

Magnetoelastic Monitoring System for Real-time Tracking of Cell Density and Growth Kinetics

by

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DISSERTATION ABSTRACT

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Cell-based medical treatments, such as stem cell therapies, have received increased attention from health researchers and medical companies due to their ability to address historically difficult or impossible-to-treat ailments such as Huntington's disease, multiple sclerosis, organ transplant rejection, auto-immune diseases, and more. However, access to high-efficiency and high-yield methods of stem cell production for therapy and research have been inhibited by a lack of robust and scalable methodologies for growing and monitoring stem cells and their attributes in clinical and industrial settings. Modern cell counting and monitoring technologies often lack the resolution and accuracy required to be effective for production and expansion of adherent cell lines, particularly in the context of smaller (< 30 L) bioreactors. While manual-sampled methods can have high accuracy, they require the detachment and destruction of the cells sampled, introducing a risk of contamination to the bioreactor, requiring technicians to perform them, and have slow turnaround times. Real-time optical and impedimetric probes are available commercially, but they exhibit sub-optimal performance in the context of adherent cells and smaller bioreactors respectively.

In this work, we evaluate the viability of magnetoelastic sensors (fabricated from commercially available Metglas 2826MB) for wirelessly monitoring the attachment and growth of human mesenchymal stromal cells (hMSCs) within *in vitro* culture systems, including 2D chamber slide cultures and 3D hollow fiber cultures. Results indicate that changes in sensor response can

be correlated with cell density on the sensor surface and cell growth activity in the culture. Experiments using a custom-built monitoring system demonstrated the capability of this system to generate temporal profiles of cell growth, which can indicate key stages of cell proliferation, including the rapid growth phase and confluence, as well as other critical events and conditions. Additionally, there was no significant difference in the density or morphology of cells after they were subjected to magnetic and mechanical stimuli from the monitoring system for 24 hours, suggesting that this method for tracking cell growth may have minimal impact on cell attachment.

The results presented in this report demonstrate that magnetoelastic sensors can be utilized to capture and analyze cell attachment and growth kinetics in a tissue culture environment. This thesis provides the foundation for a scalable sensor platform that can be integrated with bioreactors and other culture vessels to facilitate the wireless and longitudinal tracking of the attachment, growth, and confluence of anchorage-dependent cells grown *in-vitro*. In addition, this work also advances the technological readiness of magnetoelastic sensors for deployment in real-world biological and chemical sensing by investigating the use of low-cost sensor readers and improving the design and fabrication of the sensor.

This dissertation includes previously published and co-authored material adapted from Skinner W.S., Zhang S., Guldborg R.E., and Ong K.G., (2022, MDPI Sensors) [1]; Skinner W.S., Zhang S., Garcia J.R., Guldborg R.E., and Ong K.G., (2023, MDPI Sensors) [2]; and Skinner W.S., Saiz P.G., Reizabal, A., Plumley J.E., Dalton P.D., and Ong K.G., (2024, RSC Sensors & Diagnostics) [3].

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CHAPTER 1: INTRODUCTION

Increased success and expanding research for cell-based therapies have driven the demand for technologies that can monitor Critical Process Parameters (CPPs), such as viable cell density, lactate and glucose concentrations, etc., during cell culture to aid in the control and characterization of cell Critical Quality Attributes (CQAs). CQAs are established characteristics, including cell surface marker expression, adherence to plastic, and differentiation potential, used to evaluate the purity and potency of cell products. The anchorage-dependent nature of adherent cells, such as human mesenchymal stromal cells (hMSCs), can make the process of monitoring CPPs and evaluating CQAs more challenging. Viable cell density is a key parameter of interest in the expansion of cells for therapeutic applications, metabolic studies, and target protein production. Advances in on-line cell monitoring technologies, such as radio-frequency (RF) impedance, have facilitated real-time measurement of viable cell density (suitable for microcarrier culture) [4-6]. However, online methods, including RF impedance have not replaced traditional off-line methods for measuring viable cell density in anchorage-dependent cells, which often rely on measuring the DNA content, analyzing metabolic activity, or staining/detaching the cell, resulting in the destruction of the cells sampled, or having poor temporal resolution that may lead to misleading or inaccurate critical events. RF impedance monitors the electrical permittivity of the culture media. Viable cells have intact cell membranes and act as capacitors, affecting the permittivity of the culture media. RF impedance technologies are typically employed as an additional monitoring tool to track product conformance alongside traditional off-line techniques. In cases where traditional off-line methods are inaccurate or impossible to conduct, RF impedance can be used to assess viable cell density. While RF impedance demonstrates good performance in production-

scale processes, commercial users have reported poor performance of these technologies in smaller bioreactor volumes (< 30 L), such as those used in the production of cells for autologous therapies [7]. The sensitivity and accuracy of impedance-based analysis are influenced by the resistance or conductivity of the media, which can vary with changes in the concentration of biomolecules in the media.

Optical methods represent a broad category of non-destructive techniques for on-line monitoring of cell density. These methods are based on the scattering and absorption of light by cells in the culture [5, 6]. Purely optical methods have little impact on the cells and culture environment. However, they are far from ideal. Fouling of the optical surfaces by protein adsorption and the presence of microcarriers can obscure measurements. Additionally, strictly light-based methods of analysis cannot differentiate between viable and non-viable cells.

There is no technology commercially available for directly monitoring adherent cell density in small-scale (< 5 L) single-use bioreactors used in the production of autologous cells. Considering these limitations, the aim of this work is to develop a scalable sensor platform for wireless, longitudinal, on-line monitoring of the density and growth kinetics of adherent cells inside bioreactors and other culture vessels. The sensor technology is based on magnetoelastic materials, which are sensitive to mechanical loading (i.e., from cell attachment) and generate a response when probed by magnetic fields, facilitating wireless sensing of cell loading inside culture vessels and bioreactors. The overall objective of this project is the development of a scalable robust technological platform that can be integrated with multiple bioreactor platforms to monitor cell attachment and growth in real-time within culture vessels. The central hypothesis of this thesis is that magnetoelastic sensors can be embedded inside bioreactors and other culture

vessels to facilitate a remote system for monitoring cell growth with real-time feedback that reflects the growth kinetics of the bulk culture.

The goal of this project is achieved through these specific aims:

1.1 Specific Aim 1: Develop magnetoelastic sensor arrays and a scalable volumetric monitoring system

Most therapeutic stem cells can be described as adherent or anchorage-dependent cells. This class of cells requires a compatible substrate for attachment which provides support for growth and proliferation. Modern methods for characterizing hMSCs require staining and/or detachment of cells, which interrupts the growth process, can lead to a reduction in cell quality or potency, and renders the sampled cells unfit for clinical use. Development of a scalable, wireless, and label-free method for monitoring cell growth with real-time feedback can reduce cell waste and provide instantaneous data on cell number and the current stage of growth. Magnetoelastic sensors were coated with biocompatible materials and treated to support the attachment and growth of hMSCs. The performance of these sensors was improved by fabricating and testing a selection of shapes and dimensions as well as varying certain parameters of the surface treatment. The sensors were placed in biocompatible supports that could be integrated into culture vessels. Custom sensor measurement systems, including solenoids and software, were developed to facilitate the activation and collection of the sensors' signal. We hypothesized that changes in the sensor's resonance signal magnitude could be correlated to changes in cell loading and therefore cell density and proliferation at the sensor surface. This aim includes content adapted from published manuscripts: Skinner W.S., Zhang S., Guldborg R.E., and Ong K.G., (2022, MDPI Sensors) [1], and Skinner W.S., Zhang S., Garcia J.R., Guldborg R.E., and Ong K.G., (2023, MDPI Sensors) [2].

1.2 Specific Aim 2: Evaluate the sensors' ability to longitudinally monitor hMSC CQAs and CPPs in 3D.

Culture methods for adherent cells have increased in volume and efficiency by developing new approaches such as 3D microcarrier cultures and other bioreactor designs which increase the surface area-to-volume ratio. Critical Quality Attributes (CQAs) and Critical Process Parameters (CPPs) are key characteristics used to evaluate cellular identity and therapeutic potency. The current methods used to evaluate these characteristics are limited in their ability to integrate into automated systems and often require manual sampling and result in the destruction of the cells sampled. The magnetoelastic monitoring system can instantaneously indicate adherence of cells to plastic (CQA) and can be calibrated within a culture system to quantify cell density (CPP). Magnetoelastic sensors were coated with biocompatible materials and etched with oxygen plasma in a reactive ion etching system to support the attachment and growth of hMSCs. Sensors were packaged in the exposed region of a biocompatible flow cell that was designed for integration in-line with a hollow-fiber bioreactor system. Custom solenoids and software were developed to facilitate the activation and collection of the sensors' signal. We hypothesized that the cell density on the sensor in the flow cell would correlate to the density of cells on the reactor substrate, such that key features in the cell growth profile (rapid growth phase and confluence) generated by the sensor would temporally correlate with those generated by other indirect approaches such as measuring glucose consumption inside the reactor.

CHAPTER 2: BACKGROUND AND EARLY DEVELOPMENT

This chapter includes previously published and co-authored material adapted from Skinner W.S., Zhang S., Guldberg R.E., and Ong K.G., (2022, MDPI Sensors) [1] and Skinner W.S., Zhang S., Garcia J.R., Guldberg R.E., and Ong K.G., (2023, MDPI Sensors) [2].

2.1 Introduction

Magnetoelastic sensors are ideal for remote query of environmental conditions in closed and controlled systems because they can be fabricated and scaled into different shapes and sizes, functionalized with a wide array of chemical/biological sensing elements, and remotely activated and interrogated [8, 9]. Magnetoelastic materials are a cost-effective sensing platform due to the availability of raw materials (primarily composed of iron and nickel) and minimal amounts of material and processing required for manufacturing. The operation of a magnetoelastic sensor is based on its magnetostrictive behavior, which allows it to undergo cyclic mechanical vibrations at its resonance frequency when exposed to an alternating magnetic field. The mechanical resonance in turn causes the sensor to generate a secondary magnetic flux that can be detected independently (**Figure 1**). When a mass is loaded onto the sensor, the resonance frequency, magnitude of resonance, and signal phase at the resonance change due to the damping to the sensor's mechanical resonance. This change in resonance character and quality can be used to quantify the mass loaded onto the sensor's surface. Magnetoelastic sensors have been widely investigated for remotely monitoring environmental parameters, including physical quantities such as humidity, pressure, and the viscosity and density of liquids [9-11]. Detection and monitoring of chemical analytes and biologics with magnetoelastic sensors typically require functionalizing the sensor with a coating that gains or loses mass because of the presence or activity of the chemical/reaction/biologic being detected. This coating can be an enzyme or molecule that specifically binds with an analyte, a

coating that absorbs/desorbs water as pH changes, or molecules that are specifically consumed during reactions/metabolism. Functionalization of magnetoelastic sensors has increased their popularity as a platform for label-free detection [12] and has facilitated the detection and measurement of chemical analytes, such as pH [9], ammonia [9], carbon dioxide [9], calcium oxalate precipitate [13], and octachlorostyrene [14], and biologicals and biotoxins such as *E. coli*, Staphylococcus enterotoxin B, and other endotoxins [15-20].

