasonic sensors were analyzed by numerical models of the ultrasound-tissue interaction and by a stochastic treatment to infer the extracellular matrix (ECM) evolution. To reconstruct the velocity and attenuation from the recorded signals, a genetic-algorithm based inverse problem (IP) was combined with an iterative computational propagation. The ultrasonic data were validated against evolution measurements of the *in vitro* 3D chondrocyte cultures assessed by proliferation and morphological observations, qualitative and quantitative biochemical parameters and gene expression analysis. Parameters reconstructed from the ultrasonic monitoring (p-wave velocity, attenuation, density changes in the culture layer) were proved useful to indirectly determine cell culture proliferation parameters in a non-invasive manner. The significant correlation shown between glycosaminoglicans (GAG) and collagen II (Col II) expression with the elastic damping evolution of the novo ECM ($R = 0.78$; $p < 0.001$) and ($R = 0.57$; $p < 0.01$), respectively, reinforces the feasibility of using ultrasound to evaluate chondrocyte functionality. Consequently, US can be used to monitor chondrocyte proliferation and ECM formation in the context of 3D cartilage engineering.

1. Introduction

Given the poor self-healing capacity of articular cartilage, joint surface injuries are difficult to restore, leading to osteoarthritis (OA). OA is a chronic degenerative disease of the joint with limited treatment options [1]. Current research focuses on regenerative therapies such as the development of cell-based therapies. However, *in vitro* monolayer chondrocyte expansion is associated with chondrocyte dedifferentiation that results in the production of a fibrocartilage matrix with inferior mechanical properties, and a decreased expression of chondrogenic markers [2]. In fact, cartilage extracellular matrix (ECM) is a complex interwoven meshwork, with Col II and GAG as main components, which promotes chondrocyte interactions and contribute to maintain the chondrocyte phenotype. Thereby, cells deprived of ECM molecules rapidly undergo senescence or apoptosis [3]. In order to counteract chondrocyte dedifferentiation, 3D culture has been used to mimic the native tissue structure [4,5]. Therefore, in tissue engineered constructs both quality control properties and composition are essential elements to take into account in the neo-cartilage fabrication process [6–8]. The control of tissue engineered constructs

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before implantation should use non-invasive and non-destructive evaluation methods while maintaining a sterile environment. In this sense, the use of ultrasound (US) has gained greater attention as a non-invasive method to evaluate and monitor the engineered tissue [9,10].

Ultrasound consists in an acoustic energy wave that transmits acoustic pressure waves beyond the range of human hearing [11]. The proportion of sound waves absorbed by a specific tissue is directly related to that tissue density, compressional/elastic properties, as well as the acoustic scattering, which in turn depends on the ECM formation [12–14]. Based on US properties, we present here the application of this technique for monitoring the evolution of cartilage tissue regeneration in 3D polylactic acid (PLA) scaffolds seeded with chondrocytes isolated from OA patients.

On the other hand, bioreactors offer many advantages over traditional cell culture methods, such as tissue-flask or petri dishes. For instance, bioreactors enable large-scale 3D culture of cells mimicking the natural tissue development and, therefore, improving the quality of engineered tissues. Furthermore, this culture system, in which many parameters (such as medium perfusion rate; temperature; pressure; oxygen concentration. . .) can be controlled, is a powerful tool to perform studies aimed to reveal fundamental mechanisms of cell function in a 3D environment. Moreover, by automating and standardizing tissue manufacture in controlled closed systems, bioreactors facilitate the production of tissue constructs in reproducible and controlled settings with low contamination risk [15]. Here, we have combined the advantages of both technical devices and have designed a new bioreactor that allows ultrasonic real time monitoring of the biomechanical and biochemical properties of human chondrocyte-seeded 3D printed PLA-based scaffolds. Hence, we have created a robust and reproducible culture system where US non-invasive monitoring of the ingrowth tissue can be performed.

Throughout this study, a set of biomechanical viscoelastic properties of the culture were determined in real time. In addition, due to the nature of US waves, the measurements were also analyzed in terms of model parameters (M): p-wave modulus (M) and attenuation.

This study has focused on the potential of interpreting the biological phenomena inherent to a 3D cell culture through US waves analyzed by a signal processing based on the inverse problem methodology.

2. Material and methods

2.1. Cell culture

Human articular chondrocytes were isolated as previously described [4] Chondrocytes were grown in DMEM–high glucose (Sigma) Supplemented with 10% fetal bovine serum (Gibco), 50 μg/μL of l-ascorbic acid 2-phosphate (Sigma), 1% penicillin-streptomycin (Sigma) and 1% ITS (Gibco) in a 25-cm^2 cell culture flask. Cells were incubated at 37 °C humidified atmosphere containing 5% CO_2 and expanded in a monolayer for 7–10 days before the experiment.

2.2. Printed scaffold procedure

Printed scaffolds were designed with the support of BRECA Health Care Company and printed with Regemat 3D V1 bioprinter (REGEMAT 3D, Granada, Spain), based on PLA material with 1.25 g/cm^3 of density and 45 MPa of yield strength and 500 μm of pore size. Then, 50% of porosity was considered to tradeoff between the stiffness and the chondrocytes bond ability as previously shown [16]. Finally, the size of the square samples was manufactured with 4.5 mm of thickness and 17.5 mm of length and was designed with the purpose of facilitating the access to the interior of the ultrasonic bioreactor as it is shown in (Fig. 1A, B), with a magnetic piece to ensure a stable and robust support of the scaffold (Fig. 1C).

2.3. Chondrocyte culture in 3D printed PLA-based scaffolds

Printed PLA-based scaffolds were sterilized by immersion in 70% ethanol aqueous solution for 1 h, washed several times in phosphate buffered saline (PBS) and then subjected to ultraviolet light for 20 min each size. Then, scaffolds were immersed in complete medium overnight before cells were seeded. Human chondrocytes suspension containing 100.000 cells in 200 μl of medium was slowly dropped onto the surface of each scaffold and incubated in 6 well plates for 2 h at 37 °C. After that, 5 ml of fresh medium was added to each well plate. After 24 h, the scaffolds were introduced into the bioreactor and cells were monitored for 4 weeks. For biological characterization, chondrocytes-seeded scaffolds were handled identically to the US monitored specimens in a parallel twin bioreactor (n = 6).

2.4. Ultrasonic monitoring bioreactor

A “piano” shaped bioreactor was constructed of (Polymethyl methacrylate) (PMMA) material (Fig. 1D–E), with 1 MHz US transmission technology composed of a pair of Olympus transducers excited by an ultrasonic sine-burst in an Olympus pulse generator at a central frequency of 1 MHz and 10 V amplitude. The ultrasonic pulse was emitted once every 2 min to ensure that the amount of energy does not interfere in the cell culture as in [9]. Moreover, ultrasound low frequency was used. A Cleverscope oscilloscope was synchronized with an acquisition card from a PC *via* Matlab Software in-lab developed code, during 4 weeks. The biological process was monitored at regular intervals of 2 min, and the medium was automatically dispensed 3 times per day at a rate of 40 ml/week. Fig. 1F–G illustrates the employed electronic setup to conduct the experiment, while (Fig. 2A) shows the framework before launching the experiment.

