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INK-JET PRINTED STRETCHABLE SENSORS FOR CELL MONITORING UNDER MECHANICAL STIMULI: A FEASIBILITY STUDY

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Impedance-based sensors represent a promising tool for cell monitoring to improve current invasive biological assays. A novel research field is represented by measurements performed in dynamic conditions, monitoring cells (e.g., myocytes) for which the mechanical stimulus plays an important role for promoting maturation. In this picture, we applied printed and stretchable electronics principles, developing a system able to evaluate cells adhesion during substrate cyclic strain. Cytocompatible and stretchable sensors were ink-jet printed using carbon-based ink on crosslinked poly(ε-caprolactone) electrospun mats. Moreover, a customized stretching device was produced, with a complete user interface to control testing condition, validated in order to correlate impedance changes with myoblasts — i.e., myocytes precursors — adhesion. Overall system sensitivity was evaluated using three different cell concentrations and DAPI imaging assay was performed to confirm myoblast adhesion. Preliminary results showed the possibility to correlate an average increase of impedance magnitude of 1 kΩ every 15,000 cells/cm^2 seeded, suggesting the possibility to discriminate between different cell concentrations, with a sensitivity of 80 mΩ/(cells/cm^2). In conclusion, the present system might be generalized in the development of future applications, including the differentiation process of cardiac myocytes with the aid of mechanical stimuli.

Keywords: Impedance-based sensors; cell monitoring; ink-jet printed sensors; stretchable sensors; equivalent circuit modeling.

1. Introduction

In recent years, increasing attention has addressed the development of techniques able to quantitatively monitor processes related to cell cultures — adhesion, growth and differentiation —, possibly including real-time features and high specific sensitivity, for specific applications in pharmacology, regenerative medicine and tissue engineering.[1]

Nowadays, techniques for cell culture monitoring include different types of assays able to estimate, for instance, imaging of cell nuclei (e.g., DAPI), viability (e.g., Trypan Blue, Calcein-AM), metabolic activity (e.g., MTT, Neutral Red), cell differentiation (e.g., immunostaining of differentiation markers). Unfortunately, these methods are in general time- and cost-expensive, user-dependent and destructive for the sample.[2]

The idea of getting a non-invasive feedback based on real-time quantitative measurements represents a really promising path to overcome the limitations related to conventional biological assays. In this regard, impedance-based monitoring represents a valid technique, in which electrochemical sensors are used as convenient and highly customizable technology capable of providing real-time quantitative feedback on physiological processes in cell cultures.[3,4] Starting from some pioneering groups in the early '90s,[5,6] impedance-based cell monitoring has been thoroughly validated during the following decades[7,8] for obtaining, in particular, quantitative information related to cell adhesion, growth, migration and differentiation or for recording cellular electrical activity,[9] using both monopolar or interdigitated electrodes.

So far, several types of cells and culture conditions — mainly static ones — have been evaluated using this strategy. Recently, the attention has moved to monitor also dynamic cell behaviors, such as those expressed by muscle cells.[10–12] In addition

to its traditional use related to monitor cell viability, growth and differentiation, impedance-based sensors have been therefore optimized to capture fast pattern based on the contractile movement of the cell membrane[13] and are emerging as leading platforms for detecting cardiac myocytes (CMs) beating and evaluating pharmacological effects in *in vitro* assays.[14]

A peculiar feature of myocytes is related with the role played by mechanical stimulation in their development, due to the transduction of various signaling pathways induced by the applied strain. Several studies investigated the effect of mechanical stimulation on these cells, focusing on different applications, including, for instance, the complete engineering of striated muscles[15] or the possibility to induce differentiation of CMs starting from stem cells, in combination with chemical and electrical stimuli.[16,17]

In this perspective, an attractive innovative field of research addresses the development of sensors integrated in customized systems able to record impedance changes simultaneously to cell mechanical conditioning, improving the information provided by the only static setup. This would allow obtaining a non-invasive feedback from cellular maturation under a proper mechanical stimulus.

The fundamental step required in order to achieve this goal is to transfer printed bioelectronics on stretchable sensors, thus to manage the mechanical conditioning while measuring impedance changes, that can be correlated to cell culture status.

Regarding stretchable sensors, several studies have been published in particular demonstrating the usefulness of Organic Electrochemical Transistors (OECT) approach using conductive polymers (e.g., PEDOT:PSS) to monitor biomolecules and cells.[18,19]

Despite this, the combination of impedance-based measurements with stretchable surfaces has been recently performed by a very limited number of studies,[10] therefore confirming the novelty of this up-and-coming topic.

Furthermore, printed electronics could represent an interesting tool to measure impedance changes during mechanical conditioning, with an innovative approach in terms of cost reduction and of more biocompatible and suitable materials for stretchable structures,[20] compared also with traditional impedance recordings based on metallic electrodes (e.g., gold, platinum).

Among the different available printing methods, ink-jet printing technology represents a very promising technique for the fabrication of sensors for cell culture monitoring due to the interesting compromise between cost effectiveness and output in term of resolution. As shown by our previous works,[1,21] this technique allows the effective deposition of conductive inks (e.g., carbon, PEDOT:PSS) to produce interdigitated sensors on various different substrates, including materials suitable for a mechanical conditioning (Poly-caprolactone (PCL), Nitrocellulose). In particular, considering the specific application mentioned above for myocytes, the material selected has to be able to be subjected to loading/unloading cycles without unwanted buckling effects (i.e., flexure upon load during the unloading step), thus to develop sensors addressing cell cultures monitoring under dynamic conditions.[22] Further, the geometry needs to be easily customized allowing the fabrication of

electrochemical sensors addressable for different applications and optimized for cell culture routine.[23]

Therefore, the specific goal of this work was to design a complete system able to measure impedance while performing mechanical conditioning, thus to realize a platform able to evaluate the monitoring of specific cells while they are undergoing a defined mechanical strain. More in details, interdigitated sensors were realized by means of ink-jet printing technology using carbon ink — well known for affecting positively myocytes cultures[24] — and crosslinked PCL mats, which already demonstrated their cytocompatibility and optimal mechanical features.[21] Final sensors were then tested and validated into a customized system, designed to reliably control substrate strain and to continuously interface with an impedance analyzer and — without losing generalizability — by using myoblasts, thanks to their phenotypic similarity to cardiac muscle, ease of isolation/expansion and relative resilience to hypoxia,[25] therefore optimal for a feasibility study of the proposed approach.

