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New “Tribo-bioreactor” for *in-situ* monitoring of the mechanical stress transmission at the cellular level: Application in cartilage tissue engineering

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ABSTRACT

The mechanotransduction is an important aspect to provide suitable conditions for the cartilage engineering process. Therefore, a new bioreactor is developed to apply different mechanical stresses to cell/organ culture. It allows performing cell culture *in situ* with appropriate mechanical constraints according to the real-time evolution of the physical parameters with the possibility of microscopic observation. In addition to the two-dimensional mechanical stimulation of a scaffold, the originality of this device is *in situ* monitoring of the cells and rheological measurements. This study presents the design of the new prototype with the validation of its different functions. First, the mechanical characterization of different non-biological samples is performed to calibrate the rheological measurement. Then, the monitoring of fluorescent beads in different scaffolds is carried out to estimate the mechanical stress transmitted to the cells. Finally, the ability of the device to handle a cell culture of human chondrocytes seeded in a scaffold is evaluated. The combined functions of the new bioreactor open the possibility to develop new scaffolds with optimal mechanical stress transmission, which is the basis of tissue engineering of cartilage.

KEYWORDS: Bioreactor, Cell culture, Chondrocytes, Mechanical characterization, Fluorescence analysis, Collagen sponges, Agarose hydrogel

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1. Introduction

During the healthy development of living tissue, cells modify their biological behavior according to the mechanical stress they receive. Each received mechanical stimulation results in a physiological response, which define the mechanotransduction (1). It is an essential pillar of tissue bioengineering. In addition to mechanical considerations, the process of tissue bioengineering combines the use of biomaterial support (scaffold) seeded with cells and the inclusion of growth factors allowing cells to differentiate and to regenerate a specific tissue (2)(3).

Altogether, it requires choosing the ideal scaffold, controlling the cells' differentiation, and introducing the specific mechanical constraints adapted to the desired tissue-type (4)(5). Therefore, bioreactors are used as devices in which biological and biochemical process is developed under a controlled environment (pH, temperature, mechanical stresses...).

The included and fixed parameters are defined depending on the type of the desired developing tissue.

For about fifty years, a wide variety of bioreactors have been used to combine the biological constraints (scaffold and growth factors), the physicochemical constraints (compatible with an incubator, easy to sterilize, and permeable to CO₂), and the mechanical constraints (application of different mechanical stresses) to stimulate the tissue synthesis.

For the cartilage cells (chondrocytes), the transmission of the mechanical stresses has to be in 3D configuration through a scaffold. This configuration ensures the 3D spherical structure of the cells and the isotopic transmission of the stresses.

Figure 1 shows the different mechanical stresses applied to the scaffolds in cartilage tissue engineering. In the literature, other systems are presented with different forms and types of stimulation applied to the cell cultures including electric fields, ultrasound, or centrifugal forces (6)(7). These models do not represent the natural physiology of the cartilaginous tissue, hence they are not included in Figure 1.

Briefly, based on the literature, the compression combined with the direct shear is the most important mechanical stress for cartilage regeneration. It allows the synthesis of collagen type II and proteoglycans (markers of hyaline cartilage). Thereby, dynamic or static compression is applied to the chosen scaffold (8)(9)(10)(11).

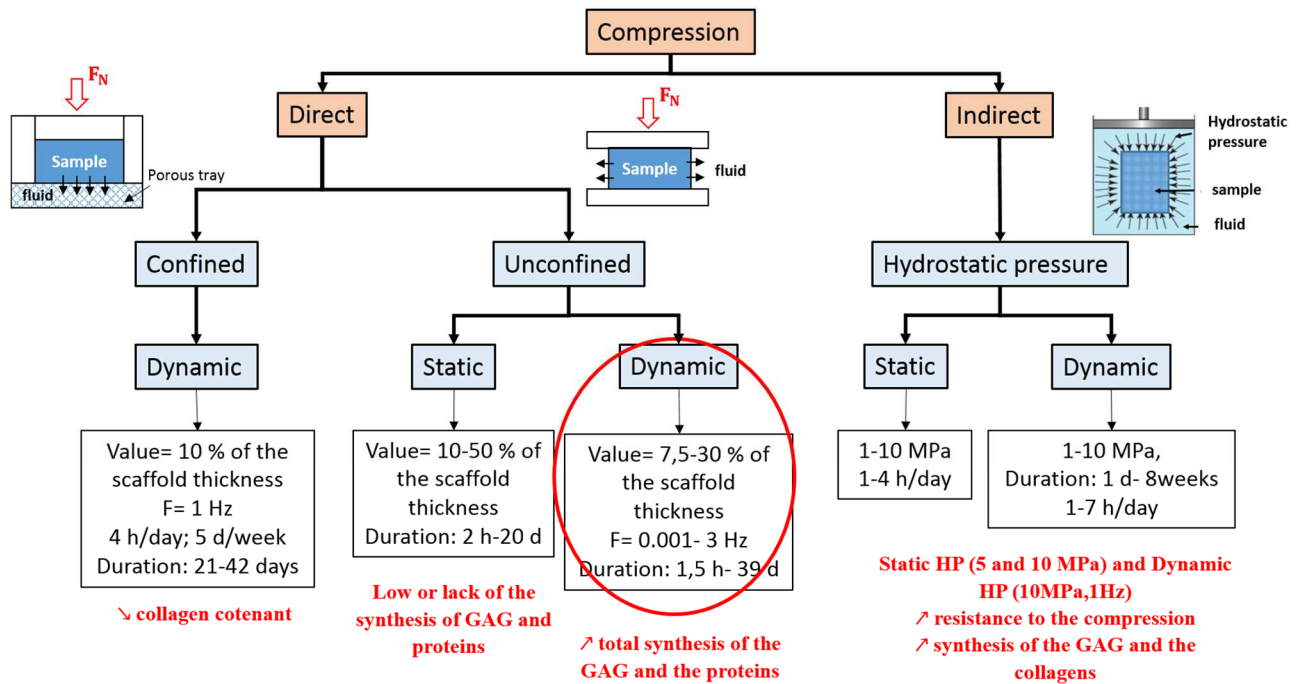


Figure 1: The different applied mechanical stresses for the cartilage regeneration based on the literature study

Furthermore, different reviews proved the efficiency of dynamic compression in comparison to static compression (12)(13)(14). Moreover, other studies demonstrated the importance of the shear stresses induced by the flow of the cell media inside the biomaterial.

Overall, the most promising results for the cartilage matrix synthesis are obtained using devices able to combine dynamic shear and axial dynamic compression (15)(16)(17) (figure 2). However, the value of the transmitted stresses to the cellular level is still unmeasurable and uncontrollable, which causes the variability of the results (14)(15)(17).

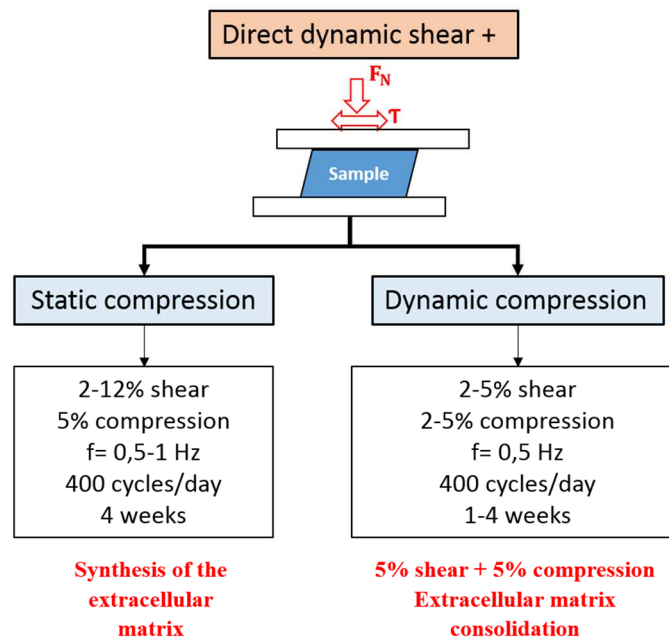


Figure 2 the obtained results with the application of the compression combined with the direct shear for the cartilage regeneration (based on the literature)

Accordingly, we developed a new bioreactor with a bi-axial load (dynamic compression and shear) able to follow *in situ* the transmitted stresses to the cells and the formation of the new tissue. Particular attention was paid to integrate the measurement of the constraints and cell visualization for local stress measurement.