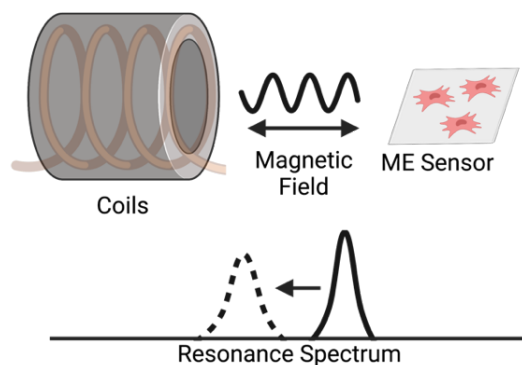


Figure 1. Illustration of the detection of cells on a magnetoelastic sensor by measuring the change in its resonance spectrum due to mass loading from cell attachment [1].

Magnetoelastic sensors are typically fabricated from amorphous ferromagnetic material, such as $\text{Fe}_{40}\text{Ni}_{38}\text{Mo}_4\text{B}_{18}$ (Metglas 2826 MB) or $\text{Fe}_{81}\text{B}_{13.5}\text{Si}_{3.5}\text{C}_2$ (Metglas 2605SC). These materials exhibit strong magnetostrictive behavior and have high magnetoelastic coupling coefficients so that energy conversion from elastic to magnetic energies, and vice versa, are highly efficient (for example, the magnetoelastic coupling factor of Metglas 2826MB is 0.98, and the magnetoelastic coefficient can reach as high as 11.7 ppm) [21-24]. Sensors made from these materials can be excited by a time-varied magnetic field, which results in cyclic stretching/relaxation of the material. Due to its high magnetoelastic coupling, the resonating material generates a strong secondary magnetic flux, which can be remotely detected and tracked with an induction solenoid.

Like all mechanical resonating bodies, the resonance of the magnetoelastic sensors is affected by its geometry, local environment, and mass-loading profile.

Improving the magnetoelastic sensor's sensitivity towards mass loading is particularly important in the context of biological/chemical sensing, which often requires the detection of very small changes in mass in the nanogram range or even lower. Previous work by a number of investigators has demonstrated the biological sensing capability of this technology with L929 fibroblast cells [25], breast cancer cells [26], and bacteria [16, 18-20, 27-30]. However, further improvement of the magnetoelastic sensor technology would be beneficial in realization of a practical mass-based cell monitoring system. In particular, we focus on understanding how variations in the sensors' geometry, position, and orientation relative to the detection system can affect the measurement quality of the resonance spectrum. Understanding and optimizing these parameters will be critical for developing this technology for use in dynamic and volumetric environments, such as a bioreactor. Multiple investigators have conducted similar studies in the pursuit of improving these sensors for mass detection: Sagasti et al. recently published a study investigating the effects of size and aspect ratio on several parameters related to the resonance and magnetic activity of the sensor [31] as well as another study determining the effects of thin polymeric coatings on the resonance behavior of the sensor [32]; Saiz et al. recently published multiple experiments detailing the effects of geometry on sensor sensitivity, including triangular and rhombohedral shapes [33, 34]; Pacella et al. have also investigated triangular-shaped sensors and non-uniform coatings as a means of increasing the sensitivity of magnetoelastic sensors [8]. Characterization of the response of the sensor was conducted to prepare for deployment in culture systems. Specifically, we investigated multiple approaches for improving the sensors' performance by altering their shapes, sizes, and orientations with respect to the applied magnetic fields.

Additionally, variations in sensitivity across the sensors' surface were investigated, and sensors of different geometries were rotated in the detection solenoid to understand how two-dimensional angular orientation impacted the sensors' resonance behavior. We also investigated the dimensional aspect ratio's impact on sensor response and determined the optimal magnetic bias field for sensors of different sizes and aspect ratios. This chapter includes content adapted from Skinner W.S., Zhang S., Guldberg R.E., and Ong K.G., (2022, MDPI Sensors) [1].

2.2 Methods

2.2.1 Sensor Fabrication

Sensors were made from a commercially available magnetoelastic material, Metglas 2826MB (Metglas Inc., Conway SC, USA) [35]. The material was purchased as a rolled, thick-film strip of 12.7 mm wide and 29 μm thick (saturation magnetization = 0.39 T, coercive force <50 A/m). A stack of 4 ME strips of about 15 cm-long were placed on top of a 0.5 mm thick steel sheet (McMaster-Carr, Elmhurst, IL, USA) that was covered in a layer of aluminum foil (Thermo Fisher Scientific, Waltham MA, USA). Rectangular sensors of 0.5 cm $\times$ 1.27 cm, square sensors of 1.27 cm $\times$ 1.27 cm, and equilateral triangular sensors with a height of 1.27 cm were cut from the strips with a high-power pulse laser (Coherent ExactCut 430) operating with a frequency of 2000 Hz, 0.05 ms pulse width, 600.0 W peak power and 60.00 W average power. Compared to the mechanically sheared rectangular sensors (average resonance frequency = 170.03 ± 0.90 kHz; $n = 10$) used in previous experiments [1, 25], these laser-cut rectangular sensors (average resonance frequency = 170.15 ± 0.43 kHz; $n = 10$) were found to have more consistent physical appearance and performance. (**Figures 2 & 3**). Manufacturing of Metglas 2826 MB involves the rapid quenching and cooling of the amorphous ferromagnetic material, which can introduce residual

stresses in the material. Shaping the material via mechanical shearing or laser-cutting can also introduce residual stress into the material. Annealing the material prior to application can reduce the variation in sensor resonance characteristics due to variations in the residual stresses in the material. The laser-cut sensors were annealed at 125 °C for 2 hours. Annealed sensors were coated with a 10 μ m-Parylene-C conformal layer via a commercially available vapor deposition coating system (PDS 2010 Labcoter, SCS, Indianapolis IN, USA). The coated sensors were then treated with oxygen plasma in a reactive etching system (March Jupiter II RIE system, Nordson March, Concord, CA, USA) for 30 s at 100 W. This coating technique was developed by Holmes et al. and employed in the fabrication of sensors for the detection of L929 fibroblast cells [25, 36].

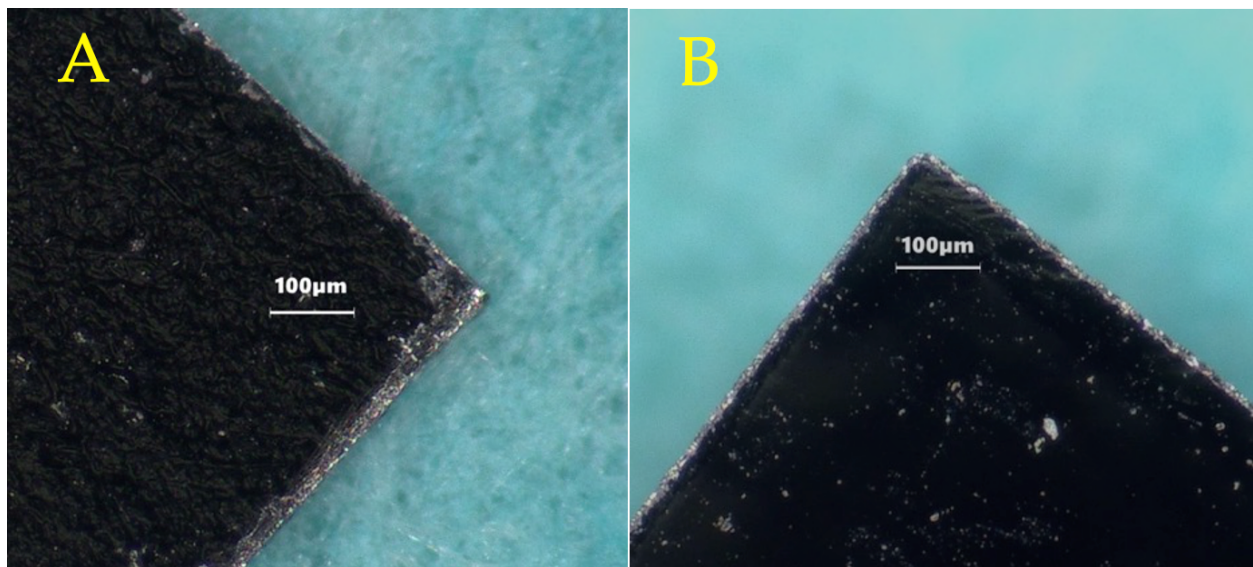


Figure 2. Microscopic images of a corner of mechanically sheared sensor (A) compared with a laser-cut sensor (B). No major differences in resonance behavior were observed between the two methods of sensor fabrication. However, the laser-cutting approach can produce sensors with consistent dimensions compared to manual shearing. (see Figure 3) [2].

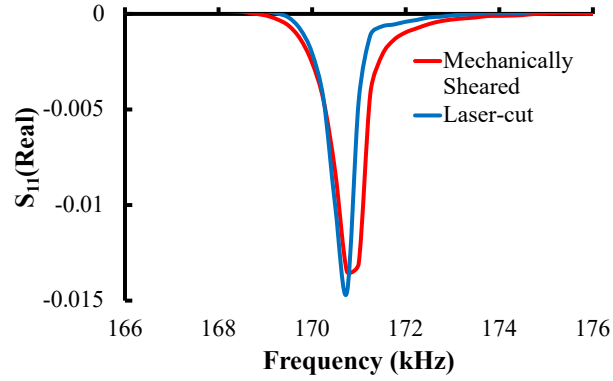


Figure 3. Characteristic resonance spectra for laser-cut and mechanically sheared sensors. The sensors share similar resonance frequencies and magnitude. The laser-cut sensor exhibits a more defined peak and higher quality factor than the mechanically sheared sensor.

2.2.2 Sensor Stage Fabrication

Sensors were suspended in wells by a grated stage with a recessed retainment area (**Figure 4**). Sensor stages were designed with computer-aided design (CAD) (Autodesk Fusion 360) and 3D printed using a stereolithography (SLA) printer (Formlabs, Somerville, MA, USA) with biocompatible and autoclavable resin (High Temp V2 Photopolymer Resin, Formlabs, Somerville, MA, USA).

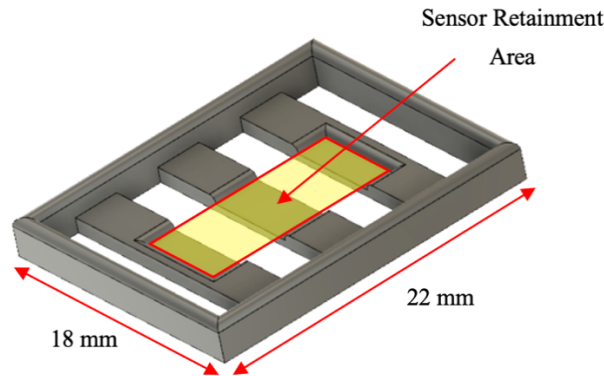


Figure 4. CAD drawing of a stage designed to retain and suspend the sensor in the chamber slide [2].

2.2.3 Detection System

As illustrated in **Figure 5**, the magnetoelastic sensors were interrogated with a custom detection system consisting of two concentric wound solenoids. The external solenoid was connected to a DC power source which supplied a DC current at 1.2 A, resulting in the generation of a DC magnetic field at 1.7×10^3 A/m. The internal solenoid, wound with 32-gauge magnet wire, was connected to a network analyzer (Keysight ENA Network Analyzer E50618, Keysight Technologies, Santa Rosa CA, USA & NanoVNA-F, Hangzhou Minghong Electronic Technology Co. Ltd., Hangzhou, China), which captured the resonance from the sensors by measuring the real portion of the S_{11} reflection coefficient at the detection solenoid's terminal. The resonance of the sensor appeared as a peak in the solenoid's captured signal. As mechanical force/stress was applied to the sensor, the measured resonance characteristics, including the magnitude, quality factor, and resonance frequency of the peak, changed. Tracking one or more of these characteristics allows the measurement of the mechanical force/stress.