2. Materials and Methods

2.1. *Sensors fabrication and conditioning system description*

Stretchable substrates were obtained as electrospun mats made of properly synthesized crosslinked poly(ε-caprolactone) (PCL), starting from α,ω-triethoxysilane-terminated PCL crosslinked through sol–gel reaction. The crosslinked structure of the PCL ensures that the electrospun maintains its fibrous morphology when heated above the PCL melting temperature ($T_m = 55°C$), as required within the ink printing procedure. Further, the adoption of a sol–gel chemistry, allows to avoid potentially toxic radical initiators. Details concerning the synthesis of the PCL precursors are reported in Ref. 26, whereas a facile incorporation of the crosslinking reactions throughout an electrospinning procedure is explained in Ref. 27. The final substrate obtained was a 50 μm thick mat, constituted by continuous randomly oriented fibers, with a diameter of about 2 μm.

Since the final aim was to monitor — with the least possible variability — the adhesion of a large population of cells, an interdigitated geometry was designed, taking into account the necessity to realize a sensor with dimensions compatible with 12 multiwell plates for cell cultures. Thus, interdigitated electrodes with 300 μm thick teeth with an overall dimension of 0.6×1 cm were printed onto previously cut regular rectangles of PCL mat. Sensors were realized by using carbon nanoparticles ink (JR DEV 79-79-9, Novacentrix; sheet resistance approximately 800–2000 Ω/sq) with a commercial ink-jet printer (Epson C88+). More specifically, in order to increase electrode conductivity, the printing process was repeated 10 times, allowing the ink to dry after every passage. After the deposition of the last layer, sensors were cured for 15 min at 110°C.

In order to validate the stretchable sensors, it was designed a system to allow the mechanical conditioning, and a customized interface to control specific strain rate and ensure the possibility to measure impedance changes over a set of

specific frequencies, during time, connecting to the impedance analyzer (HP4194A). This system was realized by integrating a one-step motor (Nanopro, NanoTec) able to reliably control the position of two easily mountable clamping tools: one fixed to the supporting rig and the other one connected with a movable bar. In order to limit the perturbation of the cell culture environment, the impedance measurements were performed by using the mechanical contact between the carbon ink of electrodes extremities and the metal clamps in the stretching device, thus avoiding any soldering issue (Fig. 1).

Regarding the acquisition system, a dedicated LabVIEW program was designed, and the impedance analyzer was programmed to acquire eight frequencies each cycle (selected due to the most significant ones indicated in the literature,[28] more

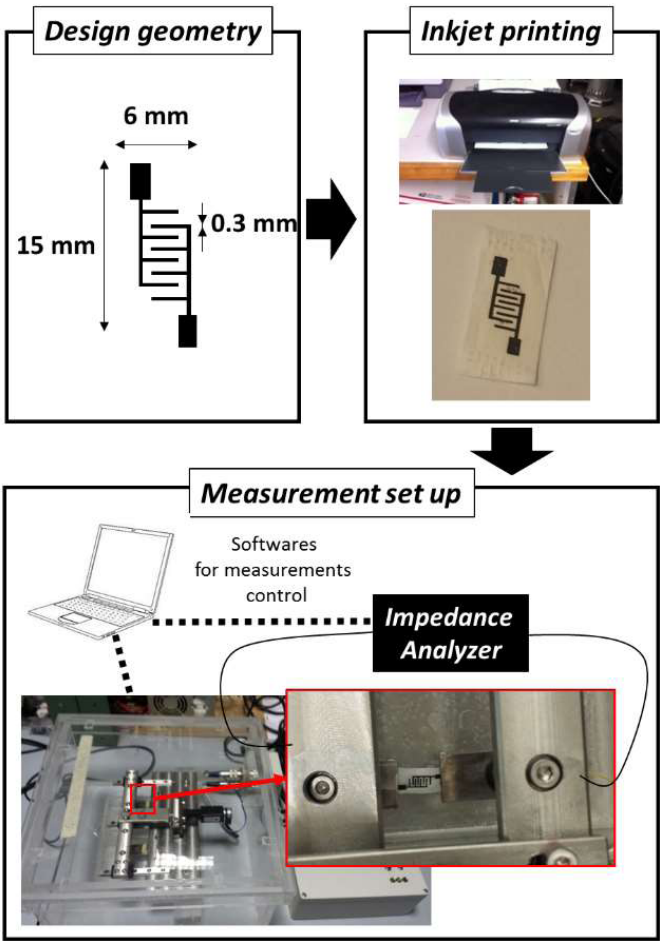


Fig. 1. Sensors and stretching device setup. Sensor geometry and final printed layout (above); system for sensors mechanical conditioning and sensors gripping clamps for measurements both in static and dynamic condition (below).

specifically: 400, 1000, 4000, 10 000, 40 000, 100 000, 400 000 and 1 000 000 Hz). The cycle is repeated every 250 ms, in order to ensure the acquisition of eight impedance sweeps for each cycle of strain (2 s).

2.1.1. *Qualitative evaluation of cytocompatibility*

Before cell seeding, sensors were washed with phosphate buffered saline (PBS) and fixed to the bottom of culture dishes using a biocompatible high vacuum grease that prevents their floating in the culture medium. After that, they were sterilized under UV radiation and finally coated with gelatin from porcine skin (Sigma Aldrich) to improve cell adhesion. In order to evaluate cytocompatibility, sensors were seeded with L6 rat myoblast (30 000 cells/cm^2) using a common complete cell culture medium and incubated for 24 h at 37°C with 5% CO_2.

Cell adhesion and vitality evaluation were performed using DAPI (4′,6-diamidino-2-phenylindole), that is a DNA-intercalating fluorescent dye. Precisely culture medium was removed and, after washing with PBS, myoblasts were fixed for 10 min with Immunofix (Bio-Optica). Cells were then incubated for other 10 min in the dark with DAPI (Life Technologies) and adherent myoblasts were observed under fluorescence microscopy (AXIOVERT 200M epifluorescent microscope, ZEISS). The testing protocol was repeatedly performed on 12 sensors, realized by using the same geometry, parameters for the printing process and batches of materials.

Images obtained were analyzed using Fiji software (an ImageJ distribution), and then compared with results from impedance evaluation.