The bioreactor was sterilized by means of both ultraviolet and an alcohol cleaning of all its pieces. Automatic nutrition was implemented via Arduino-controlled peristaltic pumps to keep a constant nourishment rate in the chondrocyte culture along the month of analysis. An intermittent flow was imposed in order not to interfere the cell settlement. These elements were assembled and configured as detailed below (Figs. 1D–G and 2A, B). Three identical, but independent experiments were carried out to assure the repeatability and statistical relevance of the outcomes. Two scaffolds seeded with chondrocytes from different patients were monitored per experiment (n = 6).

2.5. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories) was used for the determination of cell viability. Cell growth was analyzed at different time points: 0 day, 1 day, 3 days, 1 week, 2 weeks and 4 weeks. Scaffolds cultured inside the bioreactor were placed into a 6 well plate, incubated with 10 μl of CCK-8 solution per each 100 μl of media and incubated for 3 h at 37 °C. At this time, the absorbance was measured at 450 nm (BioRad Microplate Reader).

2.6. Confocal microscopy

Chondrocyte attachment, spreading and viability on PLA scaffold incubated into the bioreactor were assessed at 1 and 4 weeks using confocal microscopy. The attachment and spreading of human chondrocytes were analyzed using phalloidin-RED

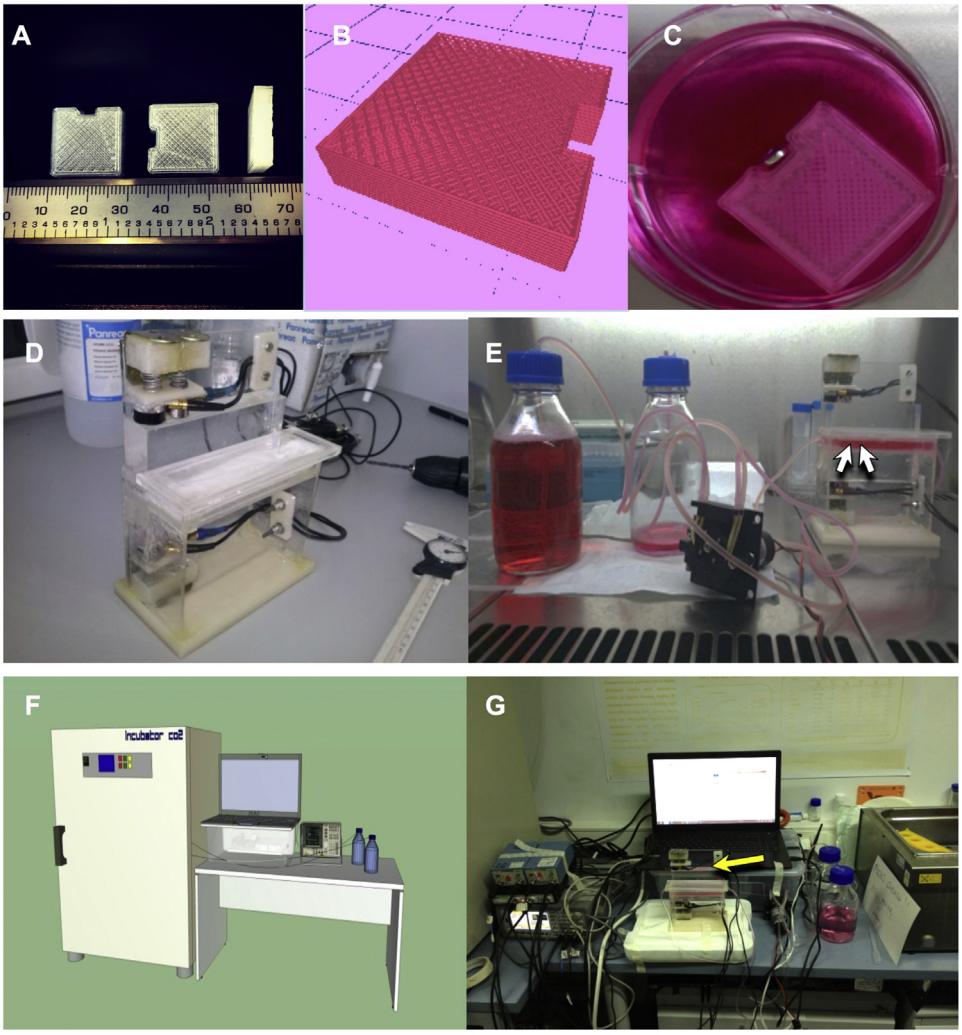

Fig. 1. Printed scaffold and bioreactor configuration. A- 3D printed scaffolds. B- 3D scaffold created with REGEMAT 3D designer software. C- 3D printed scaffold with a magnetic piece. D- Ultrasonic bioreactor. E- Ultrasonic bioreactor configuration with a peristaltic bomb to advantage nutrient flux inside the laminar chamber. F- Three dimensional representation of the experimental setup in a simulated laboratory. G- Bioreactor and electronical setup outside the incubator. White arrows indicate the localization of the scaffold and yellow arrows point to the bioreactor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to observe the expression of F-actin. For this purpose, samples were fixed in 4% paraformaldehyde for 20 min, rinsed twice in PBS and permeabilized using 0.1% Triton X-100 for 15 min. The scaffolds were pre-incubated with 1% bovine serum albumin (BSA) for 30 min and, then, incubated with phalloidin-RED for 20 min at room temperature (RT) followed by PBS washing and stained with DAPI for 5 min. The viability of chondrocytes cultured on PLA scaffolds was detected by live and dead staining using CellTracker (Green/CMFDA) and propidium iodide (PI), respectively. Briefly, samples were washed two times with PBS and incubated for 15 min at 37 °C with CellTracker Green/CMFDA (Invitrogen) and 25 μg/mL PI. Afterwards, samples were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT and stained with DAPI. For collagen II (Col II) and collagen I (Col I) staining, samples were blocked for 1 h at RT with 5% BSA in PBS and, then, incubated with Col II (SC52658, St. Cruz) and Col I (SC25974, St. Cruz) overnight at 4 °C. The next day, samples were washed thrice with PBS and incubated with the secondary antibody (St. Cruz) for 1 h at RT and, finally, were washed thrice with PBS and staining with DAPI. Cells were photographed by confocal microscopy (Nikon Eclipse Ti-E A1, USA) and analyzed using NIS-Elements software.

2.7. Environmental scanning electron microscope (ESEM) analysis

Described in detail in Supplementary data.

2.8. Biochemical analysis

For total glycosaminoglycans (GAG) and Col II analysis, *in vitro* cultured scaffold were rinsed in PBS and digested in proteinase K (1 mg/mL in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A; Sigma-Aldrich) for 16 h at 56 °C. Total GAG content was measured by the sample absorbance using dimethylmethylene blue (DMMB) spectrophotometric assay at 525 nm with chondroitin sulfate as the standard. Then, total GAG content in each construct was calculated according to the standard curve. Col II was measured using the Col II Detection Kit (Chondrex Inc). For normalization, DNA content in each construct was determined by spectrofluorometer using DAPI at 460 nm. All experiments were carried out in triplicate.