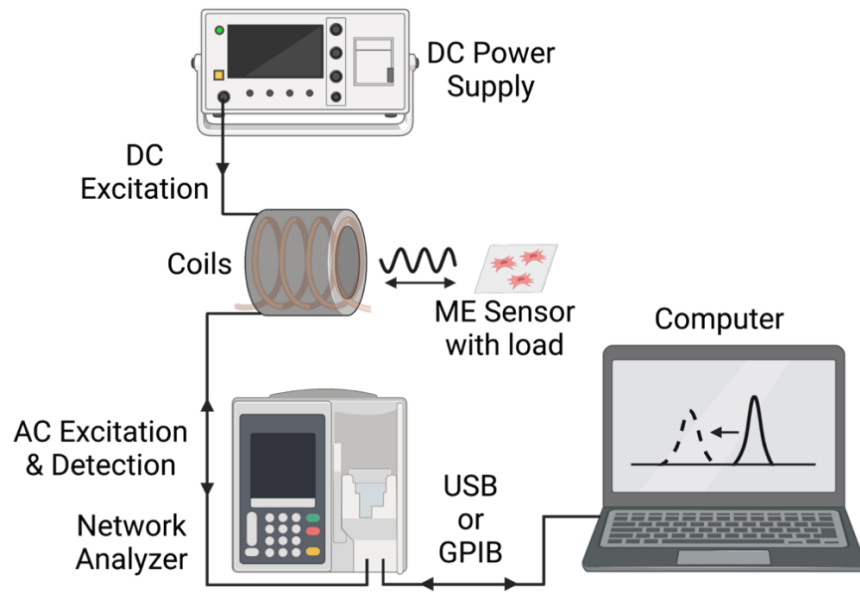


Figure 5. Block diagram of the key equipment and interfaces in this setup [2].

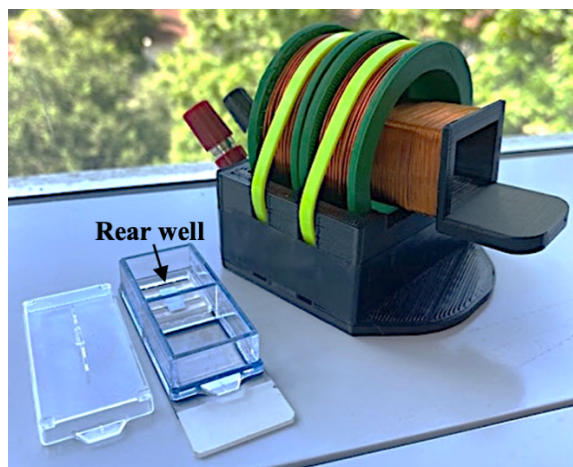


Figure 6. Photograph featuring the placement of the sensor and stage inside the chamber slide well. The detection (internal) solenoid is pulled partially out of the DC bias (external) solenoid to show detail in this image. During measurement, the detection solenoid is placed in the DC bias solenoid, such that the two solenoids are concentric. The empty/front portion of the chamber slide is not used, as the sensor is only placed in the rear chamber that is completely inserted into the detection solenoid [2].

The network analyzer and the connecting cable to the detection solenoids were calibrated together with a manual open-short-load calibration kit (Hangzhou Minghong Electronic Technology Co. Ltd., Hangzhou, China). Following calibration of the cable and network analyzer, the solenoids were connected, and the baseline signal of the solenoids were recorded prior to placement of the sensors as a background measurement. Next, a single sensor was placed in the rear well (**Figure 6**) of a 2-well chamber slide (Nunc Lab-Tek II Chamber Slide, Thermo Fisher Scientific, Waltham MA, USA) on top of the sensor stage so the sensor can vibrate freely while remaining at the same position. The chamber slide was placed inside the detection solenoid to record the resonance spectrum of the sensor inside. The background measurement was subtracted from all sensor measurements to eliminate the signal of the solenoid from the collected data.

2.2.3 Determining Variations in Local Mass-loading Sensitivity at the Sensor Surface

Three rectangular sensors ($12.7 \text{ mm} \times 5 \text{ mm}$) were cleaned with acetone and weighed with a microbalance. Following cleaning, the resonance spectrum was collected for each sensor. A mass

load was applied to the sensors via deposition of a drop of UV-curable adhesive (Dymax MD[®] Medical Adhesive 1072-M, Dymax, Torrington, CT). The interface between the adhesive drop and the sensor's surface had a diameter of 1 mm or less. As illustrated in **Figure 7**, eight locations, selected based on symmetry, were designated for the load placement.

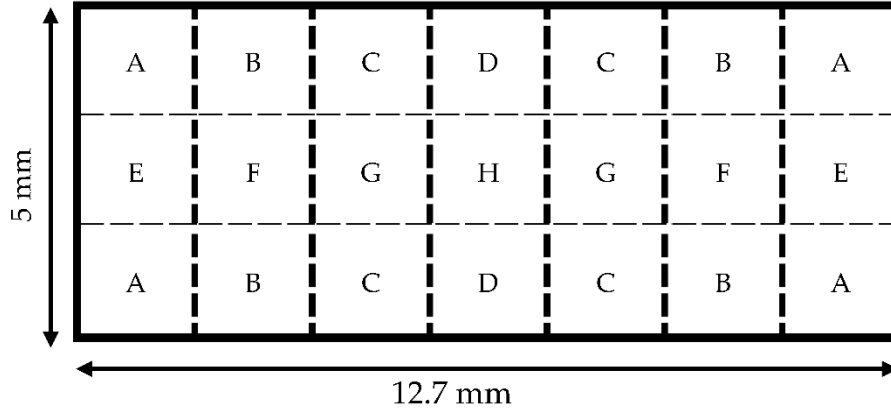


Figure 7. A diagram showing the 8 locations (A–H) where drops were deposited on the surface of each 12.7 mm × 5 mm sensor. Each letter represents a different location. With ‘H’ being at the origin center of the sensor, the rest of the letters (A–G) are mirrored across the four quadrants of the sensor surface to show symmetry [2].

Three approximate drop sizes were utilized: a “small” drop with a diameter of $0.6 \text{ mm} \pm 0.1$ mm, a “medium drop” with a diameter of $0.8 \text{ mm} \pm 0.1$ mm, and a “large” drop with a diameter of approximately $1 \text{ mm} \pm 0.1$ mm. Drops were deposited and cured under UV light for a period of 300 s. The sensors were then weighed, followed by measuring their resonances. These sensors were then thoroughly cleaned with acetone prior to the next deposition. The frequency shift per unit mass applied was calculated for each load placement and averaged among three trials.

2.2.4 Effects of Rotation on the Resonance Spectrum of Different Shapes

Sensors of different shapes were fabricated from Metglas 2826 MB (a roll of long Metglas ribbon with a width of 12.7 mm) to analyze the effect of shape on the sensors’ performance at different orientations from the applied magnetic fields. Rectangular (aspect ratio of 2.5), square,

and equilateral triangle sensors were cut to 12.7 mm along the longest dimension in their normal orientation (length of rectangle and square, height of the equilateral triangle). This normal orientation was defined as the longest dimension of the shape, which was also coincided with the longitudinal dimension of the Metglas ribbon and the applied magnetic fields. The Metglas material exhibits homogenous striations parallel with its longitudinal dimension. These striations are a result of the material's manufacturing process and can have a small influence on the resonance behavior of the sensor. Typically, having the striations aligned with the applied magnetic fields resulted in slightly sharper and stronger resonance peaks. These sensors were measured in their normal orientation (0° , **Figure 8**) and other orientations by rotating the sensors within the solenoid as illustrated in Figure 6. The maximum angular displacement investigated for the rectangular, square, and triangular sensors were 90° , 180° , and 120° , respectively.

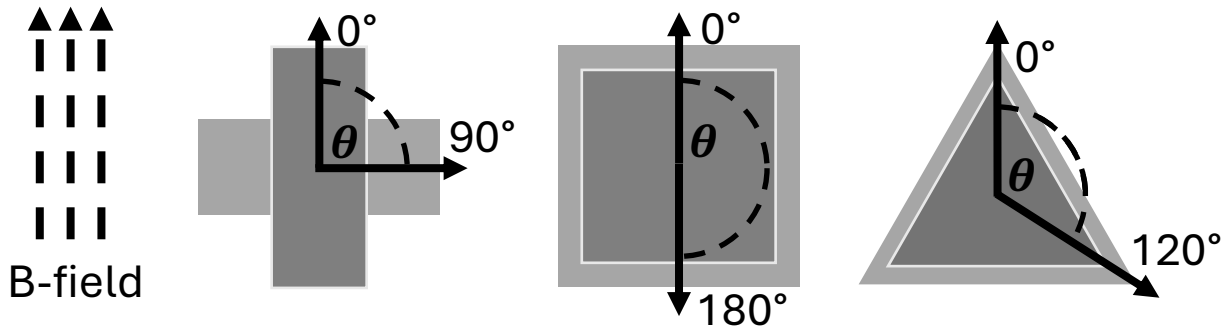


Figure 8. Schematic representation of magnetic field orientation and angles of maximum displacement in this study. The bias and activation fields are applied parallel and coincident with the 0° alignment of each shape, which is denoted by the darker color configuration. The lighter color configuration shows the same edge/corner that was initially aligned with 0° but is now aligned with the maximum displacement angle for that shape. The direction of applied fields remained the same throughout the experiments [1].

2.2.5 Optimization of DC Bias Field for Different Length Rectangular Sensors

Three sets of five sensors were fabricated into sizes of $6 \text{ mm} \times 2.4 \text{ mm}$, $9 \text{ mm} \times 3.6 \text{ mm}$, and $12.7 \text{ mm} \times 5 \text{ mm}$, such that all sensors had an aspect ratio of 2.5 (L/W). These sensors were subjected to a range of magnetic DC bias fields from $1.4 \times 10^3 \text{ A/m}$ to $3.6 \times 10^3 \text{ A/m}$ generated

from a power supply with DC currents ranging from 1 A to 2.5 A. The optimal bias field magnitude for each set of sensors was defined as the field magnitude which gave the sharpest resonance peak with the greatest resonance magnitude for each sensor. To determine the optimal bias field, the DC supply current was varied in 0.1 A increments, beginning at 1 A. At each current increase, the resonance spectrum of the sensor was analyzed via the network analyzer until the increases in field magnitude began to result in resonance peaks with decreasing magnitude. Beyond the optimal value, an over-biased sensor behaved similarly to an under-biased sensor, in that the resonance peak decreased in quality and magnitude. The under-biased and over-biased resonance behavior of the sensors were used to identify the optimal DC bias field value which resulted in a maximized resonance magnitude from the sensors.

2.2.6 Detection Solenoid Design and Fabrication

Custom solenoids were fabricated from copper magnet wire with a diameter ranging from 24 gauge to 32 gauge. The solenoids were fabricated by winding copper magnet wire onto 3D printed spools using a solenoid winding machine. Spools were wound to the maximum number of turns that could fit in two layers (24-ga = 135 turns, 26-ga = 178 turns, 28-ga = 210 turns, 32-ga = 333 turns). Thin layers of adhesive were applied in-between the two layers to reinforce the solenoid structure. SMA-style connectors were secured and soldered to the solenoid terminals before testing. Solenoids effect on sensor response was tested using a rectangular sensor (12.7 mm × 5 mm) placed in a 3D-printed retention stage in the rear portion of a 2-chamber slide.