In this study, we present the prototype of the new device called the “tribo-bioreactor”. It allows performing three main experiments: mechanical characterization for samples with different rheological properties, fluorescence analysis of cell displacement under mechanical stresses, and 3D cell culture with the measurement of applied stress at the cellular level. These experiments could be performed separately or simultaneously. This study aims to validate the “tribo-bioreactor” performance for each experiment.

2. Materials and methods

2.1. Technical consideration of the “tribo-bioreactor”

Figure 3 presents the basic structure of the new device. It solicits separately or simultaneously in compression or shear cell/organ cultures with the possibility of microscopic observation. These cultures are immersed in a specific culture medium to maintain physiological conditions. The medium flow is regulated with a peristaltic pump (Masterflex L/S).

The system (device + peristaltic pump) is designed to be placed inside an incubator to control the cell culture conditions (CO₂ level, hygrometry, and temperature) and to respect the

dimensions of the available microscope in the laboratory (Microscope Zeiss Axio Examiner) equipped with a confocal head LSM700.

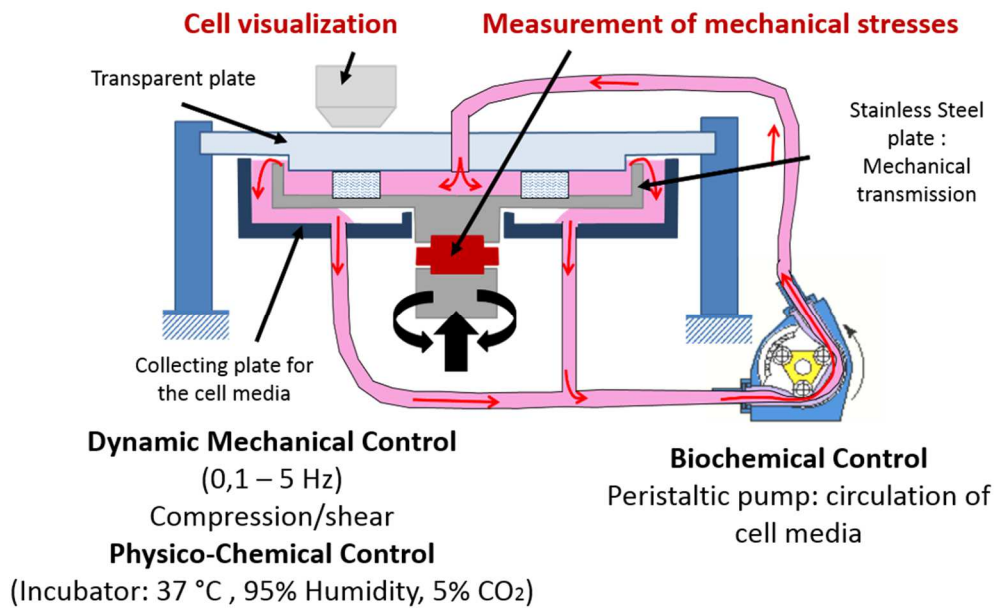


Figure 3 Global diagram of the new “tribo-bioreactor”. It allows applying mechanical stresses with microscopic observation under biochemical and physicochemical control

2.2.The concept and design of the “tribo-bioreactor.”

2.2.1. Contact configuration

The culture room of the “tribo-bioreactor” is composed of two parallel circular plates, as shown in figure 4. The transparent Plexiglas® plate allows the visualization of the samples. It is made from polymethyl methacrylate (PMMA), which is sterilizable and thermoplastic. The stainless steel plate facilitates the application of mechanical stresses.

The two plates are made with the same diameter, 50 mm. The gap between the two plates is modifiable by a lifting table in which the stainless steel plate is clamped.

To ensure biological reproducibility, it is considered essential to run several tests at the same time (3 to 10 samples). Therefore, 3 locations are made in the stainless steel plate to hold the samples. The locations are positioned 20 mm from the plate center and with a 120° equal angular distance between them. Other stainless steel plates are conceived to hold different samples (number, dimensions).

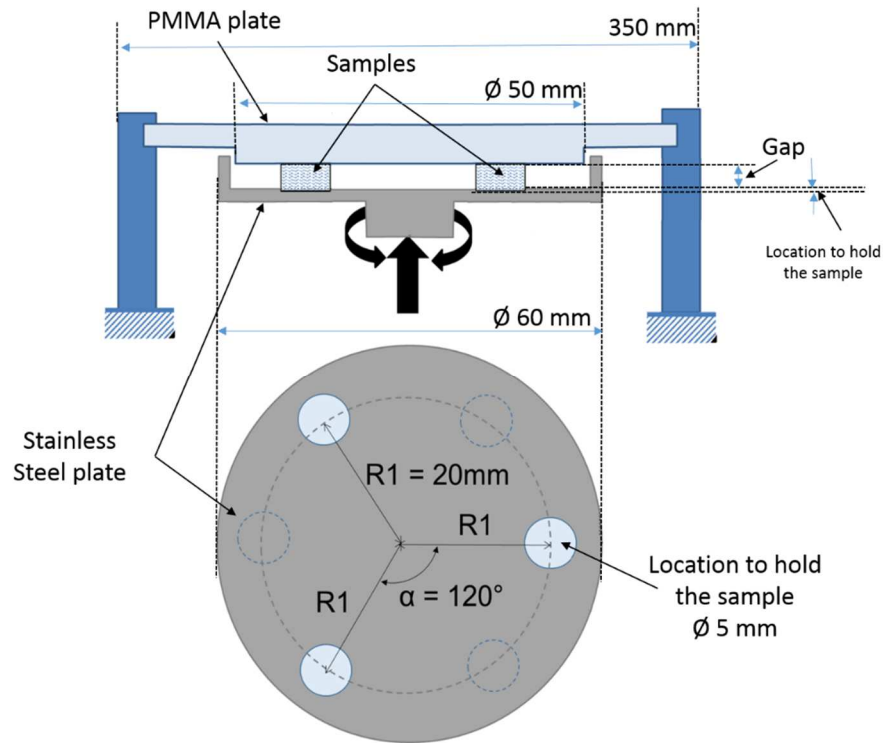


Figure 4 Contact configuration: the two parallel plates formed a cell culture chamber where the sample will be placed

An extra inferior plate made from polyvinyl chloride (PVC) is added to recover the culture medium: “the collecting plate” in figures 3 & 5. A peristaltic pump is used to renew the cell culture medium. It is connected to both sides of the culture room (up and down) through a system of pipes. Thus, the Plexiglas® plate is designed with a 3 mm central hole for the medium supply, and the PVC plate is designed with two holes (3 mm) for the medium evacuation.

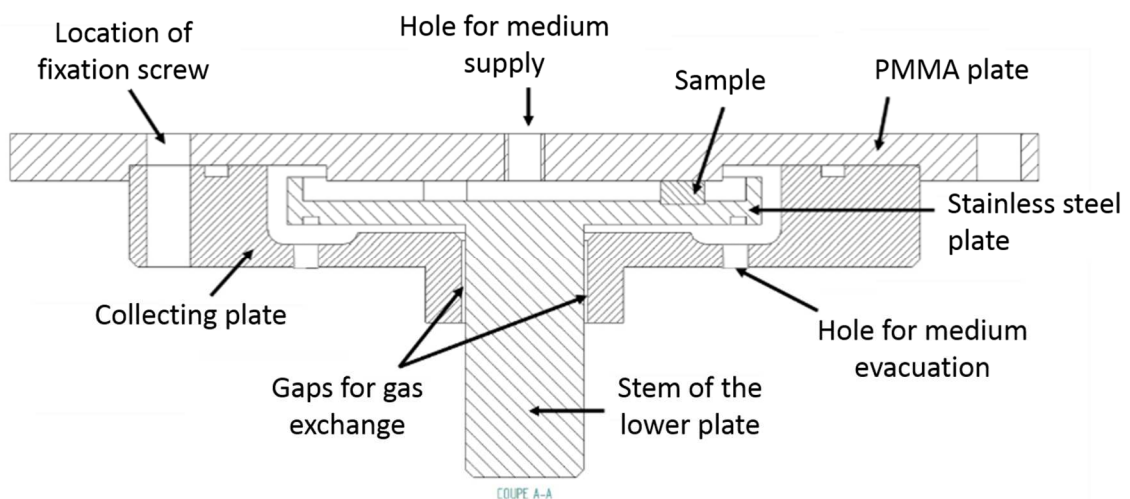


Figure 5 Cross-Section of the different plates of the “tribo-bioreactor” showing the holes used for the pipes system

2.2.2. Mechanical stresses

Piezoelectric actuators “Amplified Piezo Actuators (APA)” (Cedrat Technologies, Grenoble, France) are selected for mechanical stresses. The vertical displacement of an APA applies the compression, and the shear is applied by the horizontal displacement of another APA transformed into rotation by an elastic system (figure 6). Strain gauges measure both displacements. A piezoelectric sensor with 2-Component Force Reaction Torque Link (KISTLER, France) measures the normal dynamic force and the torque.