2.2. *Impedance-based static cell adhesion monitoring and sensors equivalent circuit*

Preliminary impedance assessment was carried out by using a standardized static measurement protocol applied before and after 24 h from cell seeding. These measurements were used to model the contribution of cell adhesion by means of an equivalent electric circuit. This analysis was specifically performed to assess the differences between fixed and live cells, in order to validate the proposed approach and optimize measurement setup. Furthermore, any possible source of interference related to the instrumentation was examined (e.g., cables, one step motor, holding device). Thus, in this phase all the experiments were performed by seeding 30 000 cells/cm^2 over 20 sensors, using a complete cell culture medium commonly used for L6 cells and incubating the sensors for 24 h at 37°C with 5% CO_2 in multi-well plates. Sterility was preserved for all the experiments when dealing with live cells. After 24 h of cell culture, 10 samples were used to directly perform impedance measurements on live cells, and 10 samples were prepared fixing the cells by using paraformaldehyde and then the impedance was measured by using the same protocol. The pre-seeding tests were carried out with medium after gelatin coating to obtain a value of reference, comparable with the measurements after-seeding. The measurements were performed by using the impedance analyzer HP4194A, keeping

the sensor in a horizontal position. For each sensor three measures have been performed, recording impedance (magnitude and phase), in a range of frequencies between 400 Hz and 400 kHz. The tests were performed for a total of ten sensors for each condition.

The same measurement was performed with live cells and with cells fixed with paraformaldehyde in order to evaluate any possible interference of the fixing procedure with the possibility to correlate changes of the electrical impedance parameters with cell adhesion. Furthermore, in order to take into consideration any difference due to the measurement setup, the impedance measurements were performed using two different setup. For live cells, keeping the sensors in a multiwell plate, the impedance was measured by means of two contact tips over the external pads, while for fixed cells, after mounting the sensor into the stretching device, the impedance was measured from two wires directly fixed in contact with the terminal pads.

For in depth analysis of impedance spectra alterations, magnitude and phase angle spectra were fitted based on an electronic equivalent circuit model for the tissue–electrode interface.[29] Parameters of the equivalent circuit were determined in a two-step optimization procedure considering static measurements performed sweeping all the frequencies in the range from 400 Hz to 400 kHz. First, the contribution of the not-seeded sensors was modeled considering a reduced equivalent circuit, without the cell-specific parameters, including electrodes and solution electrical contributes (R electrode, C dl, Z Warburg for ink diffusion, R solution and C solution). Subsequently, the cell-covered electrodes were analyzed by applying the entire equivalent circuit, thus including cell membrane/intercellular capacitance (C Cell) and resistance (R Cell) as well as extracellular resistance (R gap), determined by the fitting procedure. For fitting the impedance data acquired from the analysis the software EIS Spectrum Analyzer (EISSA1) was used.[30]

2.3. *Mechanical conditioning tests*

Although the crosslinked electrospun PCL, we used as substrate, was already investigated by means of thermo-mechanical and cyclic tests in a previous work.[25] In order to ensure the application of the correct strain to the samples and reduce mechanical hysteresis — and any possible resulting buckling, we performed a further mechanical characterization focused on the requirements of the proposed application. More in detail, by employing a dynamic uniaxial mechanical analyzer (DMA Q800, TA Instruments) under strain-controlled conditions, 100 cycles at 5%, 10%, 15% and 20% maximum strain were subsequently applied to the substrates and then two different ranges (between 5% and 15%, and between 7% and 17%) were tested in order to ensure the required 10% strain, avoiding sensor buckling. All these procedures were carried out at a frequency of 0.016 Hz in order to optimize the material response. Finally, this standardized pre-conditioning protocol was applied to the printed sensors by using the customized stretching device. In detail, printed

sensors were immersed overnight in culture medium in order to provide the same condition as required for seeded samples, washed using PBS, and then mounted on the stretching instrument. Then, the pre-conditioning protocol was performed as a training phase for the substrates, covering the entire desired strain range defined for cell mechanical stimulation. All the 15 sensors addressed to the dynamic tests were conditioned in order to ensure the repeatability of the measurements. For all the validation tests, the velocity of the one-step motor was then set to 1 mm/s, in order to ensure a frequency of 1 Hz when applying the final desired 10% strain value. These values of strain and frequency were selected in order to reproduce dynamic conditions similar to those undergone by cells during heart beating, thus aiming at future application with live cells.

Pre-conditioned sensors were then coated with porcine gelatin in order to obtain a value of reference, comparable with the measurements obtained after-seeding. Thus, after transferring each sensor in the stretching device, pre-seeding measurements were performed both in static and under mechanical conditioning, applying 10% strain, with a frequency of 1 Hz. Impedance was recorded as magnitude and phase angle, as specified in the description of the system. Particular care was put in maintaining the correspondence between each pre- and post-seeding measurement.

2.4. *Cell adhesion monitoring under dynamic conditions*

After pre-seeding measurements, all the sensors were seeded with L6 rat myoblast and incubated for 24 h at 37°C with 5% CO_2. Three different concentrations of cells were seeded (15 000, 30 000 and 60 000 cells/cm^2), thus to evaluate the ability of the sensor to discriminate among different amounts of cells, adhered on its surface. Five sensors for each concentration were considered, in order to evaluate the repeatability of the measurements.

After 24 h from seeding, sensors were prepared for impedance measurements. Precisely culture medium was removed and, after washing with PBS, myoblasts were fixed for 10 min with Immunofix (Bio-Optica). Sensors were then kept immersed in PBS until impedance measurements.

After fixing myoblasts, wet cultured sensors were placed in the stretching device and post-seeding impedance measurements were carried out at room temperature using a standardized protocol in order to correlate variations in the electrical impedance (i.e., module and phase) with cell adhesion, considering them both in static and dynamic conditions. DAPI assay was performed after impedance recording under mechanical conditioning in order to exclude the detachment of cells.

For each sensor, two preliminary impedance measurements were performed under static conditions, specifically acquiring the impedance magnitude and phase angle, in the selected frequencies.

After that, dynamic measurements were performed, recording the impedance in the same frequencies, while applying a 10% cyclic deformation to the sensor

(i.e., from 0% to 10% stretching and back). The measurements were performed by using the system (described in paragraph A) connected to the impedance analyzer. For each sensor two measures of at least 5 min each were performed, corresponding to at least 300 cycles. The same protocol was repeated for each one of the three seeded concentrations of cells (15 000 cells/cm^2, 30 000 cells/cm^2, 60 000 cells/cm^2). Tests were replicated on five samples for each condition, in order to allow a proper statistical analysis. In order to compare the results without cells and with cells at different concentrations, the impedance variation due to the mechanical stretching was neglected by considering the average value measured during the stretching cycle at each defined frequency. Using these averaged signals, the impedance contribution of cells for each concentration was estimated by subtracting the impedance magnitude measured before seeding to the one obtained after seeding. After that the values corresponding to 4 kHz (taken as reference frequency as reported in the literature[31]), were compared.