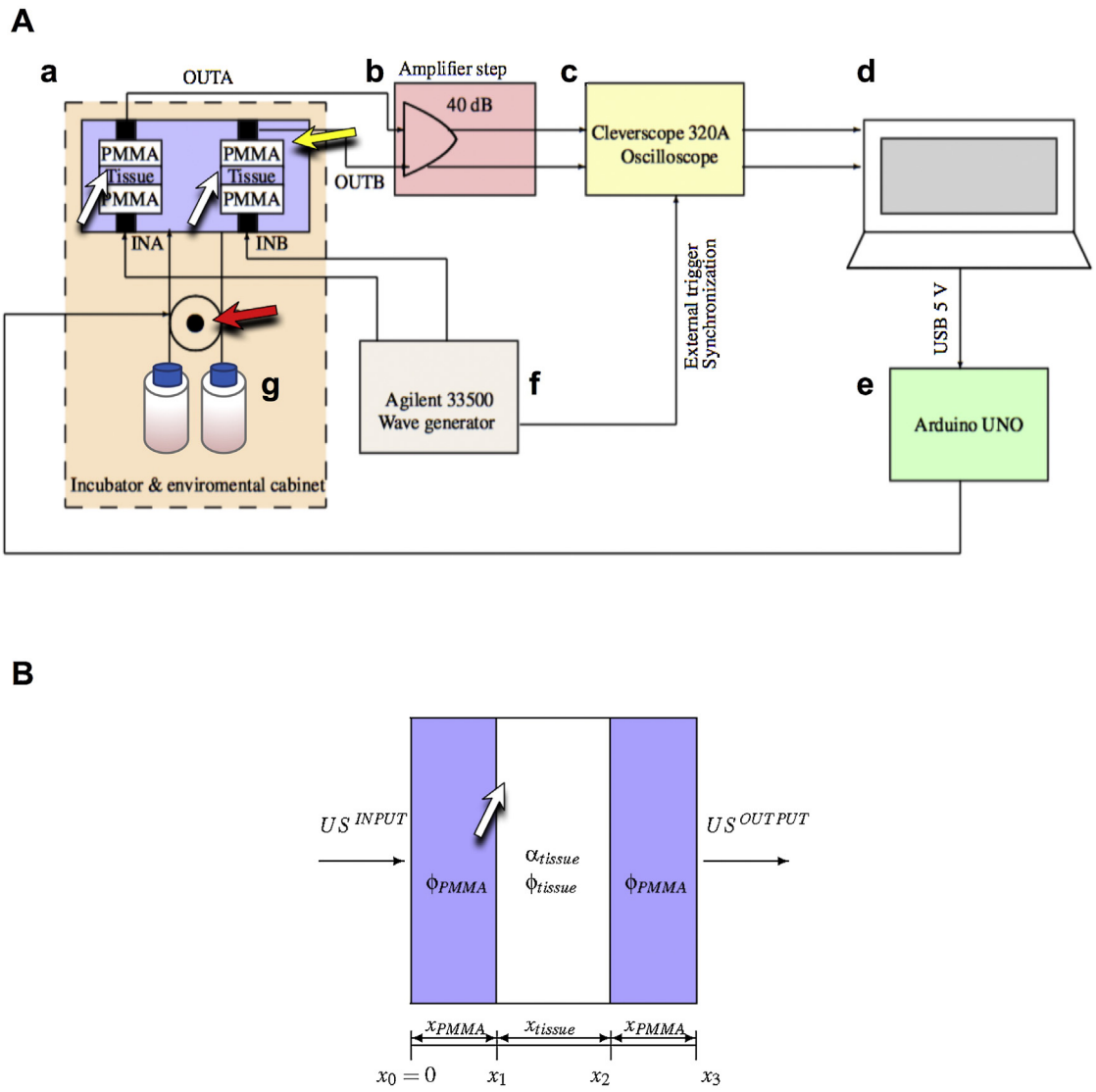

Fig. 2. Schematic representation of the bioreactor configuration and the multilayer propagation. A- Electronic configuration of the experiment with a brief explanation of the programming framework. (a) Incubator where the ultrasonic bioreactor is located, (b) amplifier step of 40 db used in the process of recording ultrasonic signals to improve the resolution via two cables OUTA and OUTB from channels A and B, respectively, (c) oscilloscope synchronized with the computer, (d) personal computer where the measurements were stored, programed, and emitted simultaneously to the synchronization of Arduino, (e) Arduino system configured to keep the peristaltic pump set up to supply the nutrients of the culture, (f) wave generator hardware to emit into channels via INA and INB cables with the configuration desired in the program, (g) peristaltic pump, bottles of nutrient and waste joined by sterile tubes. B – Multilayer propagation of the US wave through the bioreactor. US^{INPUT}: ultrasonic wave emission, ϕ_{PMMA}: delay generated by the PMMA material, α_{tissue}: decay of the US wave into the tissue, ϕ_{tissue}: delay generated by the tissue, US^{OUTPUT}: ultrasonic wave obtained after the multilayer propagation, x_{PMMA}: thickness of the PMMA layers, x_{tissue}: thickness of the tissue layers, white arrow: localization of the scaffold, yellow arrow: point to the bioreactor, and red arrow: indicate peristaltic pump set up. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. RNA isolation and real time-PCR analysis

Total cellular RNA was isolated, turned to cDNA and Real-time PCR was performed as described in Supplementary data. Primer sequences used are summarized in Table S1 (Supplementary data).

2.10. Inverse problem method background

The proposed methodology based on Inverse Problem (IP) consists of a stochastic or random model-selection used to rank which of the models are more plausible to explain the elastic wave propagation in the system and for the reconstruction of the relevant mechanical parameters during the culture reaction time. Basic concepts and formulation regarding probabilistic IP are described elsewhere [17–19]. According to these studies, a set of model parameters (MP) is related to the plausibility of a model values to be true. Statistical inference theory is used to include any information about the experimental observations and the model parameters. Then, the information about the relationship between them is computed to obtain an estimation of the plausibility of the selected process. To obtain the optimal model parameters (mainly damping and US propagation speed), firstly an initial guess is performed at the beginning of the process by genetic algorithms (GA) as a full-range random search technique. Then, as the evolution of the parameters is expected to be minor, the Broyden-Fletcher-Goldfarb-Shanno (BFGS) genetic algorithm is used as a local search in the proximity of the initial point detected by the GA (See Supplementary data).

Firstly, an initial guess is performed at the beginning of the process by genetic algorithms as a full-range random search technique.

Then, as the evolution of the parameters is expected to be small, the BFGS algorithm is used as a local search in the proximity of the initial point. In this way, it is possible to find which is the best model and which are the MP that best fits the experimental data.

As possible models, here we propose two models of energy dissipation: viscous and hysteretic. Both of them are applied in this study in order to understand the interaction between ultrasound and tissue, as they provided great performance in describing a gelation process [17]. The damping is defined in terms of the wave modulus M, which is modified from the undamped one M^0 to generate a dispersive one, which is a frequency-dependent complex modulus $M(\omega)$, where ω is the angular frequency if the modulus dispersion is represented in the frequency domain. The viscoelastic model is defined in terms of the frequency-dependent loss factor η, obtained as the ratio comprised between loss and storage modulus (see Eq. (1)), whereas the hysteretic damping (ξ) is expressed as a frequency-independent damping (Eq. (2)).

$$M(\omega) = M^0(1 - i\omega\eta) \quad (1)$$

$$M(\omega) = M^0\left(1 - i\xi\right) \quad (2)$$

Where η and ξ are the viscoelastic and hysteretic damping coefficients of tissue, respectively. Finally, it is worth pointing out the feasibility of the interpretation of these model parameters from the physical standpoint, as well as the reduced number of free parameters involved in the model.