2.2.7 Effect of Aspect Ratio on Rectangular Sensor Resonance

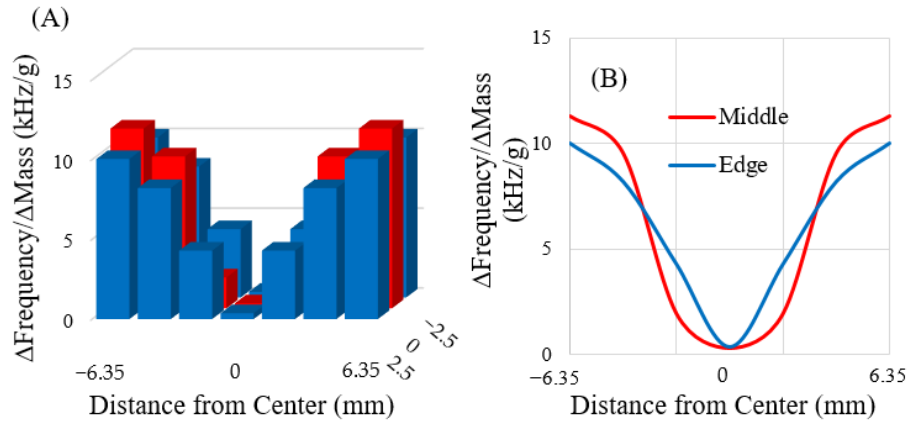
The goal of this investigation was to determine the aspect ratio for maximizing the magnitude of the sensors' response. Two sets of sensors, of length 9 mm and 12.7 mm, were fabricated into rectangles with four different aspect ratios (1, 1.5, 2.5, and 4) by changing their widths to 12.7 mm, 8.5 mm, 5 mm, and 3.2 mm, respectively. The sensors were analyzed with bias field values optimized for resonance peak magnitude and sharpness. This was accomplished using the formerly described method of analyzing a range of bias currents and selecting an optimal current based on under- and over-biased resonance behavior. The results were normalized against the surface area of the samples. Based on the results of the initial investigation, an additional group of sensors were laser-cut from a roll of magnetoelastic material to fabricate groups of magnetoelastic sensors 9 mm in length and varying in width to form groups with aspect ratios 1.3, 1.4, 1.5, 1.6, and 1.7. The sensors were analyzed in the same manner as previously described.

2.3 Results and Discussion

2.3.1 Variations in Local Mass-loading Sensitivity at the Sensor Surface

The rectangular sensors ($n = 3$) used in this experiment were expected to exhibit lengthwise mechanical resonance (longitudinal mode). Free-standing sensors were expected to have a node (minimal mechanical vibration) in the center of the material, with the most sensitive regions expected at the distal extremes. Analysis of the collected data indicated that the rectangular sensors fit the expected model: there was a region of low sensitivity at the center of the sensor (**Figure 9A,B**). If no point on the sensor is fixed and the sensor is symmetrical, the greatest resonance displacement happens at the edges furthest from the center. Hence, when the magnetoelastic sensors resonated, the maximum displacement occurred at the edges furthest from

the center. As indicated in **Figure 9**, these regions exhibited the strongest resonance signals, which were responsible for the increased sensitivity relative to other regions.



Figures 9A (left) and 9B (right). Charts illustrating the range of sensitivities that exist across the sensor surface in 3D (A) and as 2D lines along the length of the sensor (B). The rectangular sensors were assumed to behave symmetrically. The horizontal axes in both figures represent the entire length of the sensor, with the center of the sensor defined as the origin [1] ($n = 3$).

2.3.2 Effects of Rotation on the Resonance Spectrum of Different Shapes

The rectangular sensors showed the greatest variation in sensor response per degree of rotation of all geometries explored (**Figure 10A**). This was anticipated, as the rectangular sensors resonated with a longitudinal mode along the longest portion of the sensor; for the rectangle, this mode exhibits maxima at 0° and 180° . This corresponds with the expected behavior, as a rectangle's longest side can only be aligned with the direction of the excitation field at two orientations. However, another phenomenon related to the geometry of the rectangular sensors was also observed. Under a strong DC bias field, the rectangular sensors physically rotated and aligned to the direction of the applied DC bias field, conveniently positioning themselves in the ideal orientation for detection. It could be useful to take advantage of this phenomenon in dynamic and volumetric environments, where the sensors could self-orient to the ideal position for measurement.

The equilateral triangle-shaped sensors also resonated along the direction of the applied magnetic fields (longitudinal mode). Each triangle has 6 orientations in which the longest portion of the shape is aligned with the excitation field: 0° , 60° , 120° , 180° , 240° , and 300° . As a result of the increased number of orientations with aligned modes (in other words, the triangle's higher degree of rotational symmetry), the equilateral triangle-shaped sensors exhibited less variance in response per degree of rotation than the rectangular sensors (**Figure 10B**).

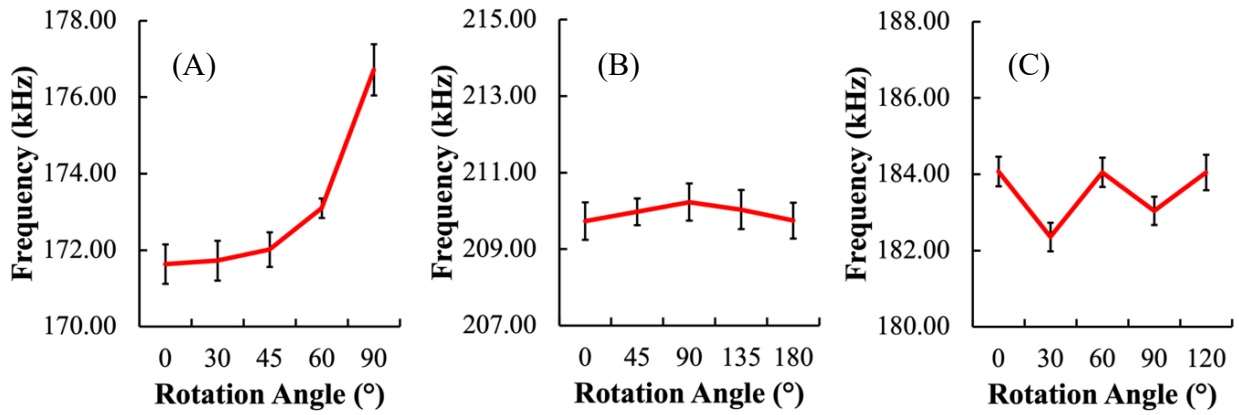


Figure 10. Changes in the sensor's resonance frequency at varying directions of magnetic field. Three shapes of sensors were evaluated: rectangular (**A**, 12.7 mm × 5 mm), square (**B**, length = 12.7 mm), and equilateral triangle (**C**, base = 14.6 mm, height = 12.7 mm). Angular increments were selected based on the rotational symmetry of the shape. ($n = 5$; error = $\pm$ standard deviation).

The square-shaped sensors exhibited the least variance in response per degree of rotation (**Figure 10C**). The square-shaped sensors also appeared to resonate with multiple modes (**Figure 11**). Specifically, the response signal at 0° rotation showed 3 resonance peaks for the square sensors. However, at other orientations, one or two of the peaks disappeared and/or appeared dampened while the third resonance peak remained mostly unchanged. Analysis of the resonance frequency and response behavior at different orientations led to the identification of one of the resonance peaks that disappeared at certain orientations as the longitudinal mode (similar to the rectangular sensors). The peak that persisted in all orientations (about 210 kHz) is the result of one or multiple modes of resonance that are similar to the radial resonance of a circular

membrane/diaphragm [37], as the peak shows relatively minimal variation, even at orientations that had substantially dampened the other two resonance peaks (**Figure 11**).

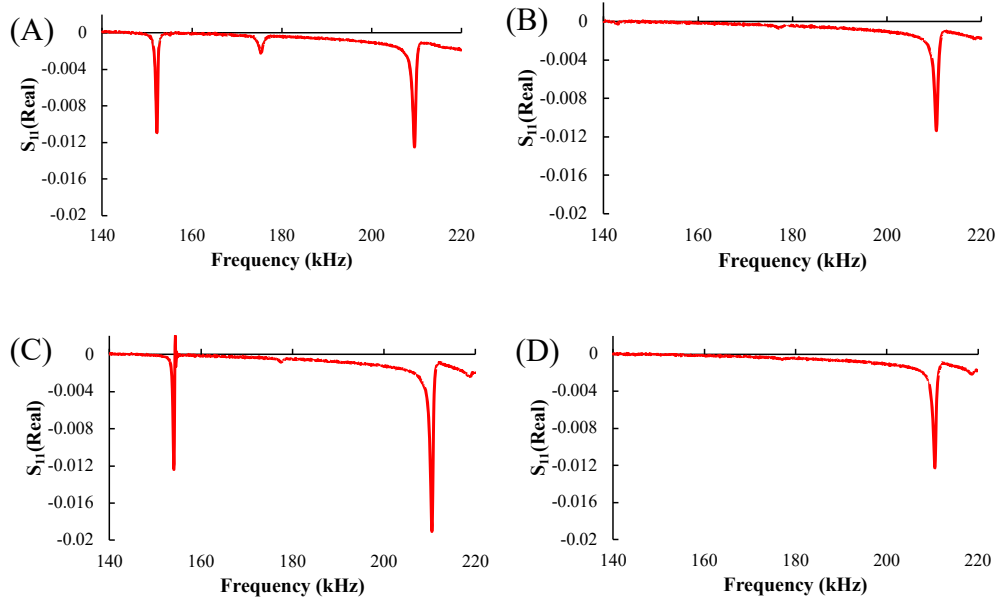


Figure 11. Plots of a single square sensor's resonances at 0° (A), 45° (B), 90° (C), and 135° (D) rotations from the normal orientation ($n = 1$).

Analysis of this study indicates that sensor geometry can be used to reduce the change in sensor response due to a rotational change relative to the direction of the magnetic interrogation field. These results also suggest that investigation of sensors that resonate with a longitudinal mode and radial-like mode might be pursued separately, as the advantages and disadvantages of these modes are mostly exclusive to one another. Sensors with longitudinal resonances can potentially be oriented with the bias field, but they exhibit the greatest change in response to misalignment from the ideal orientation. Sensors with radial-like resonance could exhibit a consistent resonance response at any orientation confined to a particular plane; however, this mode of resonance may not exhibit the same level of performance as the longitudinal mode sensors of similar dimensions. In future research, sensors might take on a variety of geometries: circular sensors, star-like sensors that are round with several triangular points along the edge, and even octagonal sensors could be

promising candidates for sensors that exhibit resonance modes similar to the vibration of a circular membrane/diaphragm.

2.3.3 Optimization of the DC Bias Field

The DC bias field is critical for increasing the amplitude of the sensors' signals to a suitable level for detection. To boost the sensor's signal, a bias field (in the form of a DC magnetic field) is required, which maximizes the sensor's magnetostrictive behavior by partially aligning the magnetic moments within the sensor. When sensors are under-biased, there are considerable cancellation effects in the rotation of magnetic moments during resonance, resulting in low net sensor vibration. When sensors are over-biased, the oversaturated magnetic moments are too strongly aligned to the bias field, resulting in minimal rotation under the agitation of the AC magnetic field. The results of the bias field optimization experiment indicated that an optimal DC magnetic bias field exists for a given sensor shape and is different for sensors of different dimensions. The results also indicated that it may be possible to predict the optimal bias field for a sensor of a particular geometry and aspect ratio if the optimal value is known for other dimensions of the same shape (**Figure 12**). In general, smaller sensors required a bias field of greater magnitude to achieve maximum magnetic flux output.

