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ULTRASONIC MONITORIZATION OF CHONDROCYTE PROLIFERATION IN A BIOREACTOR

J. M. Soto[1], R. Molina-Conde[1], G.Rus[1], J.Melchor[1], A.Callejas[1], J. A. Marchal[2], C. Antich[2],

E.López-Ruiz[2], G. Jiménez[2]

[1]Department of Structural Mechanics, University of Granada, Granada, Spain
({jmsr,rubenmolina,grus,jmelchor,acallejas}@ugr.es)
[2]Biopathology and Medicine Regenerative Institute, University of Granada, Granada, Spain
({jmarchal,cantich,gemajg}@ugr.es, elruiz@ujaen.es)

Abstract
A bioreactor has been designed to achieve a real-time testing of a tissue by means of ultrasonic signal analysis. Polylactic acid (PLA) bioprinted scaffolds were seeded with human chondrocytes. Then, scaffolds were introduced into the bioreactor and cells were monitored by an ultrasonic sine-burst of 1 MHz during 4 weeks. From a mechanical standpoint, this system is analyzed as a multilayer composed of PLA-tissue-PLA laminates and we register the output signal after crossing all them. A set of models that simulate the ultrasound-tissue interaction are evaluated by the inverse-problem formalism. Eventually, a stochastic model-class selection formulation is used to rank which model is the most suitable to describe the tissue acoustic properties. The parameters derived from this process (wave velocity and attenuation) are useful to indirectly determine other mechanical properties (i.e. Bulk modulus). Moreover, proliferation was analyzed to ascertain the proper distribution of the seeded cells.

Keywords: Inverse problem, bioreactor, chondrocytes, biomechanics, ultrasonics.

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1 Introduction

The principles of continuum mechanics are proposed alongside a formal probabilistic formulation to address the problem of characterizing mechanical properties of tissue samples based on noninvasive and non-ionizing ultrasonic measurements.
Some researchers are recently investigating the acoustic (elastic) properties of cells and soft tissue at the microscale, and envisaging the potential of ultrasound (US) as a technique to provide real-time online assessments for non-destructive tissue characterization. Brand et al. [1] conducted different analysis of the changes in the acoustic properties of cells when exposed to chemotherapy for monitoring treatment response, using high-frequency ultrasound spectroscopy and scanning acoustic microscopy. In this way, US is a powerful tool to be employed to reach a noninvasive measurement as well as a high resolution system.
On the other hand, if we move to the field of cartilage engineering, we find outstanding findings relating the feasibility of US as a monitoring tool for tissue analysis. Hattori et al. [2] developed a novel system for evaluating articular cartilage, measuring the acoustic properties of the articular cartilage by introducing an ultrasonic probe into the knee joint under arthroscopy, which successfully predicts the histological findings of degenerated cartilage. Rice et al. [3] used ultrasound data to construct cross-sectional B-scan images for qualitative observations of evolving constructs used in

tissue engineering. Moreover, US stimulation could enhance chondrogenic differentiation in a 3D culture as well as the application of a mechanical stimuli may be necessary to promote extracellular matrix (ECM) synthesis. This ECM is composed of collagen, proteoglycans, noncollagenous proteins and glycoproteins [4]. Subramanian et al. [5] states that the US stimulation induces a marked increase in the expression of chondrogenic markers, such as type-II collagen (COL2A1), aggrecan and Sox-9.
By the way, it is known that dispersive and viscous properties of tissue are strongly sensitive to tissue changes and easily unveil hidden dimensions of its micro and macrostructure. Static or slow viscoelastic mechanical constitutive laws and their values were reported by Bader et al. for skin [6], and by Ahuja for several internal tissues [7]. There are many types of uncertainty involved in the modeling of interaction between ultrasonic waves and tissue, such as excitation, material viscosity, and material heterogeneity. Consequently, this field is of a daunting complexity as there are a lot of variables to reckon with.
A probabilistic model reconstruction inverse problem is proposed based on the concept of joint probability of prior information about observation and probabilistic information introduced by the model between model parameters and observations, as put forth by Tarantola et al. [8]. The model-class selection is formulated following Beck et al. [9]. Finally, a simple formulation of the joint probability is proposed, from which either the inverse problem or the model-class selection can be derived just by extracting specific marginal probabilities.
Real-time monitorization of a tissue is a challenging problem due to the risk of losing the sterility conditions during the biopsy of the sample under test. Besides this, after the biopsy process the material must be discarded. Thus, this highly increases the amount of funding and the number of tissue samples needed by the experiment. In this work we address this by testing the tissue with ultrasound and correlating its variations with the underlying biological process of cell proliferation. Furthermore, multiple models of ultrasound-tissue interaction are proposed, implemented and contrasted against experimental observations. All assume homogeneous media with varying moduli and energy-dissipation forms that are expressed as attenuation models. Our final goal will be properly tuning of mechanical properties for producing tissue-engineered articular cartilage [5, 10]. The form and mechanical properties of articular cartilage are mainly due to ECM composition which can be monitored by US measurements. Consequently, this research will have a significant impact on arthritis treatment and other traumatic injuries.