After acquisition, the signals involved in the experiments have been normalized with respect to the peak amplitude that has been applied, considering that the system must be insensitive to changes in signal amplitude. We have also corrected a few initial offset by forcing a 0 V initial amplitude value of the US signal. Secondly, the signals have been multiplied by a rectangular window in order to delimit only the part of the signal related to the tissue layer. The main reason of this windowing is the input US wave crossing a multilayer material as is detailed in (Fig. 2B). Then, the output US signal has been registered with a transducer after crossing the three layers (with them 455 respective properties [20]) of the multilayer framework (See Supplementary data).

2.11. Statistical analysis

Data were analyzed with Matlab software program. All graphed data represent the mean ± SD from at least three experiments. The statistical analysis was tested using the two tailed Studentís *T* test. Assumptions of Studentís *T* test (homocedasticity and normality) were performed and assured by using transformed data sets log (dependent variable value + 1) when necessary. All data are presented as mean ± 95% confidence interval (CI) of three independent experiments and were deemed statistically significant for P-values < 0.01 (**) and the correlation analysis with Pearson's correlation coefficient henceforth named as *R*.

3. Results

3.1. Cell distribution and cell viability within 3D printed PLA-based scaffolds

Cells cultured in PLA scaffolds for 1 or 4 weeks were stained for actin filaments with fluorescent phalloidin. As can be appreciated in (Fig. 3A–B) fluorescence intensity increased and becomes more widely distributed after 4 weeks of culture. Phalloiding staining showed that chondrocytes attach, migrate and proliferate covering the whole surface of the scaffolds (Fig. 3B). In addition, cell viability was analyzed by CTG showing similar cell viability after 1 week (Fig. 3C) when compared with cell-bearing scaffolds maintained for 4 weeks (Fig. 3D, Supplementary video). After 4 weeks of growth into the bioreactor, chondrocytes acquired a wider and more confluent distribution within the scaffolds (Fig. 3D). Furthermore, ESEM demonstrated that chondrocytes cultured within the scaffolds presented the typical polygonal shape morphology of chondrocytes, and were firmly attached to the scaffold surface by forming pseudopodia- and filopodia-like extensions (Fig. 3E–F).

Table 1
Mathematical plausibility of each model explanation of the process in terms of damping types 1 Fractional, 2 Hysteretic and 3 Viscoelastic based on P(C) (probability in pertentage), Occam in(-log10 scale) and Certainly (log10 scale) criteria.

Model	P(C) (%)	Occam (-log10)	Certainty (log10)
1 (Fractional)	18.67	1.45	0.21
2 (Hysteretic)	38.16	2.49	0.32
3 (Viscolelastic)	43.17	3.68	0.56

3.2. Extracellular matrix formation

Production of ECM components such as Col II and GAG was quantified during 4 weeks using Col II quantification kit and dimethylmethylene blue assay, respectively. After 3 weeks of *in vitro* culture in PLA scaffolds, human chondrocytes produced a network of GAG and Col II (Fig. 4A), which are the main cartilage-specific ECM components. The GAG content significantly increased until the third week ($p < 0.01$) and, then, slightly decreased; while Col II expression highly increased between first and third week ($p < 0.01$) but a significant drop was observed after 4 weeks of culture (Fig. 4A).

In addition, qPCR analysis showed a significant increase of Sox9 ($p < 0.01$) and aggrecan (ACAM) ($p < 0.01$) gene expression in chondrocytes cultured for 4 weeks when compared with chondrocytes cultured for 1 week (Fig. 4B). Moreover, the expression of cartilage oligomeric matrix protein (COMP) showed similar values at the beginning and at the end of the experiment, while lower expression of Col I ($p < 0.01$) and Col II ($p < 0.01$) was observed at the fourth week compared with the first week (Fig. 4B). The ratio of mRNA levels of collagen type II to I (Col II/Col I) showed the differentiation status of chondrocytes (Fig. 4C).

Furthermore, immunofluorescence analysis of Col II expression showed a high staining of collagen fiber framework after 1 week of culture, in agreement with the qPCR data (Fig. 4C). On the other hand, fluorescence signal of Col II decreased after 4 weeks in culture and was restricted to certain areas of the scaffold while expression of Coll I was enhanced (Fig. 4C and D). In addition, chondrocytes cultured in the scaffold for four weeks showed a flattened fibroblast-like morphology (Fig. 4D).

3.3. Ultrasound measurements and analysis

As explained in methodology section, the viscoelastic model was determined as the most plausible, named P(C) in Table 1, followed by the hysteretic one *via* Inverse Problem (IP) with a probabilistic approach (see Supplementary material). Fig. 5A–C shows in terms of percentage the difference between channels (or samples monitored) and experiments. Each plot, from A until C are similar in percentage ranges and also in terms of trends depending on the day of culture. The variability between models could be interpreted as a difference between plausibility, note that the likehood of hysteretic and viscoelastic is almost the same as Fig. 5B–C shows. The small percentage of change of p-wave modulus between experiments could be explained since the origin of samples were obtained from different patients, however there is a common pattern in tends increasing the first week, and decreasing the rest of the time of culture with the same slope. Table 1 summarizes the p-wave modulus derived from the IP mathematical formulation with the viscoelas-