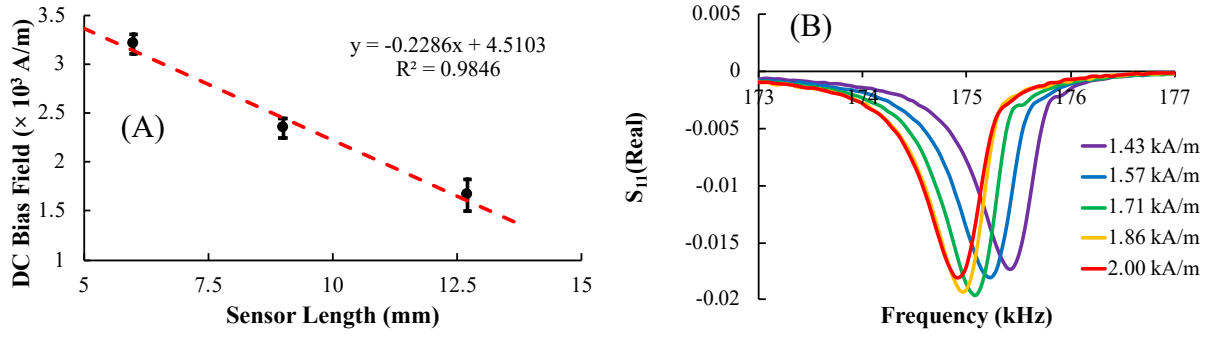


Figure 12. A plot of the results of the DC bias field optimization experiment (A) and an example of the under- and over-biasing effects on the resonance spectrum (optimal bias at 1.71 kA/m) (B). The sensors were all fabricated at an aspect ratio of 2.5 length over width. ($n = 5$; error = $\pm$ standard deviation).

2.3.x Detection Solenoid Characterization

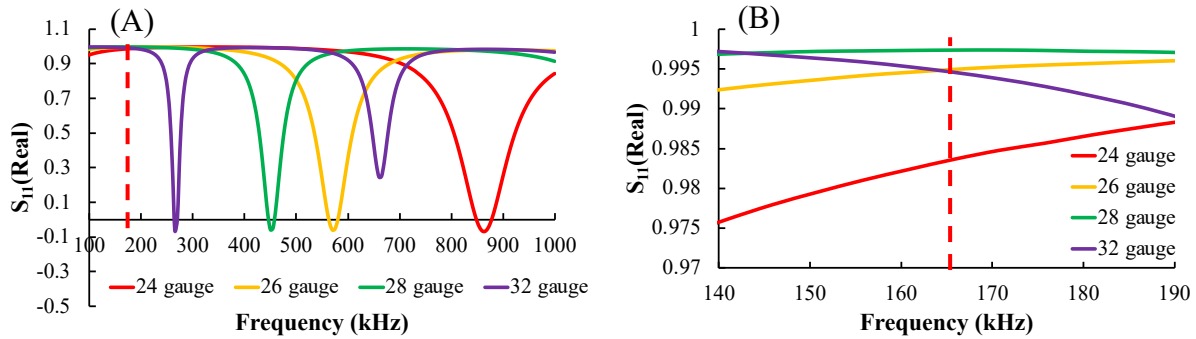


Figure 13. As wire gauge increased (wire diameter decreased) the frequency of solenoids' self-resonance increased (A). Although the self-resonance of the solenoids differed greatly, the baseline solenoid signals in the region of interest (140 kHz to 190 kHz) were similar (B). Red dashed line indicates typical sensor resonance frequency in media (~ 165 kHz).

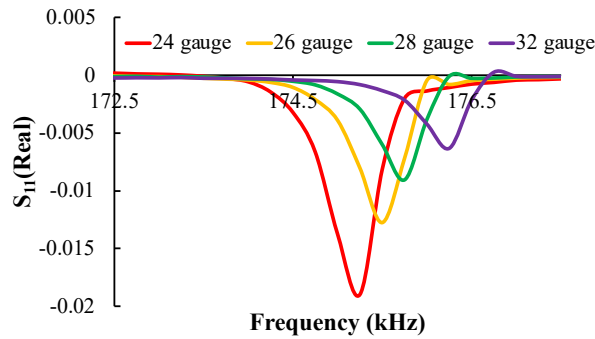


Figure 14. A 12.7 mm sensor was placed inside of a 3D printed retainer in a chamber slide. The sensor was then interrogated with a series of solenoids. As wire gauge increased (wire diameter decreased) the magnitude of the resonance peak of the sensor decreased. Additionally, it was observed that the resonance frequency corresponding to the maximum resonance magnitude increased with increasing wire gauge.

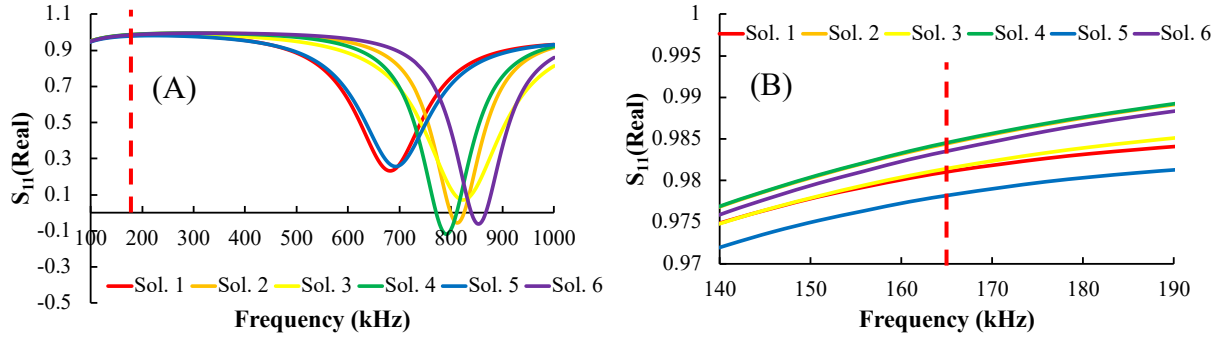


Figure 15. A set of 6 solenoids were wound with 24-gauge wire in two layers. Analysis of the solenoids' baseline signal reveals that most of the solenoids have a self-resonance around 800 - 875 kHz with similar magnitude. Solenoids 1 and 5 presented a self-resonance around 700 kHz with less magnitude (A). Although there were some differences in the frequency and magnitude of the solenoids' self-resonance, the baseline signal of these six solenoids in the region of interest (140 kHz to 190 kHz) varied by less than 1% (B). Red dashed line indicates typical sensor resonance frequency in media (~165 kHz).

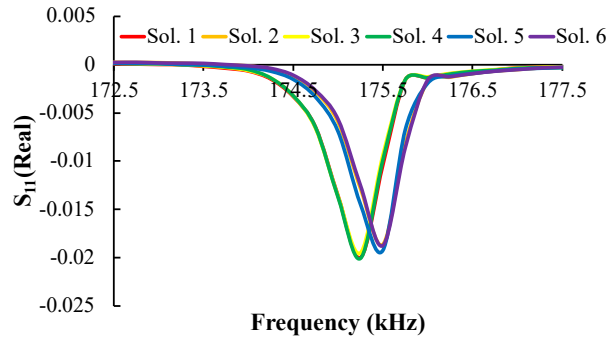


Figure 16. A 12.7 mm sensor was placed inside of a 3D printed retainer in a chamber slide. The sensor was interrogated with a series of solenoids with similar performance characteristics. The sensor generated similar resonance magnitude among the solenoids (average resonance peak magnitude = -0.0193 ± 0.0006 , $n = 6$), indicating that careful fabrication can yield solenoids with predictable performance characteristics.

The results indicated that the 24-gauge solenoid yielded the best performance characteristics with the 12.7 mm sensors. This solenoid presented the greatest signal magnitude, and the self-resonance was the furthest from the 12.7 mm sensors' resonance frequency (Figures 13 and 14). To investigate the variability in solenoid response and support future research, 5 more solenoids were wound with 24-gauge wire using same technique. Comparison of the resonance spectra captured from the 24-gauge solenoids reveals that it is possible to fabricate solenoids with predictable and similar performance characteristics. Although two of the solenoids present a self-

resonance at a lower magnitude and resonance frequency than the rest of the group, the baseline behavior of the solenoid in the region sensor resonance is very similar between the solenoids (**Figure 15**). Furthermore, the resonance magnitude of a single 12.7 mm sensor interrogated with the six 24-gauge solenoids was found to be consistent (average resonance peak magnitude = -0.0193 ± 0.0006) (**Figure 16**).

2.3.4 Effect of Aspect Ratio on the Sensor's Resonance

Of the aspect ratios investigated, the ratio of 1.5 resulted in a maximized magnitude of response for the rectangular sensor shapes of both lengths (**Figure 17**). Narrower sensors exhibited more efficient field generation, as the net magnetization of these is stronger for a given excitation field due to their smaller demagnetization factor [38]. A refined investigation analyzed a larger group of sensors with a narrower range of aspect ratios centered around 1.5. An aspect ratio of 1.5 exhibited the best balance between the sensors' self-dampening and field output (**Figure 18**). Consideration of these results suggests that an aspect ratio of 1.5 could be a good choice for general mass-based monitoring applications.

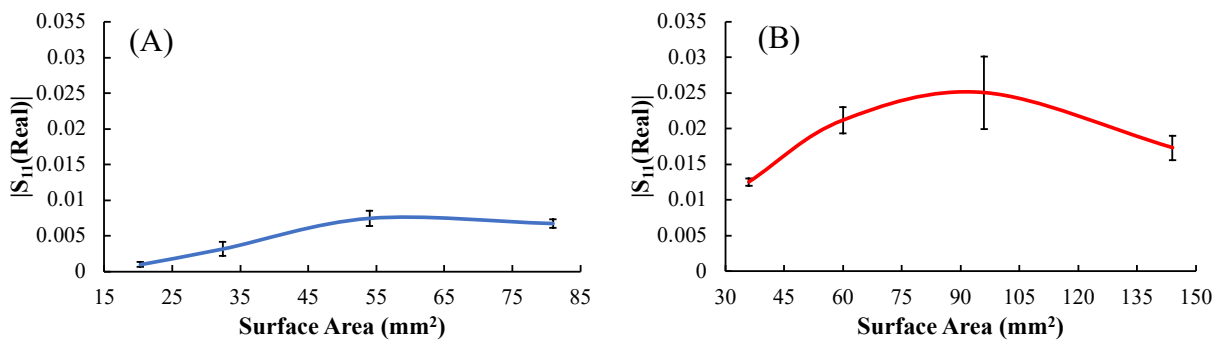


Figure 17. The responses of rectangular sensors fabricated at lengths 9 mm (A) and 12.7 mm (B) with varying widths were plotted against the surface area of those sensors. ($n = 4$; error = $\pm$ standard deviation).

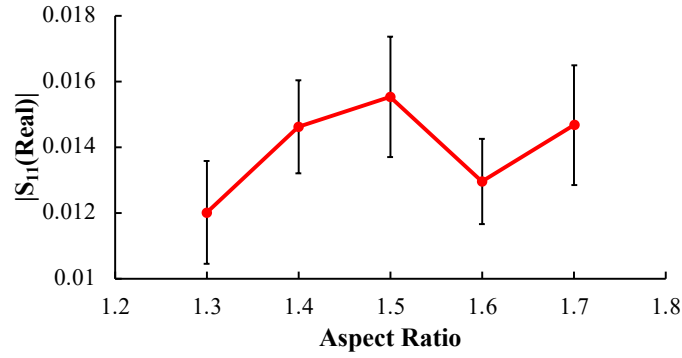


Figure 18. A chart showing the absolute value of the average signal magnitude of a set of 9 mm sensors fabricated at varying widths. Error bars represent standard deviation ($n = 67$).