The APA actuators are selected for the use of a mechanical amplifier to preload the stack of multilayer piezoelectric components and to mechanically amplify the displacement. Therefore, they provide a significant stiffness for the system and increase generated efforts.

The used elastic system is a bending bearing with flat, internal, crossed springs encapsulated in a cylindrical housing to provide precise rotation and to avoid friction losses. This bearing offers a significant lifespan when used under nominal load and rotation conditions. The rotation of the bearing is facilitated by the bending of the flat springs on which the segments are supported.

The vertical translation link is ensured by an elastic link (rod).

The piezoelectric sensor (KISTLER Type 9345B) is chosen for its characteristics, offering a sensitivity of 0.001 N for the measurement of normal force and 0.001 N.m for the measurement of torque.

Finally, the “tribo-bioreactor” is monitored by a developed LabVIEW interface (National Instruments, France) to adjust the needed load: type (compression or/and shear), the value of the APA displacement, and the frequency.

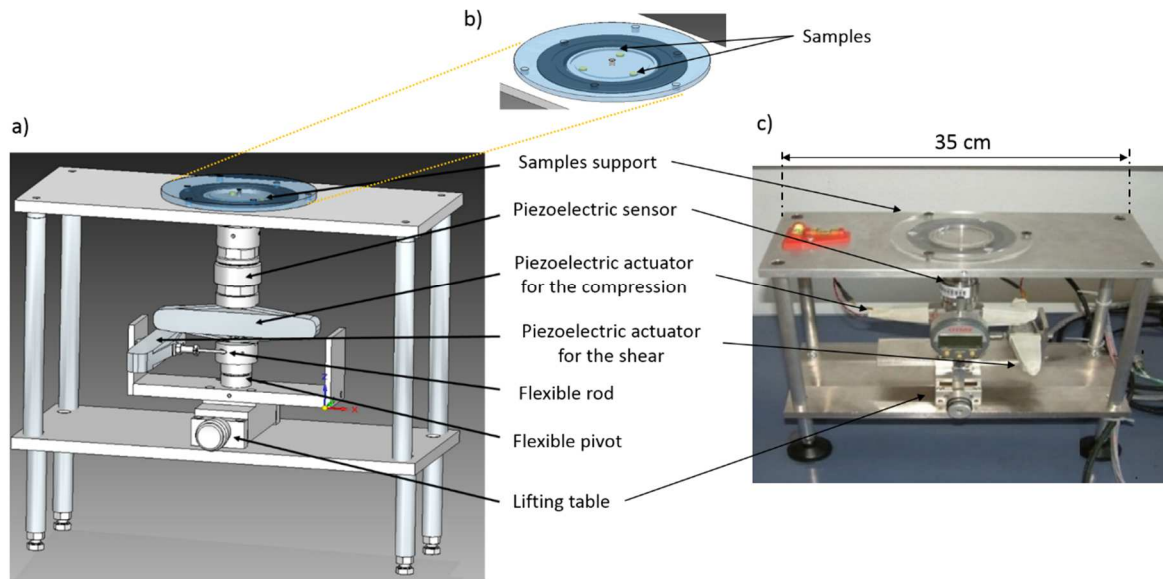


Figure 6 The prototype of “tribo-bioreactor”. a) Design with the specific components. b) The cell culture chamber is composed of two plates. c) Photography of the final prototype

2.3.Experiment I: Mechanical characterization of different samples

2.3.1. Experimental strategy

Thanks to its design, the “tribo-bioreactor” allows the mechanical characterization of the samples placed in the cell culture chamber. The combination of the applied dynamic stresses (APA) and the measured force (Piezoelectric sensor) supports the application of the dynamic mechanical analysis (DMA).

The DMA is a non-destructive technique used to characterize the viscoelastic properties of different samples (18). These properties are studied by imposing dynamic stress on the sample. The measurements obtained by the “tribo-bioreactor” are compared to those obtained by a classic rheometer.

For elastic and viscoelastic samples, the DMA allows calculating the complex modulus (E^*) and thus the elastic modulus (E'), the viscous modulus (E''), and the viscosity (η) as described in (18)(19).

For viscous samples, the viscosity calculation is based on the Stefan formula described in equation (1). It consists of squeezing a fluid between two parallel plates (20).

$$F_n = \frac{3\pi\omega\eta R^4 D}{2 * h^3} \quad (1), \text{ thus the viscosity is measured by the application of the equation (2)}$$

$\eta = \frac{F_n * h^3}{3 * f * \pi^2 * R^3 * D}$ (2); F_n is the value of the measured normal force, D is the value of the applied displacement, R is the plate radius, h is the gap between the two plates, and f is the frequency of the mechanical stress.

2.3.2. Samples and settings for the validation experiment

Three different types of samples are tested using the “tribo-bioreactor”: the bee-honey for the viscous behavior, the hydrogel discs for elastic behavior, and the agarose hydrogel for the viscoelastic behavior.

Hydrogels based on Poly(hydroxyethyl methacrylate p(HEMA) are prepared by mixing HEMA monomer (Sigma-Aldrich) with 25 % by weight of acrylic acid monomer (Sigma-Aldrich). Then, hydrogel discs (HEMA discs) are synthesized by free radical polymerization, as described in (21). HEMA discs have similar elastic properties as the articular cartilage; thus, they are selected for the testing (21).

The test parameters for the HEMA hydrogel and the bee-honey are 50 μm for the dynamic displacement, 3 Hz for the frequency, and 1 mm for the gap between the two plates. These parameters are presented as (displacement, frequency, gap) and are adjusted depending on the type of the sample and its volume.

For the viscoelastic behavior, 2 % agarose hydrogels (Sigma Agarose IX-A) are prepared. Briefly, the appropriate amount of the hydrogel powder is mixed with PBS in a beaker between 70 °C and 80 °C with stirring for ten minutes to obtain an initial concentration of 2.5 %. Then, the solution is autoclaved (22). 700 μL of the mixture is poured into 24 wells-culture plates. Agarose constructs are allowed to gel at room temperature. Then, they are punched to obtain discs with a 24 mm diameter.

The first step in evaluating the agarose hydrogel is to test the level of stress it could withstand without cracking. The gap between the two plates is 2.25 mm after applying 10 % pre-compression to the gel, the contact diameter is 24 mm. By gradually applying a displacement of 25 μm up to 300 μm with a frequency varying from 0.5 Hz to 2 Hz, the discs cracked when the displacement reached 175 μm and deformation was noticed in the signals of the force and the displacement. Therefore, the parameters for the mechanical characterization of the hydrogel agarose are fixed as (100 μm , 2 Hz, 2.25 mm).

2.4.Experiment II: Fluorescence analysis of cells displacement under mechanical stress

The fluorescence analysis is used to ensure the transmission of mechanical stress throughout a scaffold and up to the cells. It identifies the local displacement of the cells during mechanical stress. The scaffold has to be clear to allow the visualization of fluorescent cells. Alternatively, fluorescent beads are used to represent living cells.

While a dynamic compression is applied to the sample, a stack of images on the Z position is taken with confocal microscopy. The succession of the images allows following the displacement of the beads (figure 7). Therefore, a compromise between the duration of the mechanical stress and the time of the camera's acquisition is necessary. The parameters for the dynamic compression depend on the tested biomaterial.

HEMA discs are selected to adjust this technique as they have similar rheological properties as the articular cartilage. They are prepared as described previously with the addition of 15 μm polystyrene fluorescent beads (FluoSpheres TM Polystyrene Microspheres, ThermoFisher Scientific). For the testing, the parameters for the "tribo-bioreactor" test are fixed as (200 μm , 0.05 Hz, 1 mm).

For the tissue bioengineering process, it is necessary to validate this experiment using cells instead of fluorescent beads. Since HEMA discs are not convenient in the use with cells, the same test is performed with agarose hydrogels containing fluorescent cells.