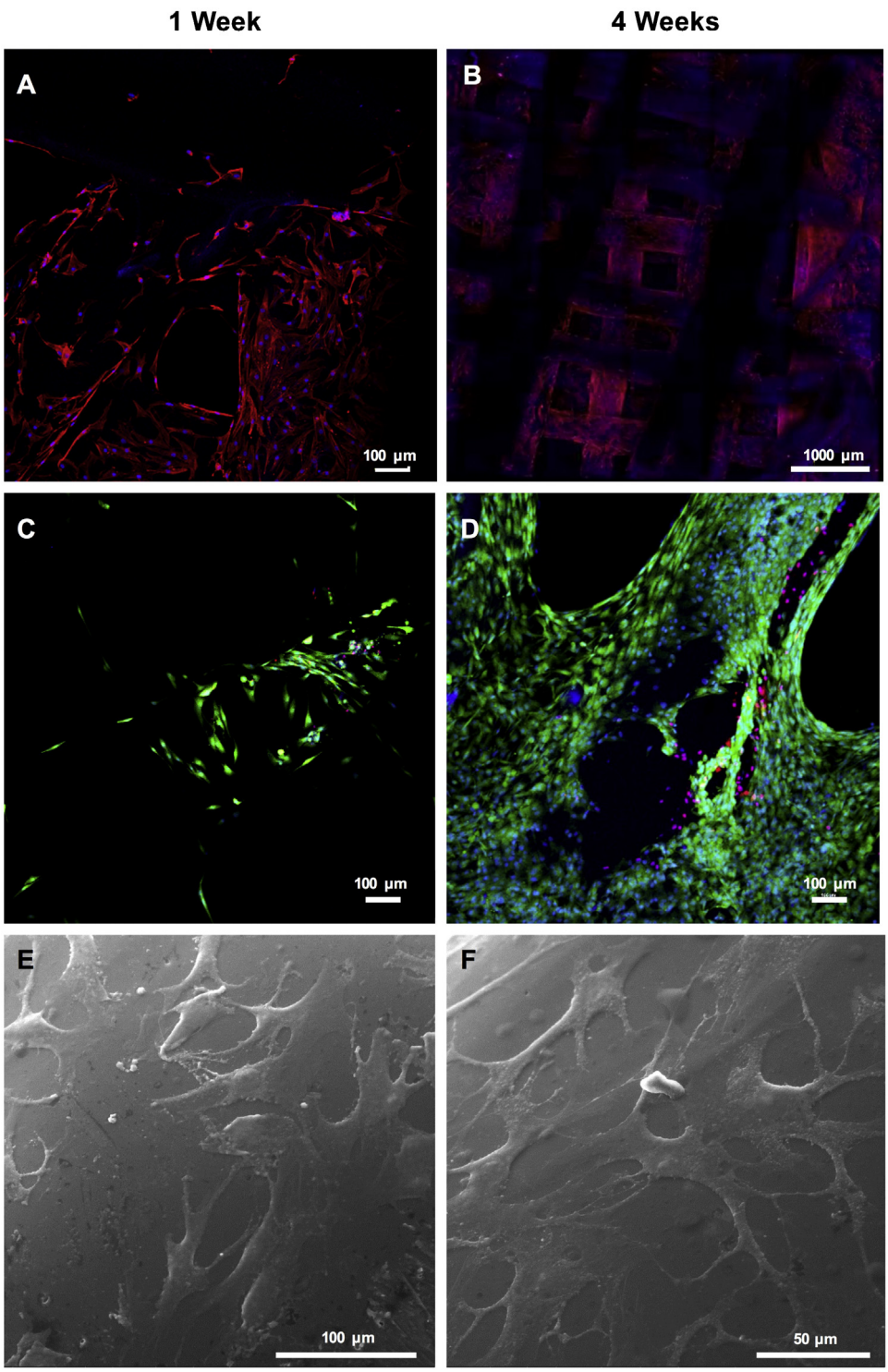

Fig. 3. Primary human chondrocytes seeded in 3D printed-based PLA scaffolds and cultured inside the bioreactor for 1 and 4 weeks. A-B – Confocal microscopy images showing F-actin stained with Red-phalloidin and the nucleus stained with DAPI (blue). C-D- Confocal microscopy images of live cells stained with green fluorescence (CTG) and dead cells identified by red-fluorescent propidium iodide staining. E-F- Scanning electron micrographs of chondrocytes growing on the PLA scaffolds after 1 and 4 weeks. Scale bar indicates 100 μm (A, C, D, E), 1000 μm (B) and 50 μm (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tic model and the hysteretic one in each of the samples, being the more plausible models, for this reason fractional model has been excluded. Both viscoelastic parameters and hysteretic parameters are correlated with ECM components showing a significant p-value in the case of damping and GAG. The Occam and certainly criteria are also considered in −log10 and log10 scales respectively. In both cases viscoelastic is still the most plausible model as in P(C) with 43.17%, the Occam shows the highest value 3.68 and analogously the Certainly with 0.56.

Viability and proliferation of chondrocytes were measured and shows that cell number increased from day 0 until 2 and 3 weeks (Fig. 6A–C). Only in one of the samples (Fig. 6C), proliferation

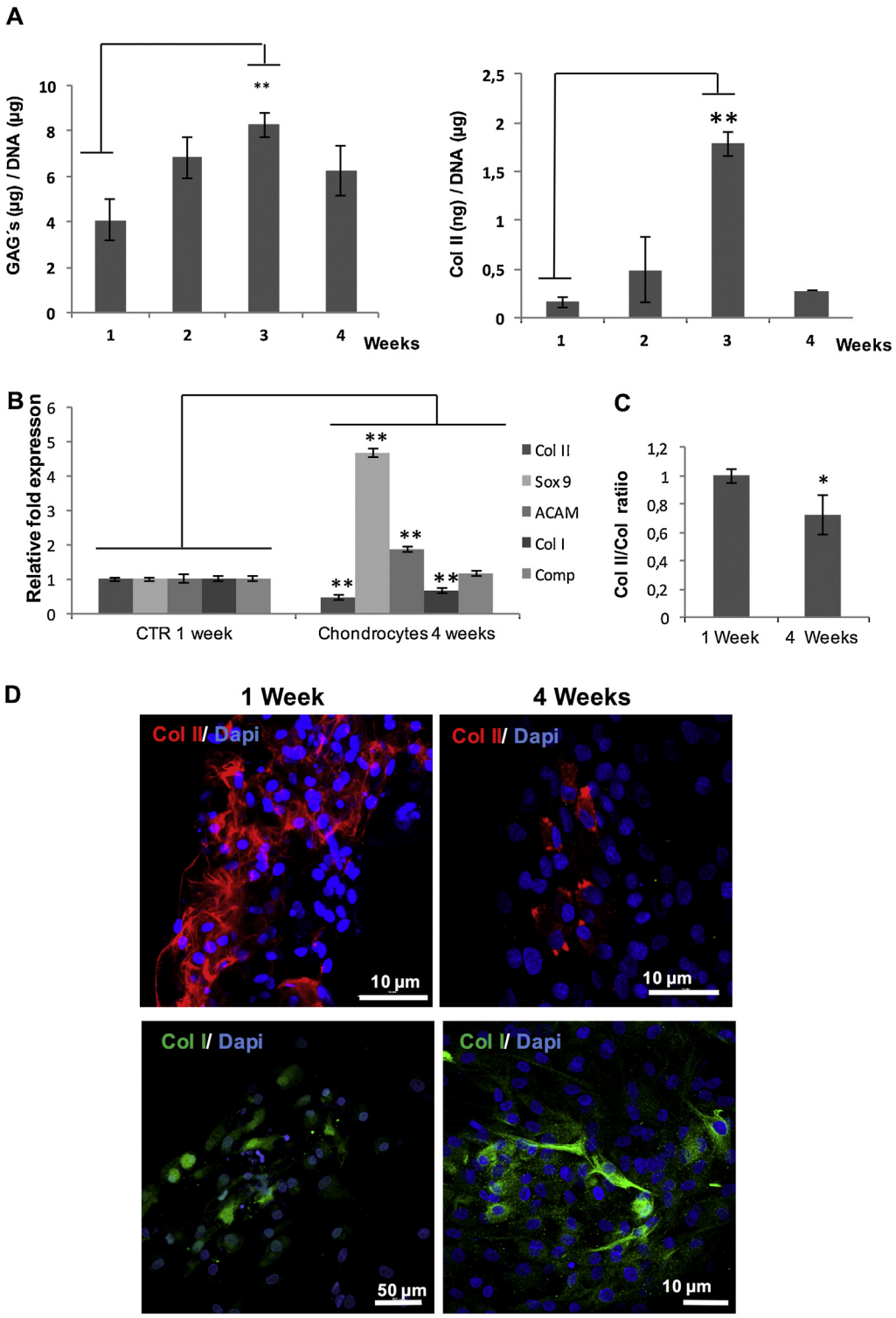

Fig. 4. Cartilage ECM characterization. A- Quantification of Col II and GAG in human primary chondrocytes cultured in PLA scaffolds. B- Real-time PCR analysis of selected chondrogenic markers in chondrocytes cultured into PLA scaffolds for 1 and 4 weeks. All gene expressions were normalized with the values of chondrocytes cultured during 1 week into PLA scaffolds. C- ratio of Col II versus Col I expression measured by real-time, quantitative RT-PCR (qRT-PCR). Results are presented as mean with standard deviation. *P < 0.05; **P < 0.01. D- Confocal images for cartilage-specific Col II staining in red and Col I staining in green can be appreciated in human primary chondrocytes cultured in PLA scaffolds during 1 and 4 weeks. Scale bar indicates 10 and 50 µm, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of chondrocytes decreased after 7 days, this may be due to the variability of the samples isolated from different patients with osteoarthritis. Moreover, cell proliferation curves fit with the range of the p-wave velocity normalization percentage with only a 0,02% of variation (Fig. 6A–C).