2.5. *Statistical analysis*

Statistical analyses were performed by using Graph Pad Prism version 5.0 (Graph Pad Software Inc., San Diego, CA, USA) and Excel (Microsoft Inc). All the numerical values are descriptively reported as mean ± standard deviation (SD). Differences (Δ) in mean impedance magnitude among different cell concentrations were determined by one-way ANOVA, followed by Bonferroni *post hoc* correction. Differences were considered statistically significant for a p-value < 0.05.

3. Results

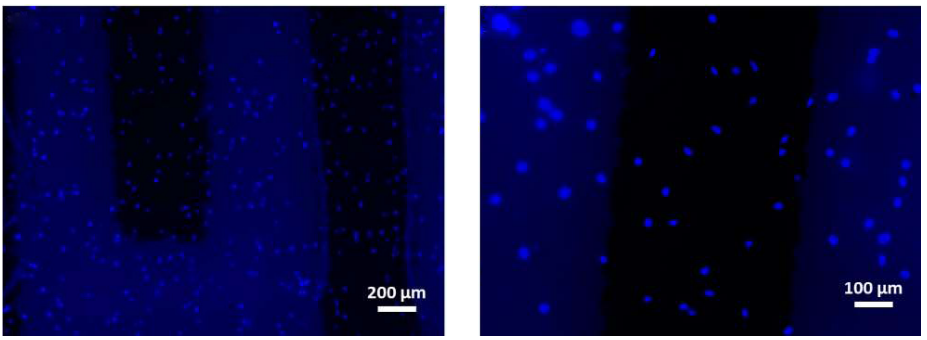
3.1. *Qualitative evaluation of cytocompatibility*

Results from DAPI assay showed a good cell viability on all the produced sensors. In particular, myoblasts appeared to be adherent and evenly distributed on the surface of each sensor, both on deposited carbon tracks and PCL mat, suggesting a good cytocompatibility of both ink and substrate (Fig. 2).

In addition, cell concentration estimated by analyzing DAPI images was about 35 000 cells/cm^2; a value which confirmed the cytocompatibility of the sensors and that was coherent with the number of cells initially seeded per unit of surface (30 000 cells/cm^2).

3.2. *Impedance-based static cell adhesion monitoring and sensors equivalent circuit*

Results obtained from static impedance evaluation before and after cell seeding suggested the possibility to correlate cell adhesion to impedance changes. In particular, the carbon on PCL sensor showed a good trend with an increase of impedance after the cell seeding compared to the pre-seeding and in agreement with DAPI assay results. It can be appreciated how the presence of the cell



(a) (b)

Fig. 2. Qualitative evaluation of cytocompatibility. Myoblasts L6 cells (30 000 cells/cm^2) seeded on sensors stained with DAPI to evaluate cell adhesion: 5× (left), 10× (right). Cells appeared adherent and well distributed on both carbon ink and PCL substrate.

changes the measured impedance in the range of 10^2–10^6 Hz, with the biggest change recorded at frequencies in a range near to 10^3 Hz in all sensors tested. Measurement performed after fixing cells showed comparable results to the ones with live cells.

Regarding impedance magnitude, a similar increase in the order of 2 kΩ could be recorded, even if with a similar response for all the frequencies evaluated. Regarding phase angle a similar trend could be recorded, suggesting the possibility to perform reliable measurements even with fixed cells.

Impedance measurements (magnitude and phase angle spectra) were fitted using the equivalent circuit-based as shown in Fig. 3, resulting in the following parameters: R electrode (around 600 Ω) and C dl (double layer capacitance, around 1 nF) for the electrode, Z Warburg for the effect of respectively of ink layers diffusion (around 50 kΩ/$\sqrt{\text{sec}}$), R solution (100 Ω) and C solution (3 nF) for the effect of electrolytic solution and R Cell (1 kΩ), C Cell (10 nF) and R gap (400 Ω) due to cell coverage.

Results from pre-seeding evaluation highlighted significant differences of baseline impedance among each sensor, with a deviation from mean of about 500 Ω. This could be attributed to the variability introduced during the printing process and during sensors mounting for setup preparation. Thus, in order to compare coherently the findings obtained before and after cell seeding, each pre-seeding measurement was compared with the post-seeding measurement performed on the same sensor (comparison for paired samples).

3.2.1. *Sensors pre-conditioning protocol and pre-seeding measurements*

Results from pre-conditioning protocol showed that the strain range from 7% to 17% can be selected as the optimal range to ensure a stable strain amplitude, (Fig. 4) after the whole aforementioned protocol.