Discs of agarose hydrogels (Sigma Agarose IX-A) are prepared as previously described. Before gelation, cells are suspended in the agarose solution with a density of 2000 cells per mL to reach a final 2 % concentration. The final agarose constructs are punched to obtain cylindrical plugs (13-mm diameter and 3-mm height). The used cells are human chondrocytes fluorescently labeled using "CellTracker Red" (ThermoFisher Scientific) as detailed by the manufacturer. The parameters for the testing are adapted, respecting the maximum load the hydrogel could undergo without cracking (150 μm , 0.05 Hz, 3 mm).

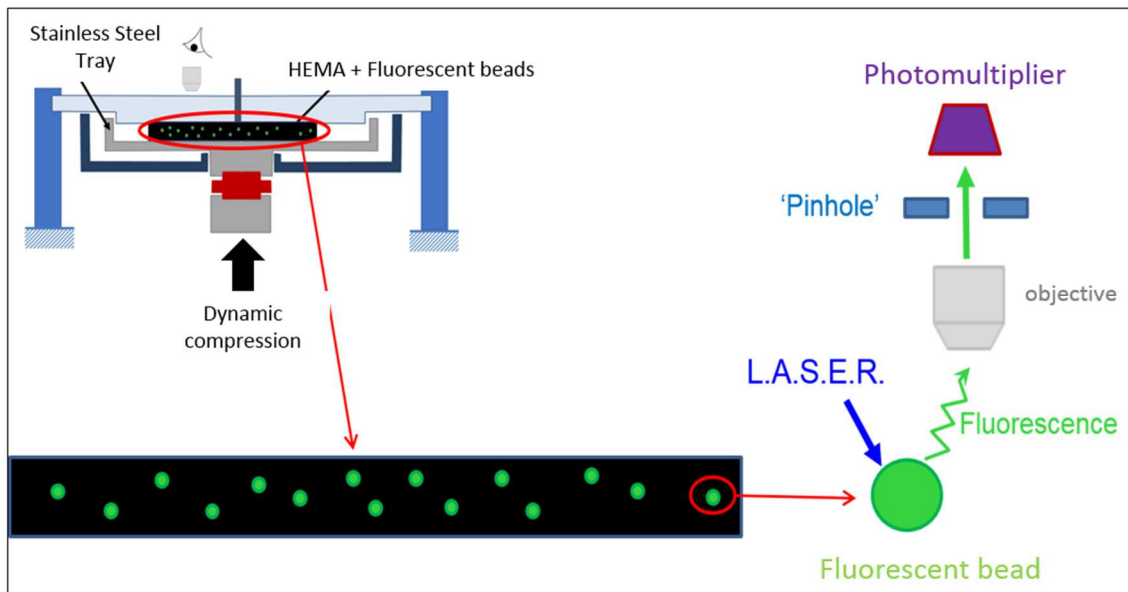


Figure 7 Fluorescence analysis principle using the “tribo-bioreactor” and the confocal microscopy

2.5. Experiment III: Use of the «Tribo-bioreactor» as a bioreactor for chondrocytes 3D culture

The “Tribo-bioreactor” is tested for cell culture using human chondrocytes seeded in collagen sponges as a scaffold (figure 8).

2.5.1. Culture of human articular chondrocytes (HACs)

Human articular chondrocytes (HACs) are isolated from macroscopically healthy zones of hip joints obtained from donors undergoing total joint replacement (n= 4; 51-79 years). The study is performed in full accordance with local ethics guidelines, national and European Union legislation regarding human sample collection, manipulation, and personal data protection (Ethics Committee for research with human samples, CODECOH: DC-2014-2325). Cartilage samples are collected after the written informed consent of the donors. Chondrocytes are extracted, as described by Hautier and colleagues (23).

Pieces of cartilage are finely cut then rinsed with PBS. Cells are obtained by two enzymatic digestions with 1 mg/mL trypsin (Sigma; ref T4799) followed by 0.06 % bacterial collagenase A (Roche Applied Science; ref 10103586001). Chondrocytes are filtered through a 70 µm sieve and then centrifuged in a control medium composed of the 1:1 mixture of DMEM with Glutamax and HamF12 supplemented with L-glutamine, 10 % of fetal bovine serum (FBS), 50 µg/mL of gentamycin (Panpharma), and 2 µg/mL of amphotericin B (Bristol Myers Squibb). The chondrocytes are seeded at the density of 1.5×10^4 cells/cm² on culture dishes and cultured in the same medium. The medium is changed and completed with 5 ng/mL of FGF-2

(fibroblastic growth factor) and 5 $\mu\text{g}/\text{mL}$ insulin, 36 hours after seeding. The medium is replaced three times per week until the cells reach confluence. The chondrocytes are then detached by the action of trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma) and again seeded at the same density (24)(25).

2.5.2. Culture of HACs in collagen sponges

Collagen sponges are provided by Symatase Biomaterials (Chaponost, France). They are composed of native bovine type I (90-95 %) and type III (5-10 %) collagen, derived from the extraction of American calf dermis. These sponges are cross-linked with glutaraldehyde to increase their stability and are beta-irradiated to ensure sterility. Sponges are then cut with a skin biopsy punch (Laboratories Stiefel) in discs with a 5 mm diameter and 3 mm thick to fit the locations made in the “Tribo-bioreactor” stainless steel plate. The pore size is about 100 μm .

The chondrocytes-collagen sponges' constructs are prepared as previously described (25) (figure 8). Briefly, chondrocytes are seeded onto the sponges at the density of 13×10^6 cells/ cm^3 , and the sponges are incubated at 37 °C for 2 hours to allow cell adhesion. A culture medium containing 10 % of FBS, and supplemented with 50 $\mu\text{g}/\text{mL}$ of ascorbic acid (sodium salt, Fluka) is then added to the wells in the presence or not of 200 ng/ mL of (BMP)-2 (bone morphogenetic protein), 5 $\mu\text{g}/\text{mL}$ insulin (Umulin, Lilly), and 100 nM thyroxine T3 (Sigma).

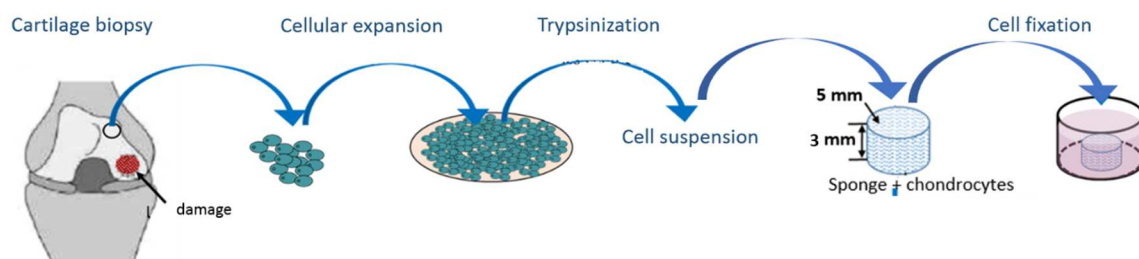


Figure 8 Different steps to obtain collagen sponges with seeded chondrocytes

The culture medium is changed every two days. Chondrocytes-collagen sponges are cultured several days or weeks *in vitro* before the “Tribo-bioreactor” test to allow cells fixation and extracellular matrix production. This period is designated as a pre-culture period. The medium circulation and mechanical constraints could cause cell detachment from the sponges. A decent pre-culture period could avoid this phenomenon.

2.5.3. Settings for the validation experiment

Several tests are performed to adjust the convenient parameters for the cell culture (presented in table 1). For each experiment, a reference sponge “REF” without mechanical stresses, cultured *in vitro*, is considered. The “REF” sponge helps to compare chondrocytes' response without and under mechanical stresses (“TriboR”). The period of cell culture is similar for the “REF” and “TriboR” sponges.

For the compression, a displacement of 10 % of the sponge thickness is applied (300 μm). For the shear, a displacement of 5 % of the sponge circumference is applied. Based on the Waldman study, both mechanical stresses are employed with a frequency of 0.5 Hz (17). The flow rate of the media circulation is slow enough not to induce hydrostatic pressure and shear effects on the samples (8 mL/min). Collagen sponges without cells are used as controls (WC). They are tested under and without mechanical stresses.