A statistical correlation analysis between the parameters from biological tests and those obtained by ultrasound was performed. The statistical analysis was carried out to explore the relationship between the proposed model and the biological parameters tested, and to asses a quantitative interpretation of the damping evolution

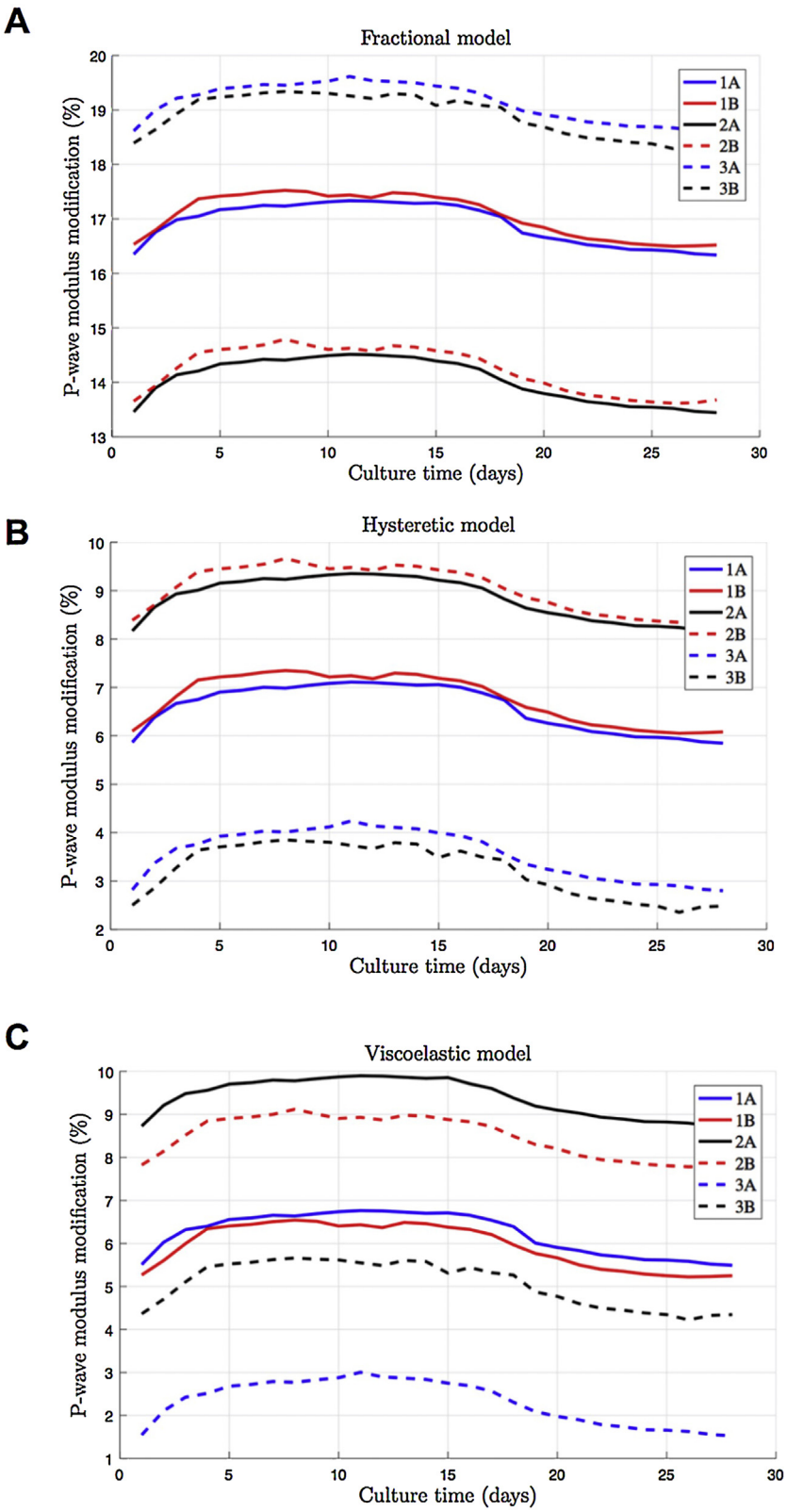

Fig. 5. Mechanical parameters depending on the physical modellization of the process. A- p-wave modulus evolution in terms of percentage for each sample explained with the Fractional model. B- p-wave modulus evolution in terms of percentage for each sample explained with the Hysteretic model. **C-** p-wave modulus evolution in terms of percentage for each sample explained by the Viscoelastic model. Culture time are 28 days in each experiment. The numbers 1, 2 and 3 represent repetitions of the experiment and capitals A and B the channels of the Bioreactor.

of the chondrocytes culture in real time. The correlation between each pair of biological variables and the damping evolution from each mechanical model of our study along with their p-value is shown in Table 2. The GAG concentration was highly correlated with the damping derived from the models $R = 0.78$ with $p < 0.001$ in the case of viscoelastic and $R = 0.7138$ in the case of hysteretic