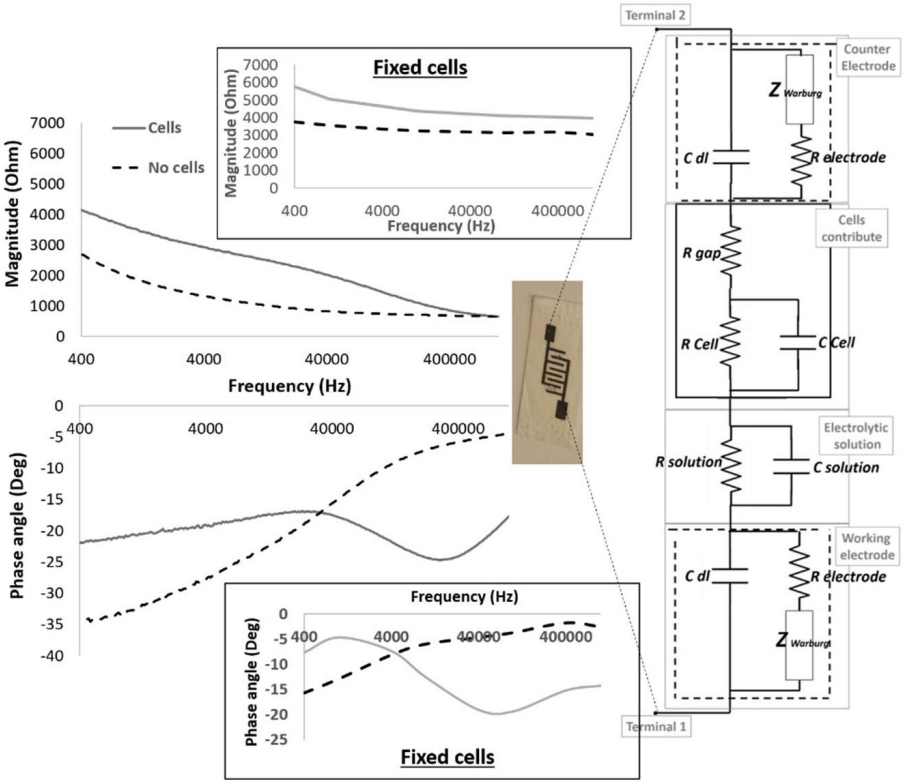


Fig. 3. Static impedance results of live and fixed cells and equivalent circuit model. Comparison between impedance module and phase angle with and without seeded cells and a schematic representation of the equivalent circuit model.

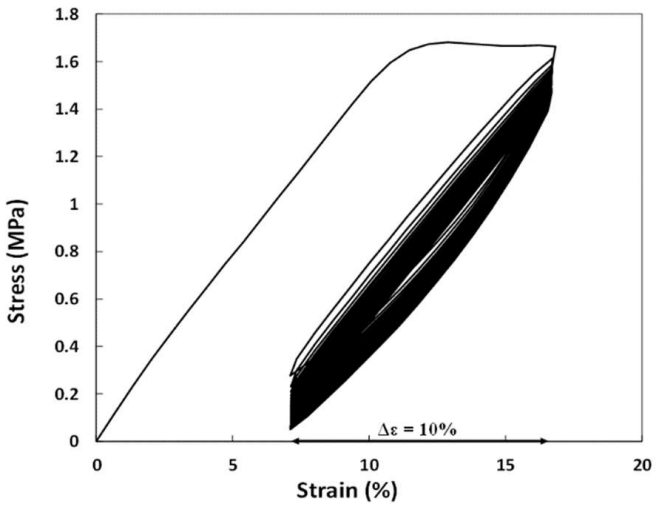


Fig. 4. Cyclic mechanical behavior in terms of stress–strain curve. Strain range from 7% to 17% after the whole pre-conditioning protocol, showing a stable strain amplitude of 10%.

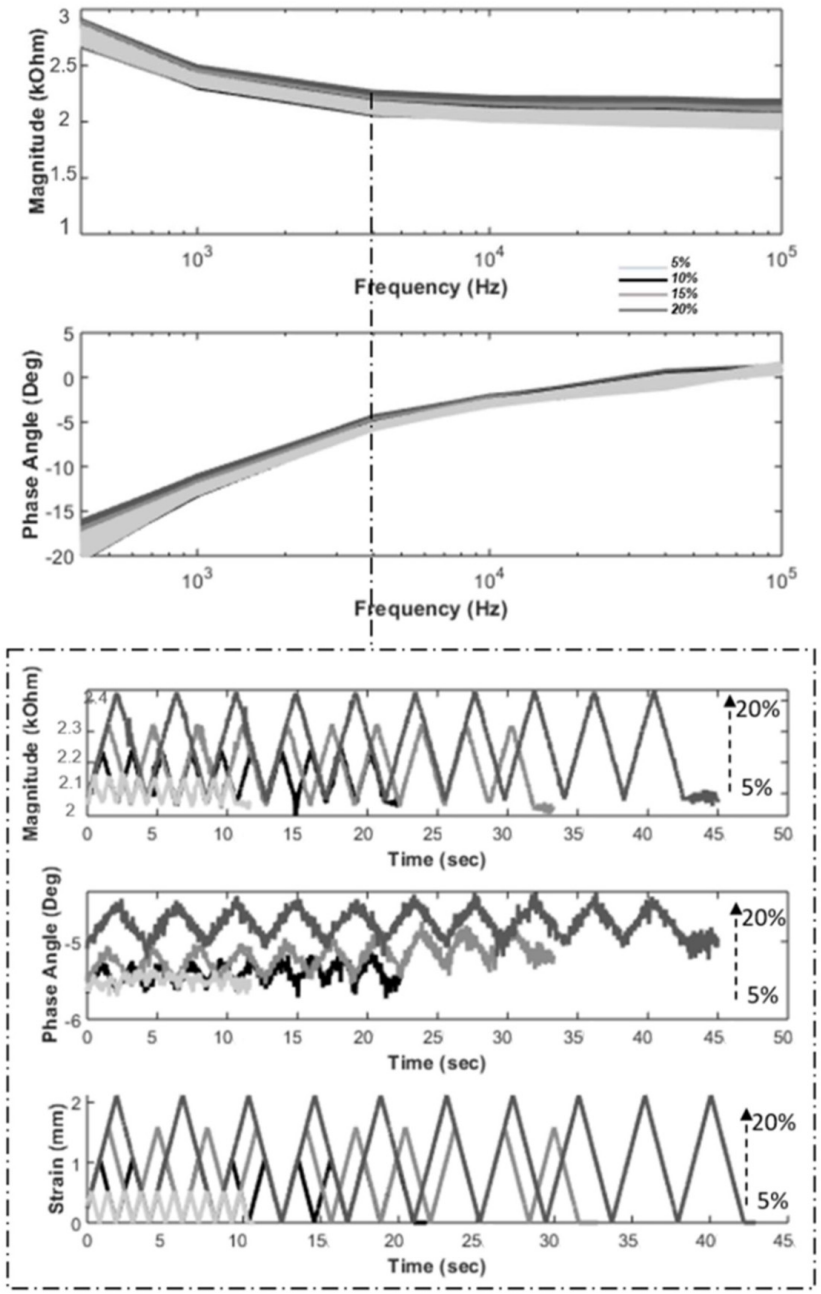


Fig. 5. Impedance variation during sensor pre-conditioning. Sensors impedance versus frequency measured during the different steps of conditioning protocol (left), Sensors impedance versus time at 4 kHz and correspondent displacement values (right).