Table 1 Tests performed using the «tribo-bioreactor» with different parameters for the duration of the pre-culture and the application of the mechanical stresses

	Test 1: Dynamic compression & shear		Test 2: Dynamic compression & shear		Test 3: Static compression	
Pre-culture period	3 weeks		4 days		2 days	
Cell culture period in the “Tribo-bioreactor”	4 weeks		5 weeks		3 weeks	
Tests	«REF»	«TriboR»	«REF»	«TriboR»	«REF»	«TriboR»
Mechanical stresses parameters	none	Compression (a) Shear (b)	none	Compression (a) Shear (b)	none	Compression (a)

(a) 10% of the sponge thickness

(b) 5 % of the sponge circumference

2.5.4. Analyzed parameters, post-test

Mechanical characterization of different sponges (“REF”, “TriboR”) is performed with the rheometer, as described in (26). Therefore, 30 % of compression is applied to the sponges with a 0.002 N preload followed by a relaxation period. Elastic modulus E is calculated based on Hooke’s law. It is given by the slope of the straight line obtained in the curve of the stress $\sigma = F/S$ (F as the normal force and S is the surface of the contact) as a function of the strain ϵ .

Also, fluorescence recovery after photo-bleaching (FRAP) is performed using the confocal microscope to evaluate the physicochemical properties of the collagen sponges. Collagen

sponges from “Test 3” are recovered and immersed in a solution of 70 kDa dextran labeled with neutral fluorescein (Sigma) at 0.1 mg/mL in PBS at 4°C for at least 1 hour (26).

Based on the method performed in the laboratory (27), the FRAP experiments are performed on the basic ZEN 2010 software for LSM 700. For each zone of the sample, a reference zone “ROI” not subjected to photobleaching is recorded.

The fluorescence intensity of the obtained experimental curve ($I_{ROI}(t)$) is divided by a correction factor to bring the I_{ROI} at the same prebleached intensity level as the reference ($I_{reference}$) at initial conditions. The correction factor is thus the ratio of the initial fluorescence intensity (pre-bleached intensity, I_0 of the experimental curve) to the reference curve. The corrected I_{ROI} is subtracted from the reference curve's fluorescent intensity ($I_{reference}(t)$) to eliminate the photobleaching due to image acquisition (26)(27). The result is represented as a curve $f_d(t)$ called “normalized difference fluorescence intensity” represented by the equation (3).

$$f_d(t) = I_{reference}(t) - \frac{I_{ROI}(t)}{I_{ROI}(0) / I_{reference}(0)} \quad (3)$$

We reproduced the same experiment for six samples (3 REF sponges and 3 TriboR sponges). as described above and in (26)(27).

At the end of the cell culture period, collagen sponges on each condition are fixed in 4% (v:v) formalin and embedded in paraffin for histological analysis. Haematoxylin-Eosin-Saffron staining (HES) is performed. It allows evaluating the structure of each sample. Also, specific immunolabelling is made for collagen type II to identify the extracellular matrix components.

3. Results and discussion

The different results are analyzed statistically. The rheological tests are analyzed using the software OriginPro of at least four measurements. Bars represent the mean and standard error of the mean (SEM). The effects of the mechanical stresses on the collagen sponges are compared to the “REF” sponges, using the Student’s t-test. In tables 2 and 3, the results are presented as mean \pm standard deviation (SD). Significant results are represented as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Non-significant results are represented as ns $p > 0.05$.

3.1.Experiment I: The “tribo-bioreactor” as a simulator for mechanical characterization

For this experiment, the “tribo-bioreactor” is compared to a classic rheometer when testing different samples with different rheological properties.

The bee-honey is first tested using a classic rheometer with a cone-plate configuration. The rheometer measurement led to a viscosity value of 4.6 Pa*s (table 2) and a phase shift value of $\pi/2$ between displacement and force signals. Using the “tribo-bioreactor”, the obtained phase shift is also $\pi/2$ confirming the viscous behavior of the sample (figure 9a). Based on the Stefan formula, a viscosity of 4.7 Pa*s is obtained. Thus, the viscosity measurements obtained with both devices are quite similar; hence the new prototype is considered as a rheometer for viscous samples.

While testing the bee-honey with the “Tribo-bioreactor”, a sliding effect between the sample and the plates appeared, causing deformation in the force signal. After numerous tests, the problem was resolved by sticking removable sandpapers on both plates.

Table 2 Mechanical characterization of bee-honey and synthetic HEMA using both rheometer and “tribo-bioreactor.”

	Rheometer	“Tribo-bioreactor”
Bee-honey: Viscosity η (Pa*s)	4.635 \pm 0.017	4.712 \pm 0.018
Synthetic HEMA: Elasticity E' (MPa)	0.2 \pm 0.05 (21)	0.220 \pm 0.001

For the synthetic HEMA, the two signals of the force and the displacement are in the same phase, which approves the elastic behavior of the sample, as shown in figure 9b. The measurements of the elastic modulus with the “tribo-bioreactor” are equivalent to those made with the rheometer, as shown in table 2. Thus, the “tribo-bioreactor” is considered as a rheometer for elastic samples.

For the agarose hydrogel, the phase shift between the measured normal force and the imposed displacement is different from 0 (elastic behavior), also different from $\pi/2$ (viscous behavior), which means that the agarose hydrogel has a viscoelastic behavior (figure 9c). After processing the signals, we obtained: $E' = 76.64 \text{ N/m}^2$ and $\eta = 2.73 \text{ Pa*s}$.

The obtained viscosity value corresponds to a viscosity value already mentioned in a previous study (28), as well as the value of the tested elasticity (29)(30). The agarose hydrogel was characterized in several previous studies consequently in this study, it was not tested with a classic rheometer. Thus, the comparison between the rheometer and the “Tribo-bioreactor” for the agarose hydrogel testing was based on the literature.

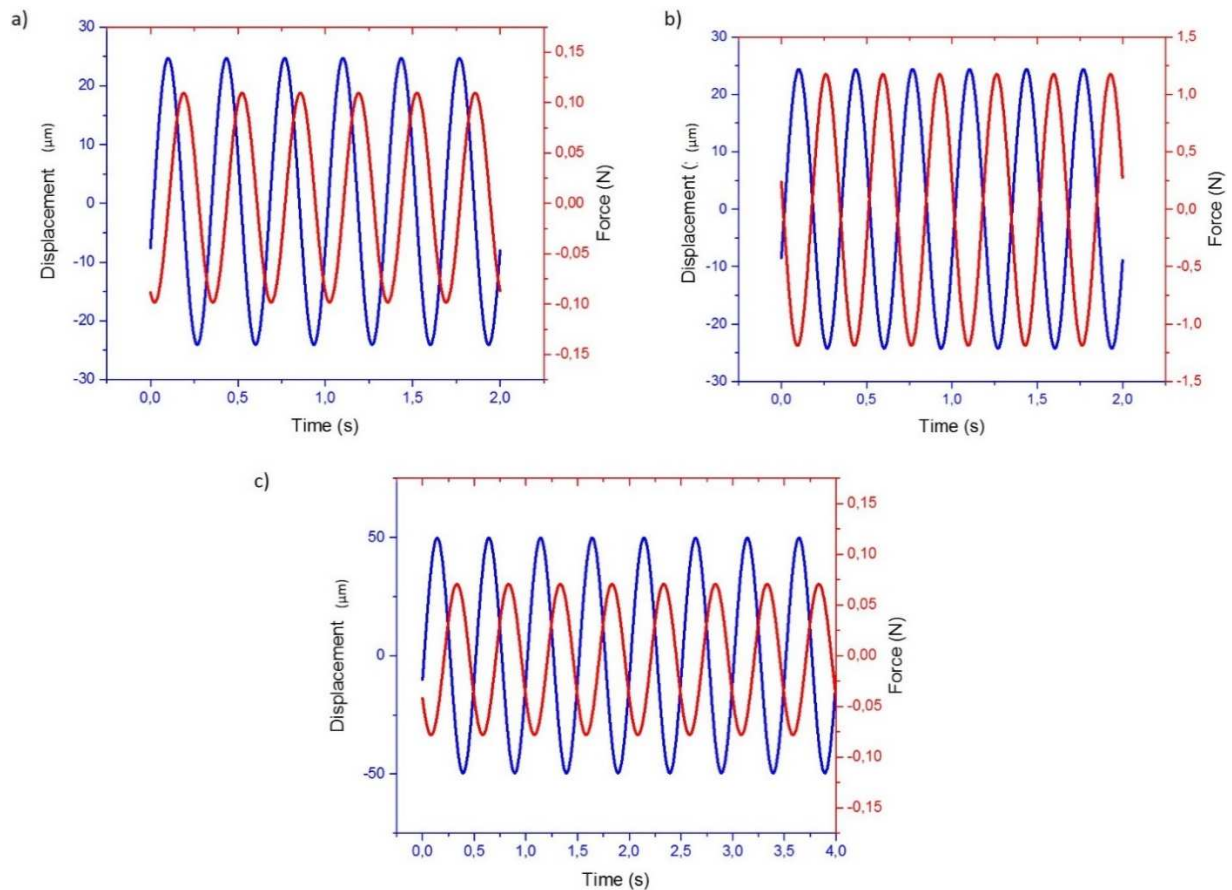


Figure 9 Obtained signals with a displacement of $50 \mu\text{m}$ and a frequency of 3 Hz : a) Applied displacement and obtained force signals of the bee-honey, phase shift $\pi/2$ b) applied displacement and obtained force signals of the synthetic HEMA phase shift π c) applied displacement and obtained force signals of the agarose hydrogels ($100 \mu\text{m}$, 2 Hz) phase shift $\neq \pi$, $\neq \pi/2$