2.4 Conclusions

Consideration of the geometry of the sensors and measurement conditions will be key factors in the development of a biological/chemical detection system for use in dynamic and volumetric environments. In the case of mass-based sensing, the ideal shape for the sensors should be a geometry that takes advantage of the knowledge surrounding sensitivity and response magnitude. Sensors with resonance modes that were similar to the vibration of a circular membrane/diaphragm exhibited the least variance in resonance frequency when the direction of magnetic fields change. For rectangular sensors, there was a specific aspect ratio which resulted in an improved response from the sensors, with each shape and size requiring a different magnitude of DC bias field to achieve an maximum resonance amplitude.

CHAPTER 3: MAGNETOELASTIC MONITORING SYSTEM FOR TRACKING GROWTH OF HUMAN MESENCHYMAL STROMAL CELLS

This chapter includes previously published and co-authored material adapted from Skinner W.S., Zhang S., Guldberg R.E., and Ong K.G., (2022, MDPI Sensors) [1] and Skinner W.S., Zhang S., Garcia J.R., Guldberg R.E., and Ong K.G., (2023, MDPI Sensors) [2].

3.1 Introduction

Increases in the success of clinical treatments using stem cells for chronic and historically difficult-to-treat ailments have generated a demand for scalable cell cultures and characterization methods that can consistently produce large quantities of high-quality stem cells [39]. To ensure the quality of cells, cell manufacturing processes often track multiple critical process parameters (CPPs), which are conditions in processes that have a significant impact on the resulting cell quality. In the cell manufacturing community, cell quality is characterized by their critical quality attributes (CQAs), which are established and quantifiable characteristics that can reliably evaluate the therapeutic potential of a cell product. Today, state-of-the-art technologies for monitoring CPPs and characterizing CQAs of anchorage-dependent cells typically require staining or detaching the cells, measuring the DNA content, or analyzing their metabolic activity [40-43]. The harsh and invasive nature of these methods increases the likelihood of errors in characterization or degradation in cell quality [44]. Furthermore, these manual, labor-intensive methods are difficult to incorporate into automatic feedback-controlled processes; thus, reliance on these methods could hinder the advancement of large-scale manufacturing of stem cells. Other non-invasive methods have been employed in 2D and 3D cell cultures to provide temporal assessment of cell culture, including techniques such as cell-substrate electrical impedance measurements and in situ microscopy and imaging [45-47]. However, methods such as electrical impedance or microscopy

are not suitable for monitoring smaller volume bioreactors (< 30 L) or adherent cells, respectively. Considering these challenges, we have developed a scalable sensor platform for contactless, longitudinal, in-line monitoring of conditions of adherent cells. The sensor platform is based on magnetoelastic materials, which are sensitive to mechanical loading (i.e., from cell attachment) and generate a response when probed by magnetic fields, facilitating wireless sensing of cells inside the bioreactor.

Magnetoelastic sensors have many advantages for monitoring cells because they can be scaled into different sizes, coated with biocompatible and functionalized materials, and be activated and interrogated remotely [8, 9]. The sensing capability of a magnetoelastic sensor is based on its magnetostrictive property, which allows it to physically vibrate and generate a secondary magnetic flux at its resonance frequency when exposed to a frequency-varying alternating magnetic field (**Figure 1**). As mass (cells) accumulates on the surface of the sensor, the resonance characteristics, including the resonance quality, resonance frequency, and magnitude of resonance, reduce in value. The changes in these resonance characteristics can be used to determine cell count in the bioreactor. Magnetoelastic sensors have been successfully used in the past to monitor biological environments and markers; for example, multiple groups have employed the magnetoelastic sensors as immunosensors to detect and identify bacterial populations of *Staphylococcus aureus* and *Escherichia coli* [18, 48], as well as other biological agents, pathogens, and endotoxins [16, 17, 19, 20, 27-29]. Magnetoelastic sensors have also been implemented in the monitoring and quantification of breast cancer cell (MCF-7) growth [26]. Recently, magnetoelastic sensors were explored for use in cell manufacturing processes. For example, our previous work demonstrated that magnetoelastic sensors can be treated to improve biocompatibility and cytocompatibility with fibroblast cells and to modulate cell properties and adhesion [30, 36, 49,

50], as well as longitudinally track cell number of fibroblasts seeded on the surface of these sensors [25].

In this study, we evaluated the viability of our monitoring system for use with human mesenchymal stromal cells (hMSCs). MSCs account for a considerable portion of the stem cell therapeutic market; therefore, the use of this technology to monitor the growth of hMSC during manufacturing could be beneficial to stem-cell based therapies by streamlining the monitoring process and providing real-time feedback for critical events, thus improving the yield and quality of cells produced. Specifically, we evaluated the sensitivity of the detection system and its ability to capture a temporal profile of hMSC growth, as previously demonstrated with L929 fibroblasts [25]. Additionally, we evaluated the effects of a gelatin coating on the sensor surface and investigated the effect of mechanical and magnetic field stimulus on the cell morphology. This chapter includes content adapted from Skinner W.S., Zhang S., Garcia J.R., Guldberg R.E., and Ong K.G., (2023, MDPI Sensors) [2].

3.2 Materials and Methods

3.2.1 Sensor Fabrication

Sensors were made from a commercially available magnetoelastic material, Metglas 2826 MB (Metglas Inc., Conway, SC, USA) [35]. Metglas 2826 MB was used because of its good magnetization (saturation magnetization = 0.39 T) and low coercivity (coercive force < 50 A/m) that result in a strong signal as well as large magnetostriction (saturation magnetostriction = 12 ppm) that makes them sensitive to mechanical loading. The material was purchased as a rolled, thick-film strip of 12.7 mm wide and 29 μm thick. A stack of 4 magnetoelastic strips of about 15 cm long was placed on top of a 0.5 mm thick steel sheet (McMaster-Carr, Elmhurst, IL, USA) that

was covered in a layer of aluminum foil (Thermo Fisher Scientific, Waltham, MA, USA). Rectangular sensors of $0.5\text{ cm} \times 1.27\text{ cm}$ were cut from the strips with a high-power pulse laser (Coherent ExactCut 430, Santa Clara, CA, USA) operating with a frequency of 2000 Hz, 0.05 ms pulse width, 600.0 W peak power and 60.00 W average power. Compared to the mechanically sheared and annealed sensors (average resonance frequency = $170.03 \pm 0.90\text{ kHz}$; $n = 10$) used in previous experiments [1, 25], these laser-cut sensors (average resonance frequency = $170.15 \pm 0.43\text{ kHz}$; $n = 10$) were found to have similar physical appearance, improved resonance magnitude, and improved quality factor (**Figures 2 and 3**). The laser-cut sensors were coated with a $10\text{ }\mu\text{m}$ Parylene-C conformal layer via a commercially available vapor deposition coating system (PDS 2010 Labcoater, SCS, Indianapolis, IN, USA). The coated sensors were then treated with oxygen plasma in a reactive etching system (March Jupiter II RIE system, Nordson March, Concord, CA, USA) for 30 s at 100 W. This coating technique was developed by Holmes et al. and employed in the fabrication of sensors for the detection of L929 fibroblast cells [25, 36].

3.2.2 Sensor Stage Fabrication

Sensors were suspended in wells by a grated stage with a recessed retainment area (**Figure 4**). Sensor stages were designed with computer-aided design (CAD) (Autodesk Fusion 360 v2.0) and 3D printed using a stereolithography (SLA) printer (Formlabs, Somerville, MA, USA) with biocompatible and autoclavable resin (High Temp V2 Photopolymer Resin, Formlabs, Somerville, MA, USA).

3.2.3 Detection System

In the earliest experiments, the magnetoelastic sensors were interrogated with a custom detection system consisting of two concentric wound solenoids, as illustrated in **Figures 5 and 6**. The external solenoid was connected to a DC power source that supplied a DC current at 1.2 A, resulting in the generation of a DC magnetic field calculated to be 1.7 kA/m. Previous research determined that 1.7 kA/m was the optimal DC bias field for the sensors of this size and detection system used in this experiment [1]. The internal solenoid, wound with 32-gauge magnet wire, was connected to a network analyzer (Keysight ENA Network Analyzer E50618, Keysight Technologies, Santa Rosa, CA, USA and NanoVNA-F, Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China), which captured the resonance from the sensors by measuring the real portion of the S_{11} reflection coefficient at the detection solenoid's terminal. The internal solenoids were wound with 24-gauge magnet wire and the external solenoid (which was used to generate the bias field) was replaced with positioned neodymium magnets to provide the optimal bias field without additional power supply; this change greatly reduced the power requirements of the system and thereby increased the number of replicates that could run simultaneously, allowing 6 solenoids in a single incubator without interfering with one another (**Figure 19**). As mechanical force/stress was applied to the sensor, the measured resonance characteristics, including the magnitude, quality factor, and resonance frequency of the peak, changed. Tracking one or more of these characteristics allows for the measurement of the mechanical force/stress.



Figure 19. Images of the longitudinal monitoring system deployed in an incubator. 6 computers loaded with custom software are connected to 6 network analyzers via USB to facilitate control and collection of data (**left**). 6 detection solenoids are distributed evenly throughout the incubator to prevent interference between the solenoids (**right**).

The network analyzer and the connecting cable to the detection solenoids were calibrated together with a manual open-short-load calibration kit (Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China). Following calibration of the cable and network analyzer, the solenoids were connected, and the resonance spectra of the solenoids were recorded prior to placement of the sensors as a background measurement. Next, a single sensor was placed in the rear well (**Figure 6**) of a 2-well chamber slide (Nunc Lab-Tek II Chamber Slide, Thermo Fisher Scientific, Waltham, MA, USA) on top of the sensor stage so that the sensor can vibrate freely while remaining at the same position. The chamber slide was placed inside the detection solenoid to record the resonance spectrum of the sensor inside. The background measurement was subtracted from all sensor measurements to eliminate the baseline signal of the solenoid from the collected data.

3.2.4 hMSC Cell Culture

Bone marrow-derived hMSCs were obtained (donor #00175, donor #00277, RoosterBio, Frederick, MD, USA), expanded, and cryogenically stored in liquid nitrogen. hMSCs (PDL 13) were recovered from liquid nitrogen, thawed, seeded at 5000 cells/cm², and cultured until

confluent. The cells were cultured in xeno-free media (RoosterBio, Frederick, MD, USA) supplemented with 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and xeno-free supplement (RoosterBio, Frederick, MD, USA). Once confluent (~72 h, confirmed via light microscopy), the cells were lifted with 0.25% trypsin-EDTA (Gibco, Invitrogen, Waltham, MA, USA), counted using an automatic cell counter (NucleoCounter NC-200, Chemometec, La Jolla, CA, USA) and then diluted with media to the target seeding density. Cells were incubated at 37.0 °C, ~93% relative humidity, and under 5% CO₂ in a microbiological incubator (HERAcell VIOS 160i, Thermo Fisher Scientific, Waltham, MA, USA).