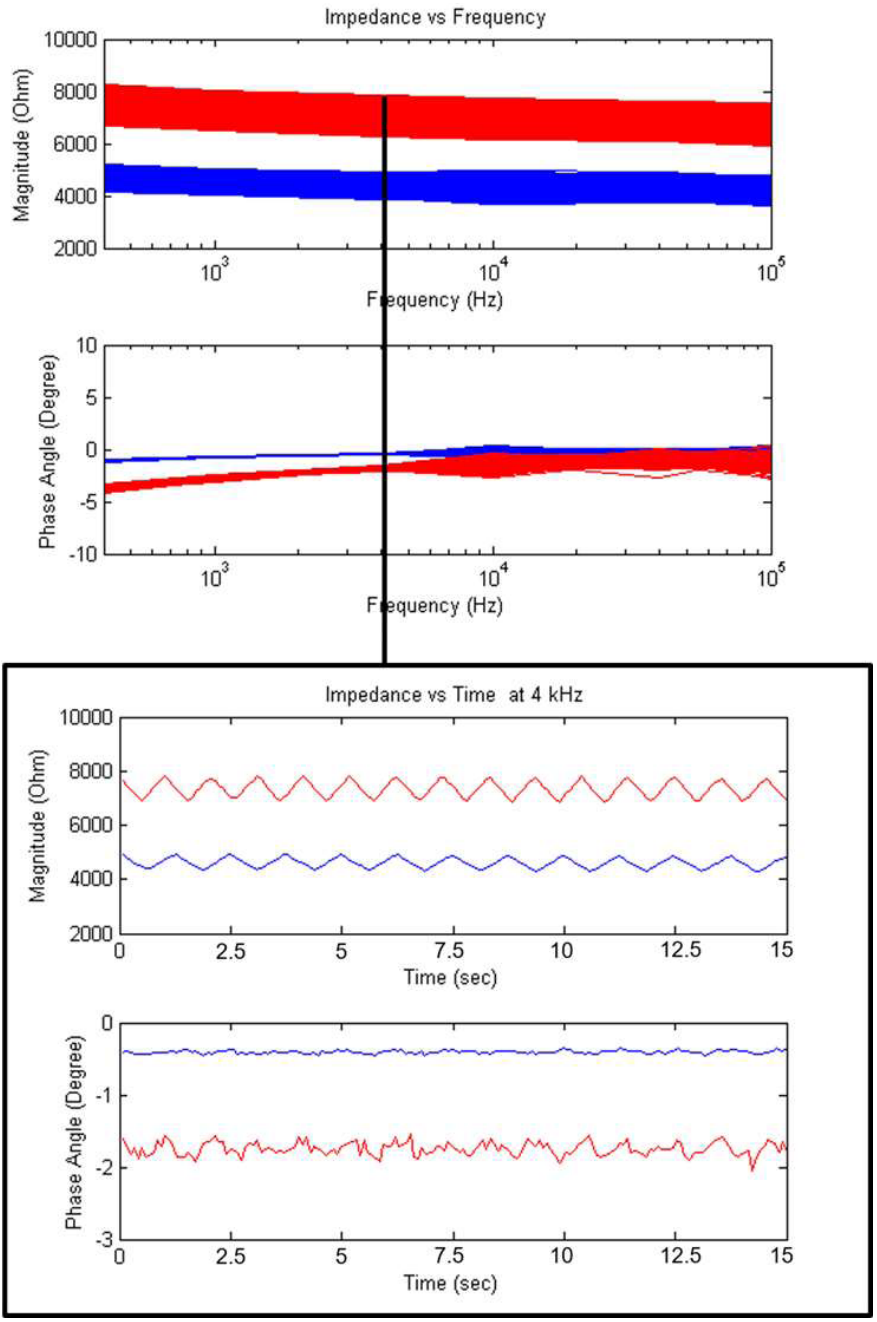


Fig. 6. (Color online) Dynamic measurements in frequency and time domain. Comparison between module and phase angle recorded from sensors prior (Blue) and post (Red) to cell seeding in frequency (left) and time (right) domain.

In fact, choosing a smaller minimum deformation (around 5%) causes a buckling effect due to the hysteretical response of the electrospun, determining, as experimentally shown, a continuous reduction in the stress level with the cycle number and the introduction of an irreversible deformation.[21]

Results from preliminary impedance measurements performed both in static and dynamic conditioning, showed a capacitive-like behavior around lower frequencies (phase angle = −20°) and a resistive-like behavior with higher frequencies (phase angle near to 0°). During the pre-conditioning sessions performed at different strain values, a variation of the impedance value (in the range of the 10%) could be identified in sync with the sensor cyclic strain (as it is highlighted in paragraph D, results section). More in detail, increasing the deformation values from 5% to 20%, the impedance variation recorded increased from the 4% to 12% of the maximum impedance value at each strain (Fig. 5).

3.3. *Cell adhesion monitoring under dynamic conditions*

Data obtained from impedance evaluation before and after cell seeding under mechanical conditioning in the range of deformation of 7–17% suggested the possibility to correlate cell adhesion to impedance changes also in dynamic conditions.

In agreement with the results obtained before cell seeding, a variation of impedance values, synchronous with the cyclic strains, was observed also considering cell adhesion (Fig. 6).

The sensors showed in particular an increase of impedance after the cell-seeding compared to the pre-seeding, and in agreement with DAPI assay results.

From sensors imaging analysis performed after the stretching, cells appeared to be still well-adherent and distributed uniformly on all the sensor surface; this finding confirmed the possibility to perform measurements under mechanical stretching without cell detachment from the sensor itself (Fig. 7(a)). Cell counting performed with Fiji software showed in fact cell concentration of 23 000, 33 300 and 80 000 cells/cm^2, likely compatible with the respective number of cells initially seeded on the different sensors.

In particular, the presence of cells changed the measured impedance proportionally to cell concentration, with a similar increase in all the range of frequencies tested, with a good repeatability for all tested sensors. The difference pre–post seeding in impedance magnitude increased consistently and linearly with the number of cells seeded. More specifically, the increase in magnitude was in the order of 1, 2 and 5 kΩ for sensors seeded with 15 000, 30 000 and 60 000 cells/cm^2, respectively (Fig. 8). Results from the ANOVA test at 4 kHz confirmed the possibility to discriminate between different cell concentrations. In particular, a statistically significant increase of Δ magnitude coherent with cell concentration could be observed comparing 15 000 versus 60 000 and 30 000 versus 60 000 cells/cm^2 (Fig. 8).