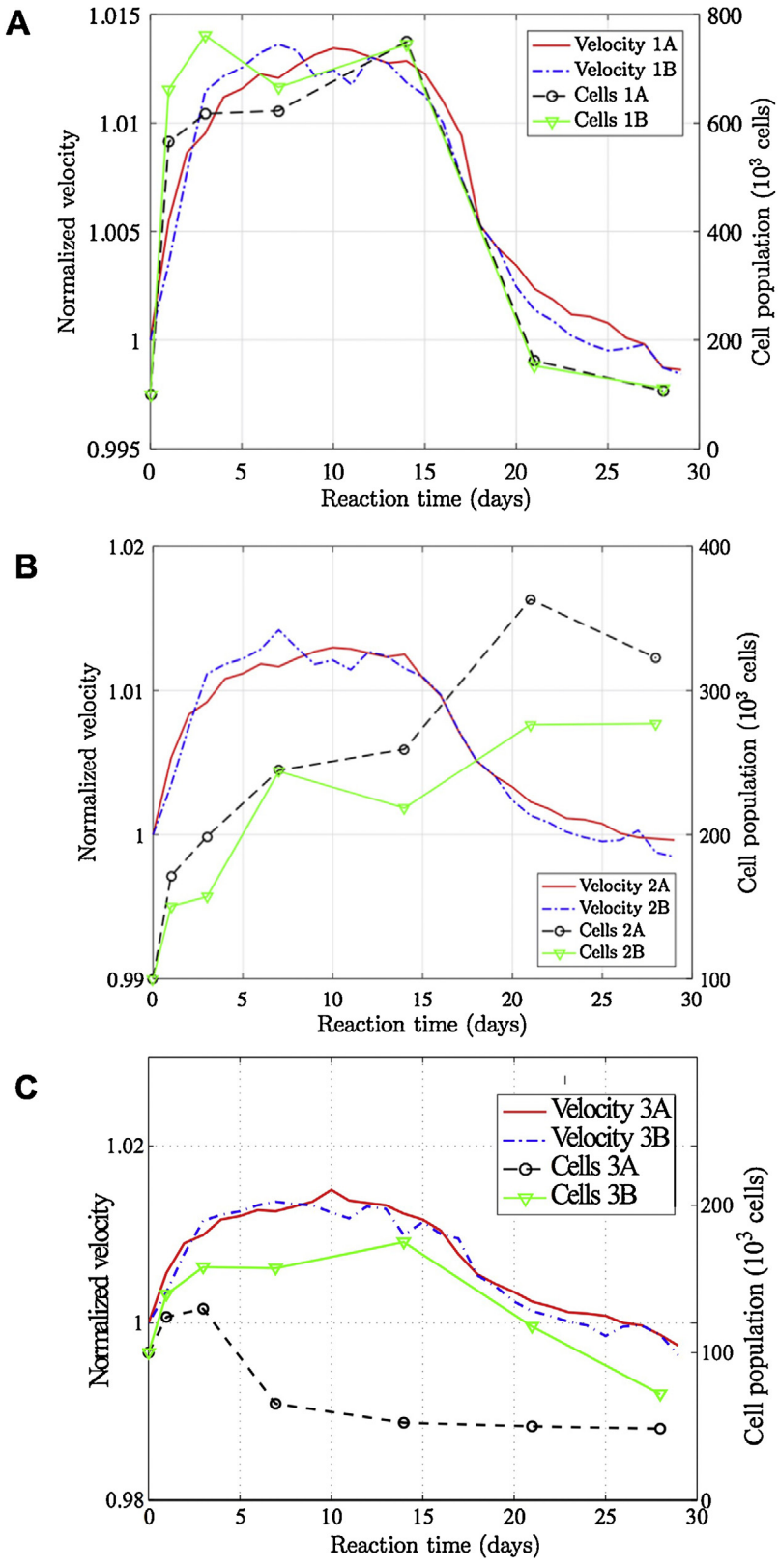

Fig. 6. Ultrasonic speed quantification and cell proliferation. A- Ultrasonic velocity and cell proliferation for the first experiment. B – Ultrasonic velocity and cell proliferation for the second experiment. C – Ultrasonic velocity and cell proliferation estimation for the third experiment. Reaction time is 28 days in each experiment. At the inset, numbers "1, 2 and 3" represent repetitions of the experiment, and the capitals "A" and "B" the channels of the bioreactor where ultrasound signal is recorded. The normalized velocity is calculated from the recorded signals of both channels and represented in the graphic as "velocity". CCK-8 assay was used to examine the cell proliferation of each group, the absorbance was measured at 450 nm (n = 3 per group). Chondrocyte proliferation curves are referred as "cells" at the graphic inset compared with the normalized velocity.

Table 2
Pearson's correlation coefficient R between Col II, shear modulus, attenuation and proliferation rates parameters.

	R	*p−Value*
Col II-*Gviscoelastic*	0.4718	0.5978
Col II-Cell proliferation	0.1881	0.3787
GAG-Cell proliferation	**0.8449**	**«0.0001**
GAG-viscoelastic damping (η)	**0.7828**	**«0.0001**
Col II-viscoelastic damping (η)	**0.5671**	**0.0039**
COLII-*Ghysteretic*	0.1130	0.5999
GAG-hysteretic damping (ς)	**0.7138**	**«0.0001**
COLII-hysteretic damping (ς)	**0.4854**	**0.0162**
GAG-*Gviscoelastic*	0.1332	0.5351
GAG-*Ghysteretic*	0.1349	0.5297
GAG-*Gfractional*	0.1233	0.5658

with $p < 0.001$, which is consistent with our hypothesis regarding that US attenuation is more sensitive to the changes in the medium than the velocity. Cell proliferation was also correlated with the amount of GAG concentration, $R = 0.8449$ with a statistical significant p-value ($p < 0.001$). Moreover, the level of Col II was statistically significant with a considerable correlation of $R = 0.4854$ with a p-value $= 0.0162 < 0.05$ for the case of hysteretic damping model and $R = 0.5671$ and a p-value $= 0.0039 < 0.01$ for the case of viscoelastic damping model. In brief, we found a high dependence between damping evolution and GAG/Col II concentrations, that was explained under the ultrasonic propagation signal.

4. Discussion

Ultrasound has been widely used as a medical tool for both diagnostic and therapeutic purposes [21–24]. In this study, we have proved that US is an effective tool to non-invasively monitor *in vitro* chondrocyte proliferation and ECM production in tissue-engineered constructs and to perform a comprehensive study of cell culture parameters such as compressibility, p-wave modulus and shear modulus. In brief, we used low-energy 1 MHz frequency transmission ultrasound mounted in a bioreactor, and the wave propagation was analyzed by a signal processing based on the IP methodology. In order to reconstruct the velocity and attenuation from the recorded signals, a genetic-algorithm and BFGS-based inverse problem was combined with an iterative computational propagation model based on the transfer matrix formalism through the multilayer mechanical system composed of PLA-tissue-PLA laminates, and then the output signal, after inter-relating all them, was registered. A pool of attenuation models (viscous, hysteretic and fractional time derivative damping) that simulate the US-tissue interactions were evaluated and ranked using a model-class selection formulation to unveil which model best describes the tissue ultrasonic propagation.

US measurements showed that p-wave speed changes were correlated with cell proliferation and ECM production. Thus, cell number and GAG/Col II increment corresponded to higher p-wave speed. From the second until the fourth week in culture chondrocyte proliferation rate decreased. Also, ECM production decreased after the third week, in agreement with other studies that have shown senescence and dedifferentiation of chondrocytes after long culture periods, accompanied by a decreased expression of chondrocyte-specific proteins such as Col II, proteoglycans and glycoproteins [3]. Here we show a decreased expression of collagen II, together with a significant reduction of Col II/Col I ratio which indicate that chondrocytes have initiated a dedifferentiation process [25,26] It is known that dedifferentiated chondrocytes show changes in cell shape [27]. We observed an incremented expression of collagen I in cells that presented fibroblastic-like morphology. Collage I has been shown to regulates fibroblastic matrix gene expression in chondrocytes [27]. Moreover, quantitative analysis showed a diminution of ACAN and collagen II protein expression following three weeks of culture within the scaffolds. Col II and GAGs decrease, main component of the ECM, also affects the mechanical stability of the chondrocytes [28], which in our model was detected by both, the hysteretic and the viscous models, in terms of a decrease in velocity and p-wave modulus. In this sense, the viscosity of the matrix induces a significant change in the stiffness of the culture, which manifests in changes in the velocity of the ultrasonic propagation. The trends were normalized in order to compare ultrasonic velocities with chondrocyte cell population per day. Attenuation from the viscous model strongly depended on the experiment, but its variations showed consistent correlations with proliferation in all the essays. Therefore, we have shown that the parameters derived from the US monitoring (p-wave velocity, attenuation, density changes in the culture layer) were useful to indirectly determine cell culture parameters (compressibility, p-wave modulus and shear modulus) in a non-invasively real time manner.