2 Methodology

The proposed methodology consists of four elements:
(1) A novel experimental setup based on ultrasound-tissue interactions is monitored in real time using a bioreactor.
(2) A selection of alternative models that simulate the ultrasound-tissue interaction by the transfer matrix formalism.
(3) A stochastic model-class selection is used to rank which of the models are more plausible.
(4) The reconstruction of the relevant mechanical parameters during the culture reaction time.

These four steps will be explained in further detail in the following subsection.

2.1 Experimental setup

The preparation of the cell culture is as follows. Firstly, polylactic acid (PLA) bioprinted scaffolds were sterilized by being immersed in 70 wt.% ethanol aqueous solution for 1 hour, washed several times in phosphate buffered saline (PBS) and then subjected to ultraviolet light for 20 minutes each size in order to ensure the sterile conditions of the construct. Then, a human chondrocytes suspension

containing 100.000 cells in 200 µl of medium were slowly dropped onto the surface of each scaffold incubated in 6-well plates for 2 h at 37˚C and 5% CO2. After that, 5 ml of fresh medium was added to each well plate. After 24 hours, scaffolds were introduced into the bioreactor and monitored by an ultrasonic sine-burst at a central frequency of 1 MHz and 10 V amplitude during 4 weeks. The biological process has been monitored at 2 minutes regular intervals, and the nutrient is automatically dispensed 3 times a day at a rate of 40 ml/week. In this way, we force an intermittent flow in order not to interfere the cell settlement. Figure 1 illustrates the employed electronic setup to conduct the experiment, while Figure 2 shows the framework before launching the experiment.

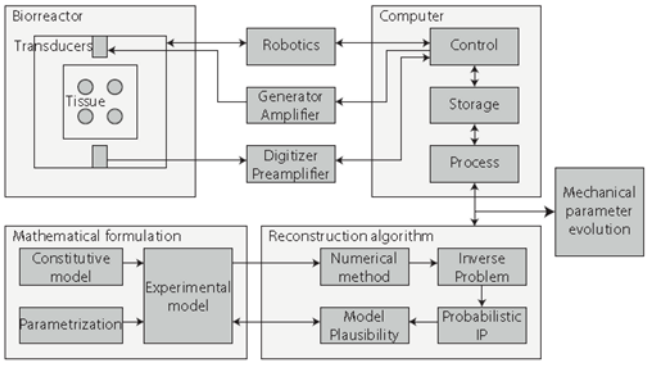

Figure 1 – Schematic experimental and electronical setup.

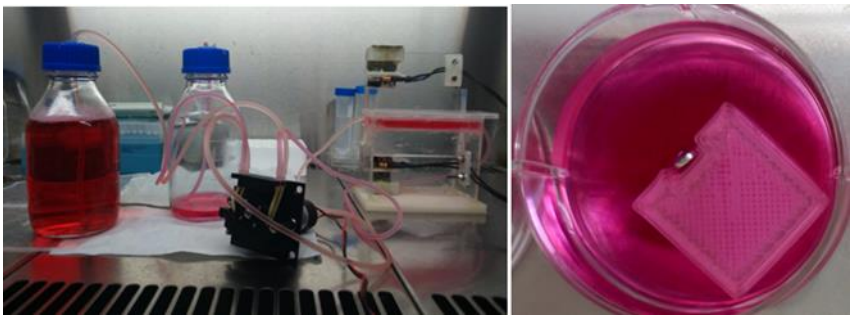

Figure 2 – Designed bioreactor (left) and bioprinted scaffolds (right) used to conduct the experiment.