3.2.5 hMSC Cell Seeding

Fabricated sensors were cleaned with 70% ethanol, allowed to dry, placed inside sterilization pouches and then sterilized with ethylene oxide gas. Fabricated sensor stages were cleaned with 70% ethanol in a sonicator (CO-Z Ultrasonic Cleaner, CO-Z supplies, Lake Forest, CA, USA) for 5 min, placed inside sterilization pouches, and autoclaved (Beta Star Small Sterilizer Autoclave, Beta Star, Honey Brook, PA, USA). A selection of sterilized sensors were placed in sterile culture dishes, covered with 0.1% gelatin solution (EmbryoMax ultrapure water with 0.1% gelatin, MilliporeSigma, Burlington, MA, USA), and placed in the incubator for 30 minutes up to 60 minutes unless otherwise stated. Sterilized sensor stages were placed inside the rear well of a two-well chamber slide (see **Figure 6**). Sensors were removed from the gelatin solution if necessary and placed inside the sensor retainment area of the sensor stage inside the two-well chamber slide. Cells were seeded at densities ranging from 5,000 to 40,000 cells/cm².

3.2.6 Sensitivity Curve

The resonance spectrum was collected by placing the portion of the chamber slide containing the sensor within the detection solenoid connected to the network analyzer (Keysight ENA Network Analyzer E50618, Santa Clara, CA, USA) and by measuring the real portion of the S_{11} reflection coefficient from 140 to 190 kHz. The position of the chamber slide was adjusted while monitoring the resonance amplitude of the sensor until the maximum peak magnitude was detected for each sensor. In a sensitivity calibration experiment, sensors were seeded with cells at a density of 5000 cells/cm² and pair a of sensors were fixed, stained, and imaged each day to determine if culture length influenced the calibrated sensors' response to cell density. This calibration experiment was performed in two parts across two weeks targeting even or odd days on either week to build a curve capturing days one through seven. As well, the longitudinal tracking method, detailed in Section 3.2.7, was employed for the calibration experiment. The difference between the initial and final measurements were compared against the cell nuclei quantified on the surface by fluorescence microscopy to generate the curve.

3.2.7 Longitudinal Tracking of Cell Loading

Six matching detection solenoids were cleaned with 70% ethanol and 10% household bleach before placing them inside of an incubator. Chamber slides containing sensors seeded with cells were placed immediately inside the detection solenoid following seeding. The detection solenoids were connected to six network analyzers (NanoVNA-F) controlled by custom Visual C# software programmed onto six laptop computers (Dell Latitude 3190, Dell Technologies, Round Rock, TX) running Windows 10 operating system. Measurements were collected once every 15 minutes by taking the average of 5 measurements captured in rapid succession.

3.2.8 Staining, Imaging, and Counting of Cells on Sensors

Following each experimental procedure, culture media were aspirated from the well containing the sensor. Next, the well was rinsed with phosphate-buffered saline (PBS) (Endotoxin-Free Dulbecco's PBS (1X) (w/o Ca^{++} and Mg^{++}), Millipore Sigma, Burlington, MA, USA). The PBS rinse was aspirated, and the cells were fixed by incubating the sensors in a solution of 4% paraformaldehyde (PFA) (32% paraformaldehyde aqueous solution, Electron Microscopy Sciences, Hatfield, PA, USA) diluted in PBS for 10 min. Following fixation, PFA solution was aspirated, and the samples were protected from light by wrapping the chamber slides in aluminum foil. Sensors were stained by covering with a solution of Hoescht nuclei stain (Hoescht 33342, Trihydrochloride, Trihydrate, Thermo Fisher, Waltham, MA, USA) diluted to 50 $\mu\text{g/mL}$ in PBS and incubated for 10 min. Following the incubation period, the staining solution was aspirated, and forceps were used to carefully invert the sensor via the stage into the empty, unused well of the chamber slide. The stage was removed; the sensor was covered with PBS, wrapped in foil, and imaged immediately. Fluorescent microscopic imaging was performed using a confocal fluorescence microscope (CSU-W1 SoRa, Nikon, Minato City, Tokyo, Japan) equipped with a 10 $\times$ magnification lens. Fifteen 1 mm $\times$ 1 mm images of the sensor surface were taken at specific locations that were kept consistent for each sensor (**Figure 20**). In the earliest experiments, cell nuclei were quantified by manually marking and counting the cells via ImageJ software v1.53t. In later cell experiments, as the density of nuclei greatly increased, custom MATLAB software was developed which could quantify cell nuclei as well as analyze the shape factor of the overall cell morphology if the appropriate stains were utilized. In later experiments, the number of images captured on the sensor surface was reduced to twelve 1 mm $\times$ 1 mm images that were captured at each of the 4 corners, the center of each edge of the sensor, and 4 images from the center of the

sensor. This change was made to streamline and simplify the imaging process while still capturing the complete distribution of cell density across the sensor surface.

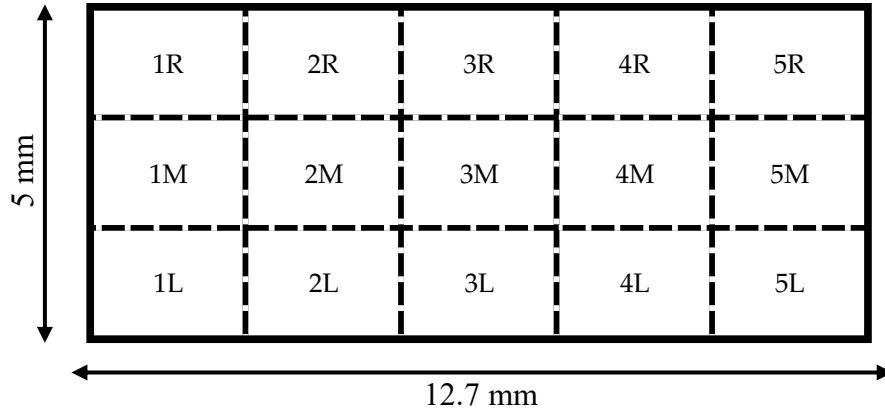


Figure 20. Diagram of regions that were targeted for imaging on each sensor. A 1×1 mm image was collected at the center for each region for a total of 15 images per sample [2].

3.3 Results and Discussion

3.3.1 Improving the Surface Treatment to Mitigate Bubble Formation and Retention

Analysis of sensor resonance profiles revealed unexpected fluctuations in sensor magnitude (~ 0.001 in reflection coefficient) that appeared in the first ~ 48 hours of sensor activity. These fluctuations were present in sensors placed in PBS, indicating that the source of this fluctuation was not due to cell activity or components in the media (**Figure 21**). Bubbles were observed to form on sensors during the seeding and wetting process, which provided a plausible explanation for the unexpected activity. Bubbles could affect the dampening of the sensor as well as its position relative to the magnetic fields used to activate and read the sensor, all of which could result in the fluctuations observed in the resonance profile. As a result, additional surface treatments were developed for the sensor and the retention stage to mitigate the formation of bubbles and improve the stability of the sensor signal.

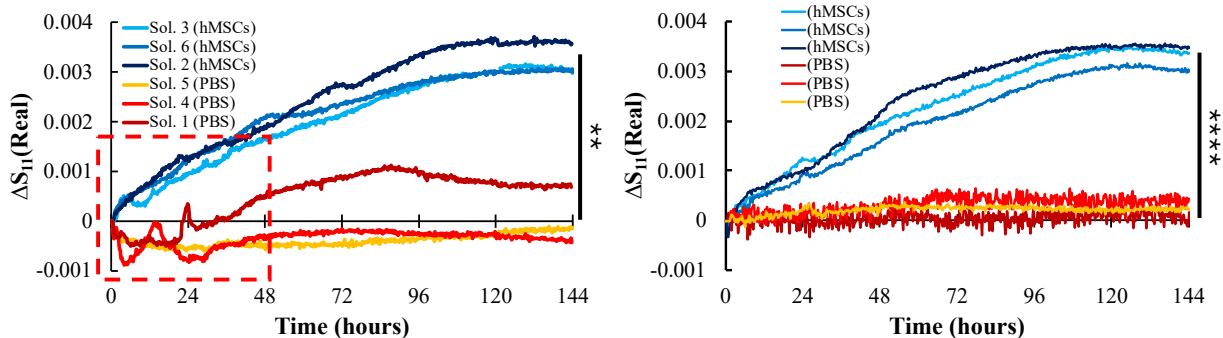


Figure 21. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) and sensor response in PBS for sensors with the original surface treatment (left, see **Figure 22** for treatment details) and the improved surface treatment (right, see **Figure 23** for treatment details). **: $p = 0.0012$, ****: $p < 0.0001$, unpaired t-test

The original surface treatment for the sensors only involves plasma etching of the top sensor surface to improve its hydrophilicity). For these single-side treated sensors, the hydrophobic surface of the under-sensor was potentially contributing to the formation and retention of bubbles. Sensors with the original surface treatment were sometimes difficult to wet or seed due to this hydrophobic under-surface and could be observed to float on the surface of the media or harbor bubbles in the space underneath the sensor, between the sensor and retention stage. To address this issue, both surfaces of the sensor and the retention stage were plasma-etched, so that all the surfaces would be hydrophilic and facilitate better wetting/seeding. This resulted in a vast improvement to the overall stability of the signal, however, acute fluctuations in signal magnitude were still observed (**Figure 22**).

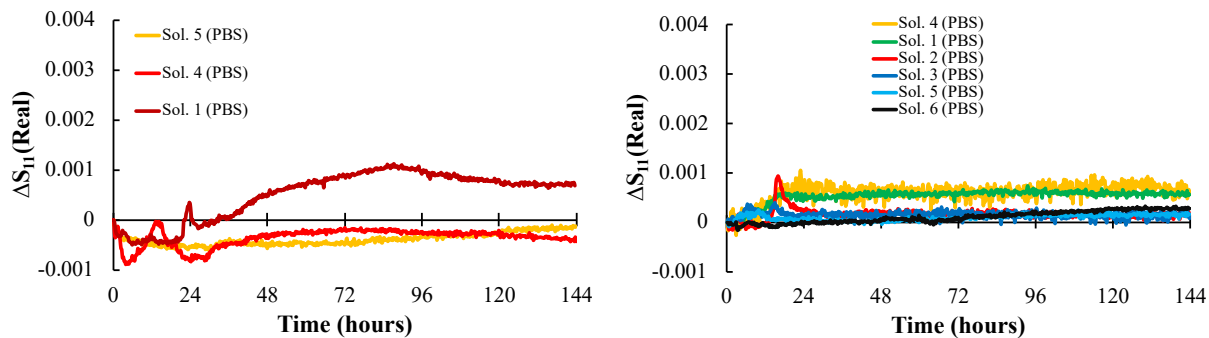


Figure 22. Sensors in PBS with the surface treatment: 10 micron Parylene-C coating on only the sensor and plasma etch on only the growth surface of the sensor (**left**); 10 micron Parylene-C coating on only the sensor, but plasma etch on both sides of the sensor and retention stage (**right**).

Inspection of the plasma-etched retention stage discovered striations (small grooves formed from the laser raster in the resin-printing process) that could be contributing to the formation or retention of bubbles. Thus, Parylene-C was also coated on the retention stages so that (1) the striations could be filled to provide a smoother surface to reduce bubble nucleation sites and (2) be confident that the hydrophilicity of the retention stages more closely matched that of the sensors, which were wetting/seeding well with the new treatments. Parylene-C of 10 micron thickness was applied on both the sensors and retention stages. The acute fluctuations previously observed appeared to be eliminated by the treatments with a more stable baseline signal in PBS and media (**Figure 23**). Comparison of these results using an unpaired t-test reveals that there is no significant difference ($p = 0.0924$) between the sensor response in PBS and media after 6 days. This suggests that adsorption of media components, including proteins, does not significantly impact monitoring system's response.