Based on the literature and in comparison with the measurements obtained with a classic rheometer, the performance of the new prototype is confirmed as a simulator for the DMA. It allows evaluating the mechanical properties of different samples with different rheological behaviors. Consequently, the “Tribo-bioreactor” is used in various research projects. It was used to evaluate the rheological properties of tumoral cell samples (prostate cancer cell lines; viscoelastic behavior) to detect the evolution of cancer and to predict its stages (27). Also, it was used to characterize the mechanical properties of different tissue-like cell-membranes developed on the surface of orthopedics biomaterials (viscoelastic behavior) (19). Likewise, it was used to characterize the mechanical properties of human cartilage samples.

3.2. Experiment II: Fluorescence analysis of cells displacement under mechanical stress

The cell tracking experiment is performed using fluorescent beads included in HEMA discs (1 mm thickness) as a first sample. The test consists of identifying the position of the beads inside the scaffold during the application of a dynamic displacement. The displacement of the beads

is followed in Z position by making 200 μm thick images stacks for 5 minutes. Several frequencies are tested from 0.01 to 0.5 Hz. The acquisition time of a single microscopic image is approximately 0.1 s. Hundreds of images are obtained for a thickness of 200 μm .

Before applying the mechanical stress to the HEMA disc, a microscopic scanning is performed to localize the position of the different beads inside the sample. To ensure a successful test, only one or a few beads are selected inside the HEMA disc. The selected beads should be isolated to not affect the contrast and the brightness of the images and not to interfere with the position of the other beads. We used a Zeiss x63 objective “W N-Achroplan 63x/0.9 M27 (FWD= 2.4 mm)” with the dynamic movement, after processing hundreds of images we estimated an error of $\pm 0, 2 \mu\text{m}$.

For the first scaffold, the selected beads are identified at the location of 150.7 μm from the total thickness of the HEMA disc (1 mm). After applying a displacement of 200 μm , the identified beads are located at position 0 μm . Thus, only 75 % of the imposed load is received by the fluorescent beads inside the synthetic HEMA (figure 10).

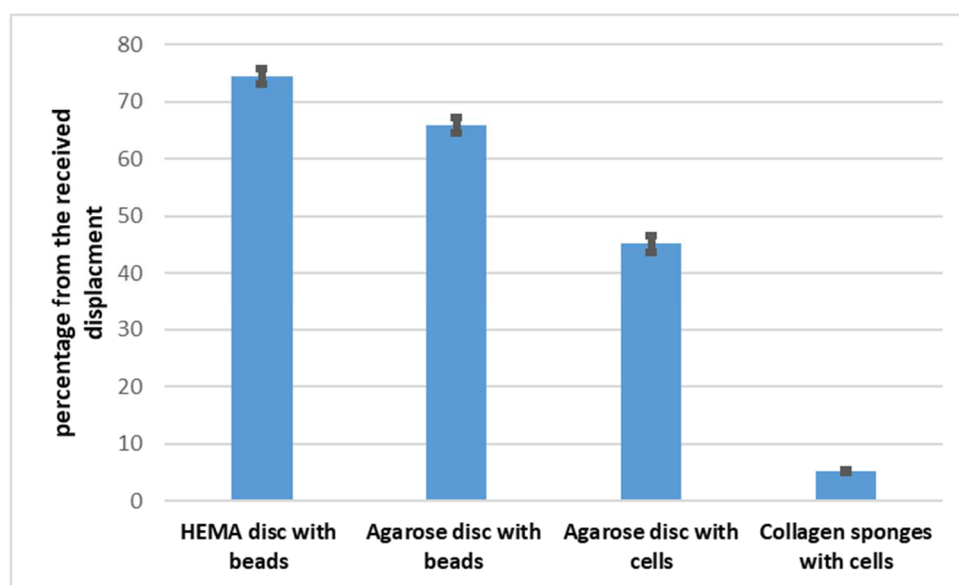


Figure 10 the amount of the received displacement by the beads/cells from the applied displacement inside the different scaffolds

In this context, the “tribo-bioreactor” allows to successfully perform the fluorescence analysis for cells displacement under mechanical stresses. This analysis is a “game-changer” in cartilage engineering and for the mechanotransduction conditions. Performing the fluorescence analysis, we are sure that even within a biomaterial, the cells still be subjected to mechanical constraints. Besides, this analysis allows us to figure out the exact level of mechanical constraints transmitted locally to the cells compared to those applied theoretically. However, the

mechanical stress transmitted could be affected by the mechanical properties of the scaffold. Thus, it is necessary to test the selected scaffold before performing the cell culture.

Therefore, the same experiment is performed with agarose hydrogel containing fluorescent cells. The parameters for the testing are adapted, respecting the maximum load the hydrogel could undergo without cracking. The agarose hydrogel is tested with two different configurations either containing fluorescent beads or cells (chondrocytes).

As showed in figure 10, the cell tracking showed that the agarose hydrogel allows the transmission of only 66 % of the imposed displacement when containing fluorescent beads and only 45 % of the imposed displacement when containing cells. This result confirms the effect of the scaffold stiffness on the transmission of the load and whether it contains beads or cells. Indeed, the mechanical characterization confirmed that the HEMA disc had a higher elastic modulus in comparison to the agarose hydrogel disc.

Also, the mechanical transmission of the applied constraint inside the collagen sponges was estimated as less than 10 %.

The collagen sponges had the lowest level of mechanical stress transmission, however, they are selected for the biological validation of the “Tribo-bioreactor” (cell culture) because of their interesting use from a biological point of view and for a direct clinical application.

Overall, the application of the fluorescence analysis made it possible to follow successfully the local displacement of cells/beads inside the biomaterial. Due to this context, it is necessary before launching a cell culture to evaluate the mechanical transmission for the selected scaffold to adjust the applied parameters (31)(32).

3.3.Experiment III: Use of the “tribo-bioreactor” for chondrocytes 3D culture

The main challenge for 3D cell culture with the “Tribo-bioreactor” is to keep the cells inside the collagen sponges (selected as a scaffold for this part of validation). Therefore, three tests with different pre-culture periods are performed to identify the appropriate time for a better cellular attachment to the collagen sponges.

The sponges from test 1 were subjected to 3 weeks of pre-culture and 4 weeks under mechanical stresses (10% of compression and 5% of shear).

The sponges from test 2 were subjected to 4 days of pre-culture and 5 weeks under mechanical stresses (10% of compression and 5% of shear).

The sponges from test 3 were subjected to 2 days of pre-culture and 3 weeks under 10% of compression.

Overall, the HES staining showed a good cellularization of the sponges at the end of each pre-culture period, irrespective of the pre-culture period (figure 11).

We noticed the collagen fibers of the sponge (in brown/dark red) alongside the thinner fibers (in pink/purple) also cell nuclei (the small purple circles) which represent the cell synthesis.

The HES of Test 1 (3 weeks of pre-culture, 4 weeks of culture with compression and shear) showed that a long pre-culture period (3 weeks) did not allow cell development inside the total structure of the sponge. Indeed, many empty areas were detected in the sample. This result could be related to the obtention of a denser cellular zone at the periphery of the sponge (figure 12). Therefore, for the remaining tests, the pre-culture period was reduced: 4 days for Test 2 and 2 days for Test 3.

For Test 2 (4 days preculture, 5 weeks under dynamic compression and shear), the application of the direct shear to the collagen sponges generated a sliding effect between the surface of the sponges and the “Tribo-bioreactor” plate which disturbed the transmission of mechanical stresses inside the sponge. Therefore, for Test 3 (2 days preculture, 3 weeks under dynamic compression), only dynamic compression was applied to the sponges, they received only the shear applied by the flow of the culture medium circulation.