It is worth pointing out that keeping sterile conditions is necessary to avoid the risk of the contamination. Particularly, the bioreactor has been sterilized by means of both ultraviolet and an alcohol cleaning of all its pieces. Then, the sterility is maintained using an Arduino-controlled peristaltic pump to keep a constant nourishment rate in the tissue (a chondrocyte culture in this case) along the month of analysis. This process was conducted 6 times to assure the repeatability and statistical relevance of the outcome. In this way, we create a set of 6 assays (labelled as 1A, 1B, 2A, 2B, 3A, 3B) where the number refers to the experiment and the letter is the position of the scaffold in the bioreactor. As described in the Section 4, there is a biological variability among tissue samples but these differences are considerably lower when cells come from the same patient and are kept under the same environmental conditions.

2.2 Theoretical background regarding numerical mechanical models

Three alternative models are tested to idealize the removal of energy by dissipation or radiation: viscous, hysteretic and proportional to integer time derivatives of the particle movement [14]. The

viscoelastic (α^vis), hysteretic (α^hys) and fractional time derivative damping (α, β) constants are defined by the models from Eq. (1) and (2),

$$M^*(\omega) = M^0\left(1 - \omega\alpha^{vis} - i\alpha^{hys}\right). \quad (1)$$

$$M^*(\omega) = M^0 \cdot (1 + b(i\omega)^{\beta})/(1 + a(i\omega)^{\alpha}). \quad (2)$$

Where M is the frequency-dependent complex modulus $M^*(\omega)$. The mathematical model described above is approximated by a semi-analytical model of the wave interactions within multilayered materials based on the transfer matrix formalism (TMF) (following the methodology of Bochud et al. [14]). Moreover, the bulk modulus (K) of all traversed materials (both PMMA layers and tissue) is related to Young's modulus (E) and compressional waves speed (c_p).

2.3 Fundamentals of probabilistic inverse problem

In this section we describe the basic concepts regarding probabilistic inverse problem in agreement with Tarantola et al. [8] formulation. In this way, the solution is not a single-valued set of model parameters (MP) but a probability density function (PDF) that will be referred as p(MP) thereon. These MP have possible values within a subset of L possible values. By the way, PDF values are related to the plausibility of the model values to be true, scilicet, the degree of agreement between experimental and numerically-obtained parameters.
Statistical inference theory is used to include any a priori information about either the experimental observations (O), the model parameters MP and the model class (C). The information of idealized relationship between them O = O (MP) is computed by a numerical model belonging to C. The former are defined by means of the probability densities to prior (labeled with 0 superindex) data: $p^0(O)$, $p^0(MP)$ and $p^0(C)$. On the other hand, the information about relationship between observations and model (labeled as m superindex) is provided by the following PDF function: $p^m(O, MP|C)$. The a posteriori probability p (O, MP, C) of the possible model MP is obtained jointly with the observations O and class C as follows,

$$p(O, MP, C) = k_1 \left(p^0(O, MP, C) \cdot p^m(O, MP, C)\right) / (\mu(O, MP, C)). \quad (3)$$

Where $\mu(O, MP, C)$ is the noninformative density function and k_1 plays the role of a normalization constant. The probabilistic model is given by its PDF that, after some hypothesis regarding independency of processes and uniformity of prior information (see [11] for further details) is obtained by the marginal probability from Eq. 4,

$$[p\ (MP)]_{C=C_i} = k_3 \int p^0(O) \cdot p^m(O|MP, C) dO. \quad (4)$$

Where k_3 is a normalization constant employed to satisfy the theorem of total probability in the aforementioned equation. Finally we aim at finding the probability p(C) that could be interpreted as a measure of plausibility of the suitability of model class C. The value of p(C) is provided by Eq. 5:

$$p(C) = \iint p\ (O, MP, C)\ dMP\ dO. \quad (5)$$

2.4 Genetic and BFGS algorithm search

The maximization of p (MP) for monitoring the evolution of the chondrocytes population is carried out by two sequential algorithms. 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 Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm is used as a local search in the proximity of the initial point.
Particularly our goal here is to determine both attenuation parameters from our three models and the P-wave speed that can be written as follows,

$$c_p = \sqrt{E(1-\delta)/(\rho(1+\delta)(1-2\delta))}\,. \tag{6}$$

Where δ is the Poisson coefficient and ρ is the density of the medium in which the wave is propagated. To show the suitability of this approach, Figure 1 shows a typical recorded ultrasound propagation overlaid with one model-based simulation.