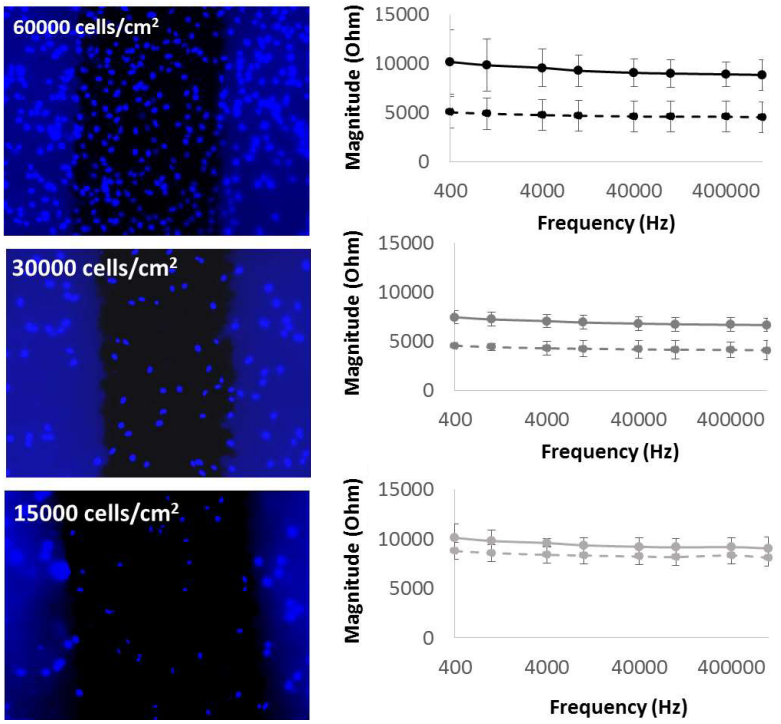


Fig. 7. DAPI versus impedance-based dynamic measurements. Results from DAPI imaging after dynamic measurements for the three cells concentrations (a). Impedance evaluations under mechanical conditioning, performed before and after 24 h from cell seeding, averaged for each frequency drafted lines referred to pre-seeding tests, solid lines to post-seeding cells after cell adhesion (b). Difference between impedance post and prior to cell seeding versus frequency (c).

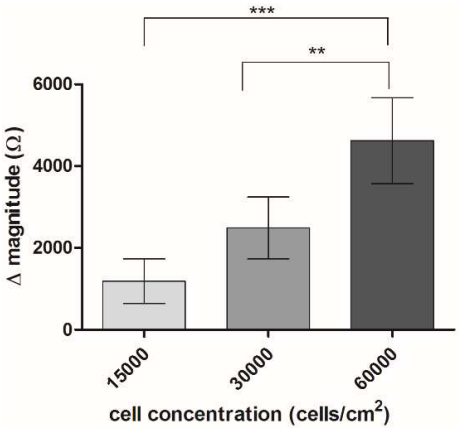


Fig. 8. ΔMagnitude between impedance module post and prior to cell seeding at 4 kHz for different concentration evaluated (15 000, 30 000 and 60 000 cells/cm^2). The statistical significance was represented as follows: $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

One of the main hurdles regarding fields like tissue engineering, pharmacology and regenerative medicine is represented by the possibility to quantitatively monitor in real-time cell adhesion, growth and differentiation processes. This chance would allow to engineer cell cultures, improving the well-accepted model for drug testing and pathology development.[32] Nowadays, testing methods are often invasive, time- and cost-consuming and, in general, destructive.[33]

Interesting developments are given by impedance-based sensors, in which electrochemical sensors are used as convenient and highly customizable technology capable of providing real-time quantitative feedback on physiological processes in cell cultures.[3,4] Following the basic principle of measuring the quality of the cell barrier through changes in the impedance, cell proliferation, barrier function, cell junctions and cell motility are investigated using a constant alternating current with a given frequency.[34] Mainly, the materials used until now in the realization of this type of sensors are noble metals (i.e., gold or platinum), for both two-dimensional (2D) electrodes and sensitive elements (mainly nanoparticles or wires) in three-dimensional (3D) environment.[35–38] In the last decade, technologies related to printing electronics, often complementary to organic electronics, caused a great innovation in the field of sensors and electronics for biotechnological applications, both in terms of reduction of production costs and in term of more biocompatible and suitable materials for stretchable structures.[20] In this field, the use of electrochemical sensors coupled to stretchable printed electronics opens a number of interesting paths in order to perform continuous monitoring of cell properties and behavior, without affecting standard culture conditions and even addressing excitable cells, such as muscle cells.[39]

In this picture, the main finding of this work was the validation of the use of an ink-jet printed sensor — compatible with cell culture routine and with mechanical stretching — for the specific assessment of the adhesion of myoblasts but winking and aiming in future developments at monitoring CMs reliably obtained during growth and differentiation from iPSCs.

The choice of carbon-ink and PCL-substrate was specifically performed after a deep analysis, assessment and comparison of several inks and materials.[1] Carbon and carbon nanotubes, among cytocompatible materials, are an optimal solution to record and stimulate contractile cell cultures and furthermore they have been demonstrated to promote CM differentiation, useful for future applications of the very same setup.[24] Furthermore, PCL represents one of the most promising materials in tissue engineering, with a set of properties which respond to the specifics requirements for compatibility, mechanical stretching and printability. Further, in order to address the presented application and overcome specific drawbacks related to thermal stability (i.e., loss of fibrous morphology in case of a non-crosslinked PCL), a stable crosslinked structure of PCL was obtained following the specific protocol described in Ref. 27. This ensures the possibility to treat the material above T_m during the ink-printing step without the loss of its micro-fibrous structure. As suggested in a previous work,[21] the proper pre-conditioning performed on the sensors before cell culture and the choice of the stretching range in which the sensors operated, allowed to apply the desired 10% strain amplitude without any evidence of buckling during unloading phase. Here-performed DAPI assay confirmed the cytocompatibility of the proposed solution, which included both carbon ink and crosslinked PCL.

Concerning electrical characterization, a significant increase in impedance magnitude and a change toward a capacitive-like behavior of the phase could be appreciated both in static and dynamic conditions due to cell adhesion. Regarding the reported averaged results, the observed increase of impedance magnitude remained almost constant for the whole range of frequency, without any evident peak at specific frequency; this "smoothing" effect was probably due to the fixing procedure, whose contribution remained in any case equal for every considered concentration. However, considering a subset of single measurements, interestingly the Δ magnitude between not-seeded and seeded sensors appeared to be increased for frequencies in a range between 4 kHz and 100 kHz, similarly to the results obtained on live cells and reported in Fig. 3. This finding is in perfect agreement with several results reported by literature.[28,31,40] In particular, most of the data has been recorded at an AC frequency of 4 kHz since this frequency has been found to be the most sensitive for the most frequently used cell lines. At this frequency, in fact, the contribute of cells to impedance variation can be measured without interference of cables or other sources.[31] However, this frequency has been shown to be different depending on the morphology of the specific cells. In other words, the difference between impedance of cell-free and cell-covered sensors is bigger at the frequency where the cells block the current most effectively, i.e., acting as insulating material.