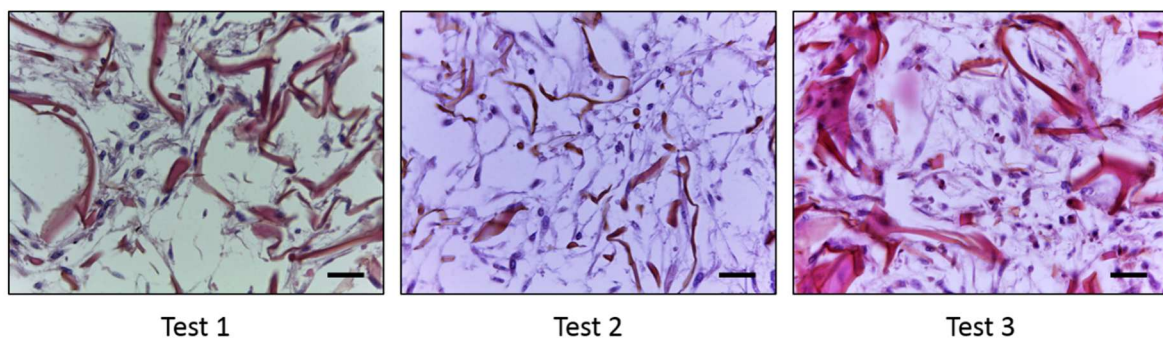


Figure 11 Hematoxylin-eosin staining of “TriboR” sponge. Collagen sponges were seeded with articular chondrocytes, pre-cultured for 3 weeks (test 1), 4 days (test 2), or 2 days (test 3) before placement in the “tribo-bioreactor” and compression. Scale bar = 50 μ m

Moreover, for the three tests, the chondrocytes submitted to mechanical constraints synthesized a more abundant cartilaginous matrix compared to the “REF” condition (without mechanical stresses). Indeed, immunostaining revealed an accumulation of type II collagen, the most abundant protein found in native cartilage, in collagen sponges cultured in the “Tribo-bioreactor” under mechanical stresses (Figure 12).

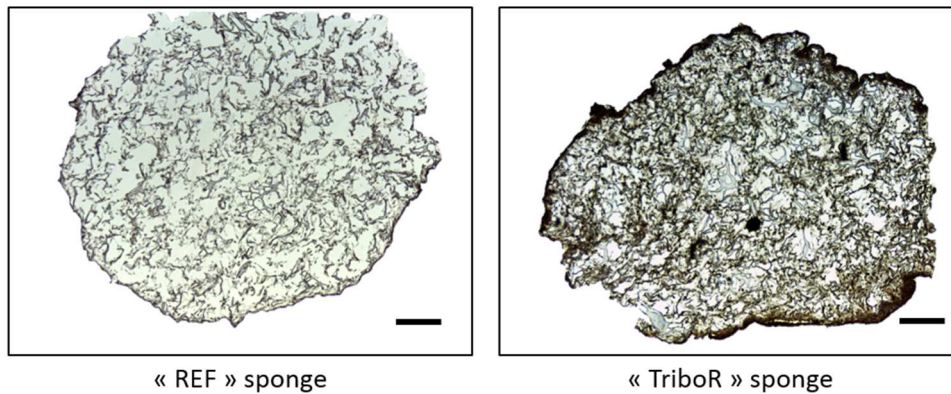


Figure 12 Type II collagen immunostaining of "REF" sponge compared to "TriboR" sponge from Test 1. Collagen sponges were seeded with articular chondrocytes, pre-cultured 3 weeks before placement in the "tribo-bioreactor" and submitted ("TriboR" sponge) or not ("REF" sponge) to mechanical constraints. Scale bar = 500 μm

For the mechanical characterization, it was not possible to process the signals obtained with "Tribo-Bioreactor" from Test 1 and Test 2 because of the combination of the compression and the shear in addition to the sliding effect noticed in Test 2.

Only data from Test 3 were subjected to a DMA, an increase in the elastic signal was noticed after 3 weeks of dynamic cultures (figure 13a).

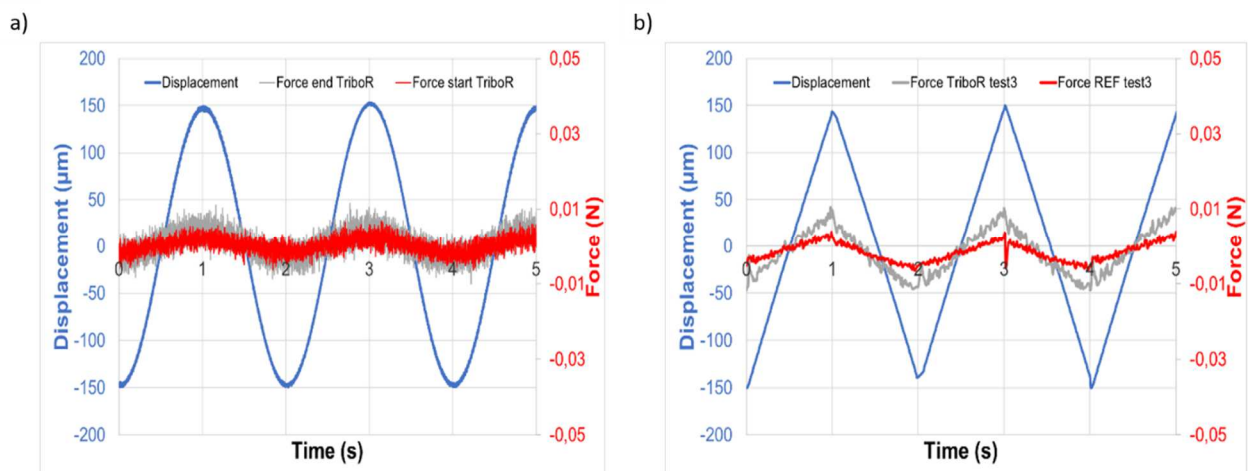


Figure 13 Mechanical characterization of collagen sponges after 3 weeks of cell culture: a) curves obtained from "Tribo-bioreactor" test, b) Curves obtained from the applied compression using the rheometer

Subsequently, it was interesting to compare the mechanical properties of the different sponges from the three tests. At the end of the cell culture, the sponges were removed from the "Tribo-bioreactor" and tested in the rheometer with quasistatic compression (figure 13b).

With the rheometer, three independent experiments were performed, and the calculation of the elastic modulus E gave acceptable accuracy (figure 14).

For a better standardization, results are paired: data obtained with "REF" sponges are compared to "TriboR" sponges. As expected, the DMA highlighted the importance of mechanical stresses. Indeed, the "TriboR" sponges were more elastic or more resistant than "REF" sponges except

for “Test 2”, where no significant difference is detected. Collagen sponges without cells (“WC”) were also used to assess the elastic modulus of the scaffold.

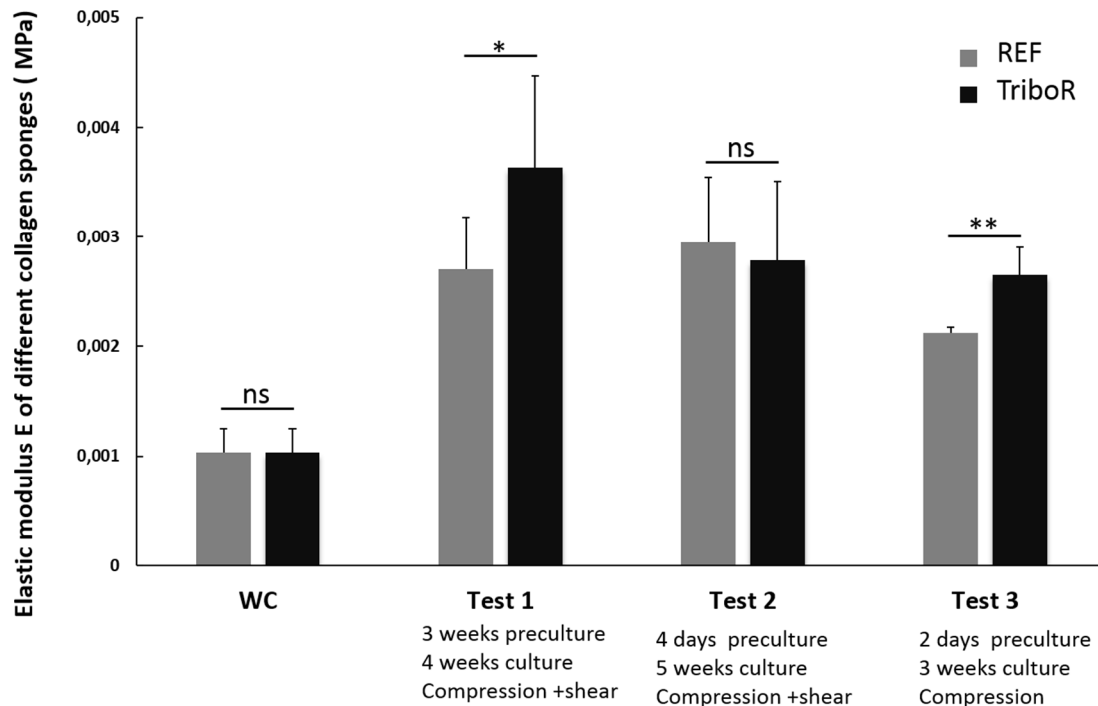


Figure 14 Mechanical properties of collagen sponges without cells (WC) or with chondrocytes cultured under or without mechanical stresses. Rheological tests on the sponges were performed with a rheometer, and the elastic modulus E was calculated. The elasticity of the “REF” sponges was compared with “TriboR” sponges. * $p < 0.05$, ** $p < 0.01$ ns: no statistical difference