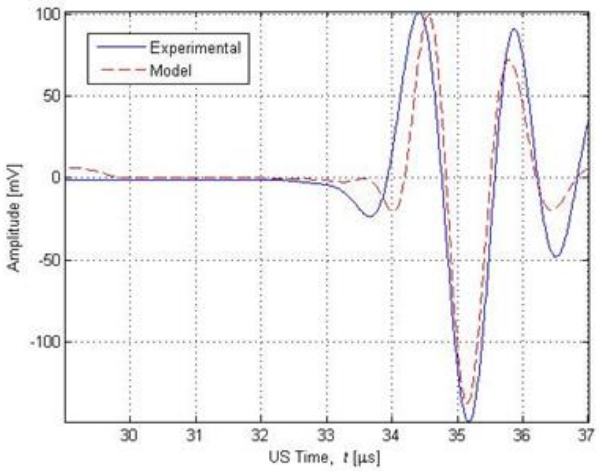

Figure 3 – Typical captured and simulated ultrasonic signals at the bioreactor transducers.

3 Experimental results and discussion

With the procedure from the previous Section we determine that the viscous model is the most plausible, followed by the hysteretic. This fact is due to the higher number of parameters in the hysteretic model (4 parameters) in contradistinction to the viscous one (only 2 parameters). Consequently, viscous model is simpler and fits properly with the experimental data.

Figure 4 summarizes the P-wave velocity derived from the IP with the viscous model in each of the 6 assays, while Figure 5 contains respectively the viscous attenuation parameter.

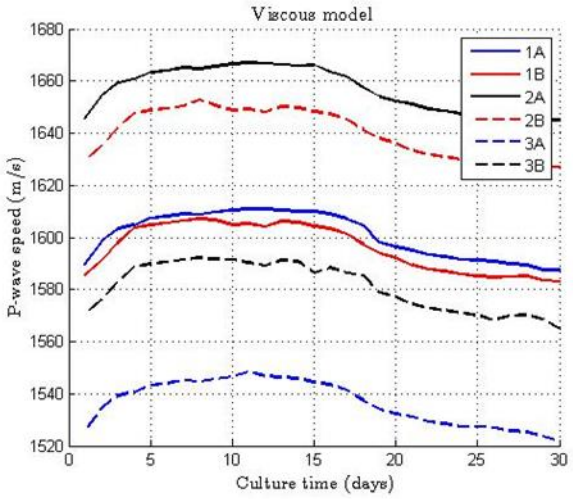

Figure 4 – P-wave speed depending on the culture time (viscous model).

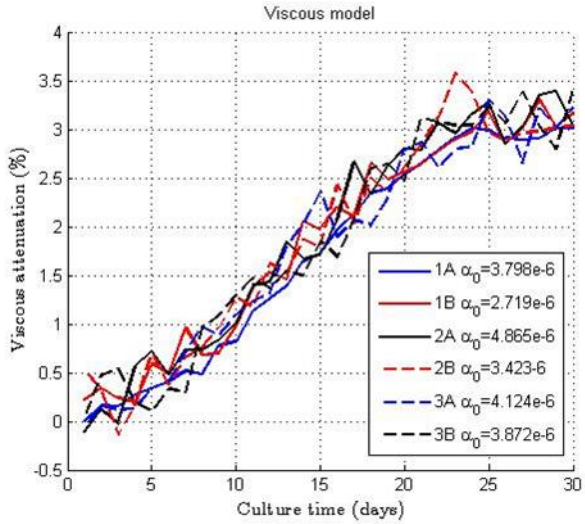

Figure 5 – Attenuation parameter α derived from viscous model (% of variation respect to the initial value α_0 of each culture).

Moreover, we obtain the cell proliferation curve (See Figure 6). The evolution of cell population during the experiment can be explained as follows:

(1) Firstly, chondrocytes need an accommodation period to set themselves on the scaffold and adhering to it. At the start of the experiment, the cells are in suspension and require approximately 2 hours to stick to the scaffold.