Several findings in the literature demonstrated that even higher frequency, superior to 40 kHz, are interesting in order to gain information related to cell adhesion and spreading of epithelial and fibroblasts cells.[7] Similarly, other works highlighted 100 kHz as optimal value for sensing 30 μm diameter cells, suggesting that also cell size and morphology should be considered in defining the optimal sensor in order to obtain the highest value of sensitivity.[28] Therefore, these findings represent an interesting starting point to use this technique even as a method able — for instance — to investigate myoblast aggregation in myotubules by identifying a shift in the frequency, in addition to the increment of module. The equivalent circuit obtained highlighted that the effective cell coverage can be electronically schematized with the introduction of a RC parallel circuit, in agreement with the literature.[4,29] Moreover, the Warburg contribute added may be discussed and justified taking into account the interaction between electrolytic solution and the multiple ink layers.

The optical analysis of DAPI assay performed after mechanical conditioning appeared in agreement with impedance results. More specifically, cells imaged after cyclic strain appeared adherent and uniformly distributed, suggesting the feasibility of the presented technique. In addition, the images obtained from fluorescence microscopy after the tests gave positive results also about the status of the materials, underling a good mechanical adhesion of the ink (no delaminated areas) and an optimal strength of the substrate under fatigue (no visible cracking areas).

Concerning identified limitations, before cell seeding, a high variability in impedance was observed among the different tested sensors. This suggests that the proposed technique should be better standardized, above all concerning the printing process and setup preparation. This limitation was however overcome in this study by performing a paired samples comparison, i.e., measuring the impedance for each sensor before and after cell seeding.

The choice of using fixed cells for the validation of the proposed method needs to be discussed in more details. By analyzing scientific literature, we specifically found that the fixing procedure primarily act on cell ultrastructure[41] or on nucleic acids[42]; both these effects are of low relevance for the scope of our work, since they minimally affect the cell impedance characteristics. As highlighted in a recent paper by Hobro and Smith[43] — that compared the morphology of live cells with aldehyde-fixed ones — the use of aldehydes for cell fixation results in relatively minimal changes to the cells, if compared to those provided by organic solvent-based methods. Furthermore, this choice was performed only after evaluating the possible effects introduced by formaldehyde on impedance measurements in static condition. As shown in Fig. 3, the reported analysis highlighted a similar increase in impedance magnitude and a similar trend for phase angle in both the conditions. Thus, fixed cells were considered as an acceptable compromise for assessing the feasibility of the proposed method and the ability of the system to correlate changes of impedance to different numbers of cells attached on stretchable sensors under static and dynamic conditioning, validating the overall setup.

Of course, despite this, the results obtained will need to be discussed for any future use of the present setup for long term monitoring of live cell growth and differentiation. Studies over longer term periods will be required to confirm the possibility to preserve the sterility or any long-term effect of the mechanical conditioning on cell attachment to sensor surface. Thus, despite the aim of this work was just to properly demonstrate the possibility to use this approach for monitoring cell adhesion, specific design considerations have been performed for future applications with live cell cultures. The stretching system have been properly designed with autoclavable materials, with a petri like cover, in order to be able to preserve the sterility even for long term cell cultures. Furthermore, the part addressed to mount the sensor has been designed able to host four different sensors, with separated wells for allowing cultures with different culture medium and avoid cross contamination. Interestingly, impedance analysis performed on dynamic and static conditions are reliable and comparable. This represents an important starting point in order to confirm the possibility to adopt this kind of stretchable sensors and the presented setup to monitor cell growth on a long period of time, and translating this same experiment to other kind of cells such as CMs.

The results obtained from the dynamic evaluation of the sensors confirmed the possibility to apply strain in the physiological ranges of around 10%, well known in the literature as the strain induced in CMs by the physiological rhythmic heart beating. Further, numerous studies show that this mechanical conditioning might promotes structural and functional maturation of mouse and human CMs, if associate with a series of complex other stimuli.[16,39]

5. Conclusions

In conclusion, we presented a system addressed for evaluating cell adhesion under mechanical conditioning by means of impedance-based measurements. To reach this aim, stretchable sensors were specifically designed and realized using ink-jet printing technology, depositing carbon-based ink on stretchable PCL substrates; further, a stretching device was implemented and customized with proper interfaces in order to control both mechanical and electrical parameters during dedicated testing.

Without losing generalizability, the results here presented validated the overall methodology by using myoblasts as testing cell lines. Successfully performed experiments confirmed the possibility to use the provided solution in order to obtain information about cell adhesion (differences in impedance magnitude between pre- and post-seeding) and cell concentrations by recording an increase of impedance magnitude of about $80\,\mathrm{m\Omega/(cell/cm^2)}$. Moreover, the results from the comparison between pre- and post-seeding allowed us to detect specific frequency ranges that in future works could be used to develop a dedicated wireless portable electronic device for continuous monitoring of cell impedance for specific cell cultures.

Particular care should be put in reducing the variability related to the printing process and in setting up the culture, improving the portability of the overall system (maybe with wireless modules) and — in a perspective of providing a complete self-standing bioreactor — considering also the control of culture parameters, including temperature and humidity.

Overcoming these limitations would allow the use of this system to continuously monitor the dynamic behavior of several types of cells, including CMs during differentiation process, mimicking physiological conditioning, thus to support and improve traditional methods.

Author Contributions

S. T. and M. M. equally contributed to this work conceiving, designing and performing the experiments, analyzing data, writing the paper; M. L. S. performed the part of the experiments related to cells seeding; M. B. and M. S. designed the stretching device and the tool for data acquisition; M. S. and N. F. L. contributing in designing the experiments and reviewed the paper; N. I. contributed in optimization of the mechanical protocol and reviewed the paper; C. G. and M. L. F. contributed in realizing the crosslinked PCL electrospun; P. D., D. U. and E. S. reviewed the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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