In addition to the mechanical characterization, FRAP experiments are performed on “TriboR” and “REF” sponges from Test 3. The data showed that the distribution of the fluorescence is different depending on the sample:

- 1) Figure 15C shows the uniform fluorescence distribution of 70kD dextran-fluorescein inside the cell culture medium which served as the hydration solution of the sponges
- 2) Figure 15D shows the fluorescence which marks the fibers of the collagen sponges (REF sponges). The cells were not noticed because of the fibrous interface created around the sponge which did not allow more penetration into the sponge
- 3) Figure 15E shows the fluorescence which marks a uniform extracellular matrix around the cell clusters (dark areas). Also, in the background the fibers were noticed, however, they were covered by the cells and the synthesized extracellular matrix.
- 4) Figure 15A shows an example of the two obtained FRAP curves. The fluorescence diffusion was faster in TriboR sponges than in REF sponges. We also noticed the bi-exponential behavior which could be caused by a fast diffusion of the trapped culture

medium in the macroporosities of the sponge and by a slow diffusion at the level of the neo-synthesis of the cartilage.

- 5) A statistical analysis of the different diffusion times was performed (9 curves per sponge, 3 REF sponges, and 3 TriboR sponges, Test 3). Figure 15B shows that the REF sponge had a big difference between the two diffusion times, whereas the detected difference in the case of the TriboR sponges was not significant. This difference between the REF and TriboR sponges could be caused by the different structures of the two sponges. The TriboR sponges had probably a more homogenous structure compared to the REF sponges. This observation is in coherence with the results showed by figures 15D and 15E. These figures showed a homogeneous appearance of the TriboR sponge in which the fibrous matrix of the sponge seemed to be well incorporated into the neo-synthesized matrix, however, in the REF sponge, the fibrous areas were noticed isolated next to empty areas.

Overall, these results confirmed the role of mechanical stresses in the synthesis of the cartilage-like extracellular matrix and the capacity of the new device to handle a cell culture for four weeks without any contamination, further analysis for viability were performed with another team of the same project confirmed the innocuousness of the test (33).

Consequently, the performed different cell cultures presented an experimental validation of the “Tribo-bioreactor” as a bioreactor for cell culture. However, the stiffness of the collagen sponges was not suitable enough for a convenient mechanical stress transmission. Consequently, most of the synthesis of the cartilaginous matrix was at the periphery of the sponge. Therefore, it is considered to change the biomaterial scaffold for future experiments: agarose hydrogel.

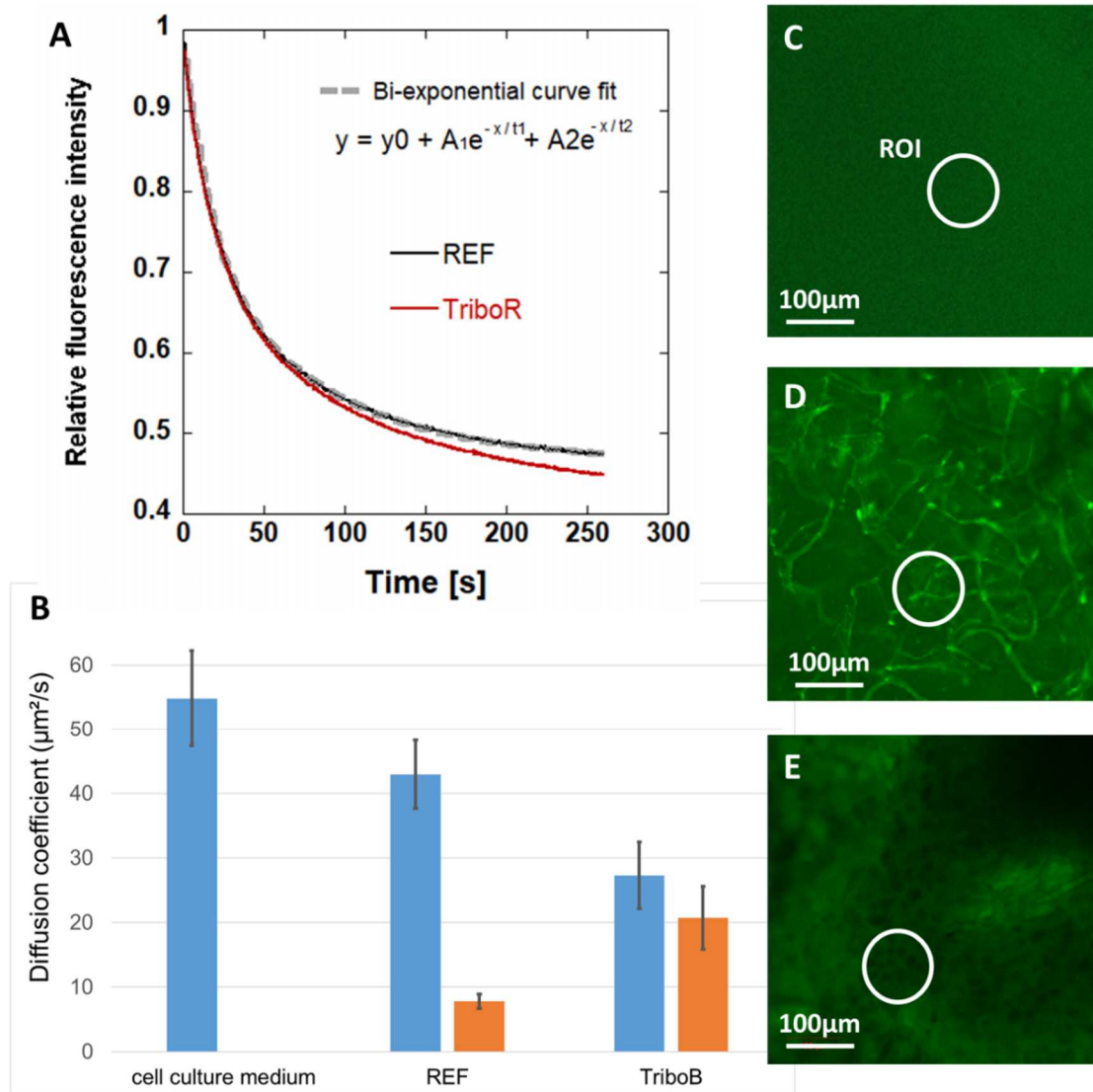


Figure 15 Different obtained data from the application of the FRAP experiments with the confocal microscopy

4. Conclusion

The primary purpose of this study is to develop a new simulator for tissue engineering, which combines biological, physicochemical, and mechanical constraints with the possibility to measure these constraints. A designed prototype presented a simulator with bi-axial loading allowing to follow the tissue development *in situ*. The originality of the “tribo-bioreactor” is allowing at the same time: (1) the control of the normal and tangential mechanical stresses applied to cell cultures, (2) the measurement of these stresses, (3) the control of the transmission of mechanical stress to cells. Each of these tests is independent and can be performed separately. However, the “tribo-bioreactor” can combine the three different tests in one. Briefly, it is

possible with the new device to perform a cell culture with appropriate mechanical constraints according to the real-time measurement of the physical parameters.

The performance of this new device may be promoted in the field of cartilage tissue engineering. It undergoes the limitation of other simulators as it can measure the transmitted load at the cellular level. Other tests are ongoing to improve the use of this prototype in cartilage reconstruction.

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DISCLOSURE STATEMENT

All authors declare no conflict of interest and confirm that none of them has disclosures.

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