(2) Then a matrix of type II collagen (COL2A1) is synthesized by chondrocytes to create a fibre mesh of proteins as well as glycosaminoglycan (GAG) branch-like structures immersed in collagen matrix (ECM). The larger levels of cartilage specific COL2A1, the higher quality the engineered tissue

provides. Indeed, the consistent and positive correlation between chondrocyte density and the amount of ECM has been demonstrated [5].

(3) The matrix continues growing until the cells come in synaesthesia phase and its development stops.

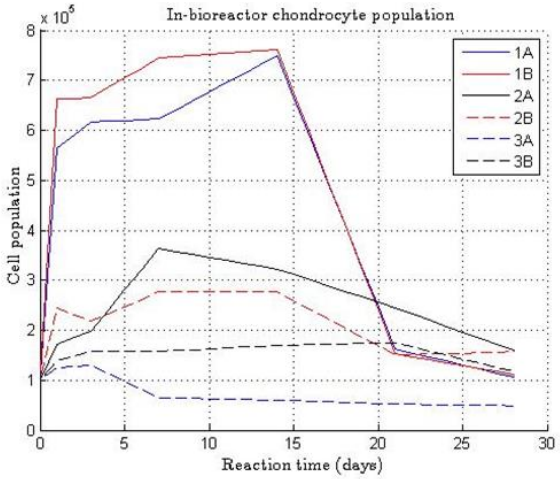

Figure 6 – Cell proliferation in the 6 assays.

We conclude that this cell proliferation agrees with the changes that suffers the P-wave speed. The higher number of cells, the higher speed we obtain during the initial growth of the population. Nevertheless, this behavior changes when the cell population decreases. On the other hand, attenuation from viscous model strongly depends on the experiment, but its variations are similar in all the assays: the larger the culture time is, the higher the attenuation is. The explanation for this last phenomenon could be the waste product in the medium.

Finally, to validate this outcome, the cell proliferation was analyzed and confocal images of the scaffolds were taken (See Figure 7) in order to ascertain the proper distribution of the seeded cells in the scaffolds. In these images living cells are stained in green while dead cell nucleus stained by propidium iodide in red.

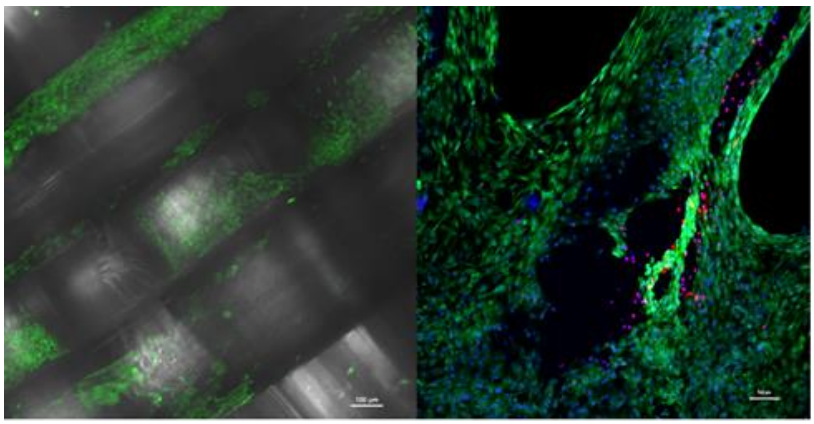

Figure 7 – Confocal microscope images after 1 (left) and 3 weeks (right) of culture in the bioreactor.

4 Conclusions

To reconstruct the velocity and attenuation from the recorded signals, a genetic-algorithm and BFGS-based inverse problem is 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 we register the output signal after crossing all them. A pool of attenuation models (viscous, hysteretic and fractional time derivative damping) that simulate the ultrasound-tissue interaction are evaluated and ranked using a model-class selection formulation to unveil which model best describes the tissue ultrasonic damping. The parameters derived from this process (P-wave velocity, attenuation, density changes in the culture layer) are proved useful to indirectly determine histological parameters non-invasively in real time. Furthermore, we underline the relevance of avoiding temperature changes in the culture because the reconstructed speed would suffer from severe peaking effects due to environment instabilities.
In conclusion, ultrasound is sensitive to the evolution and quality of the tissue under test. A clear correlation is observed among velocity, attenuation and the development of the culture. Thus, the usefulness of this technology in the field of regenerative medicine appears promising. Ongoing works will propose a biologically plausible explanation of the changes in the culture taking into account more medical information such as a genetic profile analysis.

Acknowledgements

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