The applicability of US to study *in vitro* cell culture parameters of tissue engineered cartilage tissue has recently been investigated [23]. Chondrocyte seeded scaffolds were cultured in an US integrated bioreactor with acoustic energy at frequencies around 5 MHz, then a simplified mathematical model based on Biot theory was developed trying to model the propagation of the incident US wave through the scaffolds. Finally, the damping of the US was assessed and the model analysis was connected to cellular responses [23]. Other authors have shown the use of US to non-destructively evaluate the mechanical properties of maturing TE cartilage constructs in a sterile bioreactor [29–31]. Furthermore, our approach adds a new perspective of the IP methodology in this field, which, together with a real time mechanical characterization of the tissue contributes at the acquisition of a consistent dataset. In addition, M. Rice et al. characterized the cartilage matrix evolution in poly (ethylene glycol) (PEG) −based hydrogels with an automated computer system in three times of flight measurements mapping the culture stiffness [9]. The main contribution of our signal processing methodology compare to this study, resides in the power of the IP procedure which methodology provides simultaneously a set of experimental parameters that supports the fit of the viscoelastic model as the most plausible model.

This procedure has not been explored previously and provides a new *criteria* to understand and control the 3D cartilage evolution modellization at real time.

Moreover, it has been shown that scaffold microstructure has a high impact in the development of the new tissue. In fact, current scaffolds for cartilage tissue engineering are often designed with a total porosity of 48–95% and pore diameters ranging from 50 to 500 μm [32]. Here, we printed PLA scaffolds with 500 μm of pore size and 50% of porosity and proved the exponential growth of chondrocyte throughout the scaffold volume until the third week of culture. As previous studies have shown that topography of scaffolds have an influence on ECM remodeling processes and matrix organization [33,34] and can also be validated in biomechanical terms [35], the potential use of ultrasound to quantify cell response to nanoscale geometry would be of great interest for future studies.

5. Conclusion

To advance towards an effective therapy for cartilage repair, TE is demanding new methods to control culture systems. Improved strategies to monitor *in situ,* non-invasively, and in real time chondrocyte proliferation and ECM formation are needed.

Nondestructive tissue quality assurance procedures such as US evaluation is presented as an interesting candidate to ensure the quality and functionality of the formed tissue, as an accurate non-

invasive procedure that can be automated. This enables a significant step for bioprinted treatments to meet the requirements by the regulatory agencies and move from lab bench to bedside. In this vein, we have presented a novel bioreactor system that allows the integrated ultrasound monitoring of 3D scaffolds for controlled formation of cartilage ECM and growth of chondrocytes, which provides a real-time information about human primary chondrocytes functionality in a non-invasive way. In particular, mechanical parameters reconstructed by ultrasound were correlated with the amount of ECM components measured at the scaffolds, which were shown in terms of repeatable and consistent trends over the time in culture and among the cell populations. The mechanical evaluation of the ultrasonic monitoring in terms of p-wave modulus were explained according to fractional, hysteretic and viscous models which reflects a good and statistically significant correlation between attenuation in both models and GAG and Col II. Moreover, the IP procedure provides a set of experimental parameters that generate evidence about the most plausible viscoelastic constitutive models.

Henceforth, bioreactors combined with ultrasound could help, not only *in vitro*, to achieve a controlled environment for a real-time monitoring of the neo-cartilage TE formation processes, but also potentially to monitor, in a clinical context, the treatment effectiveness by evaluating the progression of the patient lesion improvement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.snb.2018.03.152.

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Biographies

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Elena López Ruiz is a PhD at the University of Granada and an Associate professor at the Department of Physiology, Anatomy and Molecular Biology at the University of Pablo de Olavide, Sevilla. She received her PhD in Regenerative Biomedicine in Jaén University (Spain) in 2014. During her PhD she focused on the differentiation of adult stem cell toward cardiac and cartilage linage. She was granted with a Marie Curie fellowship to carry out a research internship at Bath University, UK. Following her PhD, she conducted 6 months research at the Interdisciplinary Nanoscience Center (Inano), at Aarhus University. Her research interests focus on regenerative medicine therapies including the use of decellularized tissue and 3D bioprinting, cell differentiation, and also the treatment of cancer through the study of therapies targeting tumor cancer stem cells.

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Gema Jiménez is a PhD at the Department of Human Anatomy and Embryology of the Faculty of Medicine (University of Granada). In 2016 he defended his PhD in Biomedicine (UGR), focusing on biomedicine regenerative/tissue engineering to treat cartilage lesions. During her PhD she did a pre-doctoral stay of 5 months (2015) at the University of Minho (Portugal). Her knowledge in regenerative biomedicine covers cell differentiation, polymers screening, 3D cultures and new technology approaches. On the other hand, she works in parallel on the field of cancer, studying new methodologies to establish an early diagnosis and more personalized treatments. Her research not only focuses on the basics, but is also clearly translational, with the possibility of diagnostic application and therapeutic use.

Cristina Antich is a PhD student under the supervision of Prof. Marchal. She holds a degree in Biomedicine (University of Barcelona, Spain). She cursed a MSc degree in Tissue Engineering (University of Granada, Spain). Her research field is regenerative medicine, mainly using 3D bioprinting. In her PhD, supported by predoctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU2014-04417), she has focused on the development and characterization of suitable biomaterials to be used in tissue engineering and its application to bioprinting as a bioinks.

Macarena Perán graduated with a B. S. in Biology and a M.S. in Biochemistry and Molecular Biology in 1996 from the University of Málaga, Spain. She moved to the Neuroscience Department at Durham University, UK, where she was awarded with a Marie Curie Fellowship and graduated in 2000 with a Ph.D. She moved back to Spain and completed a postdoctoral program in the Faculty of Medicine at Granada University. In 2005/2006 Macarena went to Bath University, UK, Prof. David Tosh lab. In 2011 Dr. Peran spent a year as a visiting scientist in the Salk Institute for Biological Studies, California, Prof. Juan Carlos Izpisua-Belmonte lab. Actually, Dr. Peran is Reader in Anatomy, University of Jaen, Spain and belongs to the Institute for Regenerative Medicine and Pathobiology (IBIMER). Dr. Peran actual research interest is the development of new anti-cancer drugs against cancer stem cell and the induction of cell differentiation with application in cell therapy.

Jose Manuel Baena is currently pursuing his PhD under the supervision of Prof. Rus and Prof. Marchal. He holds degrees in industrial engineering (Polytechnic University of Valencia) and in mechanical engineering (TU Braunschweig), focused on laser technologies and Biomechanical Engineering. His research experience spans from customized design of prosthesis to medical devices (Institute of biomechanics of Valencia). In 2011 He founded BRECA Health Care leading the use of new technologies in Health Care. He has authored the book “Emprender en carrera – Racing entrepreneurs” www.emprenderencarrera.com and has been awarded the Fernando Alonso program by Cajastur to study at Oxford Brookes University, UK.

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Guillermo Rus started his research on computational mechanics at the Universidad de Granada (UGR, 1995), where he disputed the PhD thesis on Numerical Methods for Nondestructive Identification of Defects (2001), providing defect search algoritms and sensitivity computation with Boundary Elements. He applied these experimentally at the NDE Lab at MIT (USA) as a Fulbright Postdoctoral Fellow, rendering novel robust quantitative approaches to ultrasonics and impact testing. He started up the NDE Lab as a lecturer at the UGR, 2003, focusing on bioengineering applications such as orthopaedic implants debonding at University College London and Universite Paris VI, or biomaterials at the Nanomaterials Technology Laboratory and Institute of Bioengineering of Alicante, or on advanced materials for civil engineering, such as FRP damage state monitoring with Hanyang University, Seoul, probabilistic problems with Caltech, or prognosis with NASA.