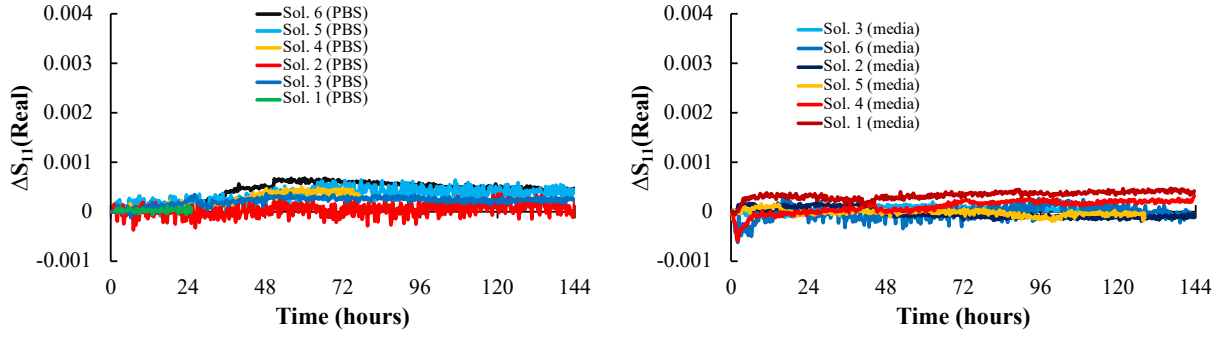


Figure 23. Sensors with the surface treatment: 10 micron Parylene-C coating on sensors and retention stages, plasma etch on all parylene coated surfaces in PBS (**left**) and in xeno-free culture media with xeno-free supplements (**right**). There was no significant difference between the response magnitude of the sensors in PBS and media after 6 days. $p = 0.0924$, unpaired t-test.

The 10 micron Parylene-C coating on the sensors and retention stages with plasma etching treatment on all coated surfaces was selected as the surface treatment to continue with in further research and development. As well as developing this new treatment, we also began warming the sensors and retention stages in PBS to culture temperatures as an additional precaution against bubbles and signal fluctuation. Not only are the sensors sensitive to changes in temperature, but changes in temperature of the liquid surrounding the sensor can also cause gases to become undissolved from the liquid and contribute to the formation of bubbles. Warming the sensors and retention stages in PBS greatly improves the wettability of surfaces (as they are already wetted by the PBS) at the time of seeding and reduces temperature shock to the sensor and culture media at the time of seeding.

3.3.2 Heterogeneity of Cell Distribution on Sensors

The distribution of hMSCs on magnetoelastic sensors formed a consistent pattern of the highest cell density at the center of the sensor, with a decreasing gradient toward the edges (**Figure 24**). This gradient distribution of cells is the result of a short period of growth (24 h), the

chosen seeding density, and the colony-forming behavior of hMSCs. Research by Neuhuber et. al. demonstrated that the plating (seeding) density of MSCs has an effect on the growth pattern and kinetics of the cells [51]. The group observed that cultures seeded at high density were evenly spread, where cultures seeded at lower densities grew in colonies. In the case of our sensor, the seeding conditions and duration of culture facilitated the consistent formation of a colony in the center of the sensor. Selecting a higher seeding density or increasing the duration for which the cells are allowed to grow could result in a more homogenous distribution of cells on the sensor surface. There were no observed differences in cell distribution gradient between the resonated sensors and non-resonated control sensors, indicating that the vibrations due to the sensor resonance did not contribute to the cell density distribution.

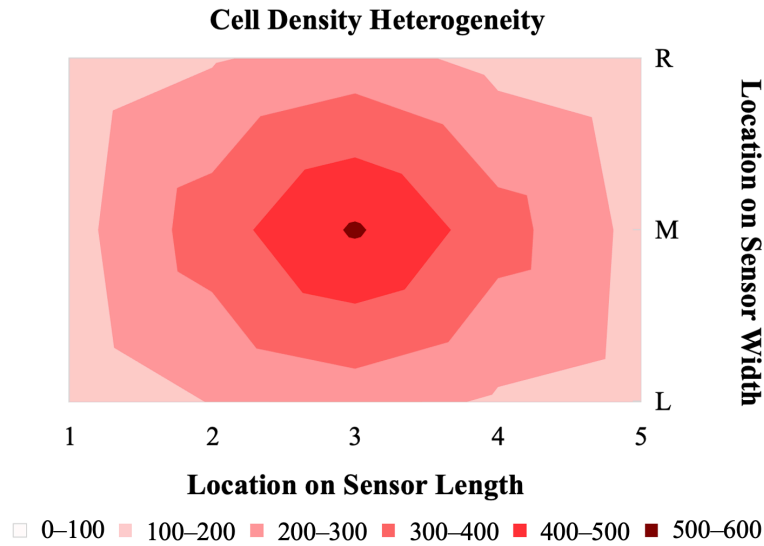


Figure 24. A 2D map representing the heterogeneity in cell density across the sensor surface after 24 h of growth and following resonance measurements at 0 and 24 h ($n = 5$) [2].

3.3.2 Evaluation of Sensor Sensitivity

Magnetoelastic sensors, like other physical resonance sensors, exhibit concurrent variation in the resonance amplitude and frequency. In air and other dry conditions, the signal-to-noise ratio

of resonance sensors is high, and tracking the frequency can yield more sensitive performance for monitoring damping by mass. In liquid environments, the signal-to-noise ratio is lower due to damping and viscoelastic effects of the liquid; in liquid environments, the resonance amplitude provides a more accurate and robust response to damping [52]. Comparison of the shift in resonance frequency against the shift in resonance amplitude with respect to cell loading reveals that the shift in resonance amplitude is a more accurate predictor of cell density than the shift in resonance frequency (**Figure 25**). Based on these results, the resonance amplitude was chosen as the parameter to track for monitoring cell growth.

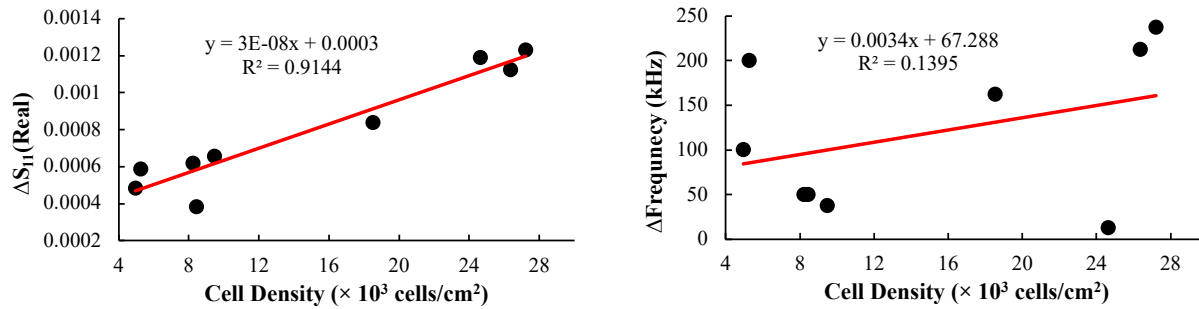


Figure 25. Curves comparing the change in the real portion of the S_{11} reflection coefficient at the resonance frequency of the sensor (**left**) and the change in the resonance frequency (**right**) to the average density of cells imaged on the sensor surface. These cells were seeded at a range of densities from 5,000 to 40,000 cells/cm² (RoosterBio donor #00175) and the nuclei were quantified 24 hours after seeding.

As shown in **Figures 26 and 27**, the linearity of the calibration curves suggests that there was a direct relationship between the total number of cells attached to the sensor surface and changes in the real portion of the S_{11} reflection coefficient at resonance. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formulas: $\text{LOD} = 3.3\sigma/S$ and $\text{LOQ} = 10\sigma/S$, where σ is the standard error of the regression intercept and S is the slope of the regression line. According to data collected using different seeding densities in a 24-hour period (**Figure 25**), the $\text{LOD} = 6,570$ cells/cm² and the $\text{LOQ} = 19,900$ cells/cm². These values give some indication of the sensor's performance during the early stages of culture, as the culture period was

only 24 hours. According to data collected over a 7-day culture, the LOD = 19,300 cells/cm² and the LOQ = 58,400 cells/cm², which give a better indication of the sensor's performance during the culture period recommended by the supplier for expansion. The relatively high coefficient of determination for the calibration curves suggests that the monitoring system is a good candidate for further development and implementation in larger cell-culture systems.

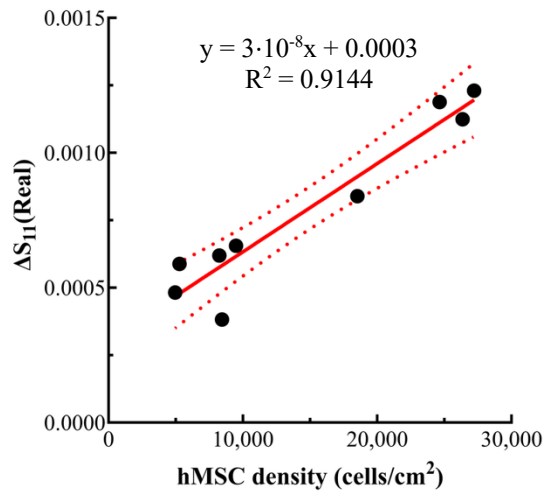


Figure 26. A curve comparing the change in the real portion of the S₁₁ reflection coefficient at the resonance frequency of the sensor to the average density of cells imaged on the sensor surface. These cells were seeded at a range of densities from 5,000 to 40,000 cells/cm² (RoosterBio donor #00175) and the nuclei were quantified 24 hours after seeding. 95% confidence bands for the regression analysis are shown. The linear relationship suggests there is a direct relationship between changes in signal magnitude at resonance and cell density on the sensor surface.

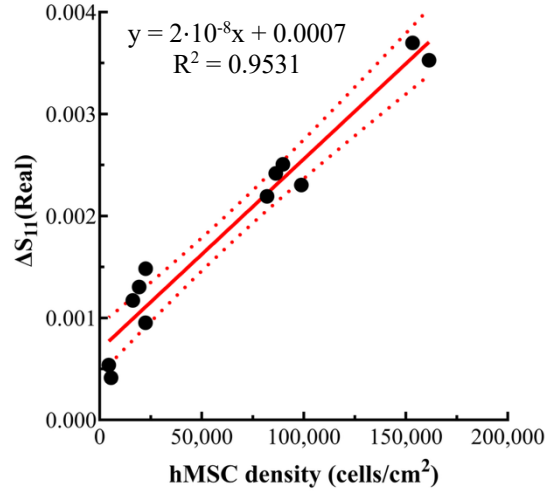


Figure 27. A curve comparing the change in the real portion of the S_{11} reflection coefficient at the resonance frequency of the sensor to the average density of cells imaged on the sensor surface. These cells were seeded at 5000 cells/cm² (RoosterBio donor #00277) and the nuclei on a pair of sensors were quantified at 48 hour increments, up to 7 days. The experiment was conducted in two parts across two weeks to facilitate replicate samples. 95% confidence bands for the regression analysis are shown. The linear relationship suggests there is a direct relationship between changes in signal magnitude at resonance and cell density on the sensor surface.

3.3.3 Longitudinal Tracking of Cell Loading

As depicted in **Figure 28**, a steady rise in sensor response corresponded to the loading of cells, their increasing mechanical attachment to the surface, and proliferation. As cells approached confluence on the sensor surface, the rate of change began to plateau. This behavior highlights the value of this approach for monitoring cell culture, as this technology can track cell growth in real time, non-invasively, and remotely. This technology also has the potential to indicate conditions of interest in cell manufacturing, such as cell attachment and confluence, by correlating acute changes in the slope of the longitudinal plot with the observed onset of those conditions.

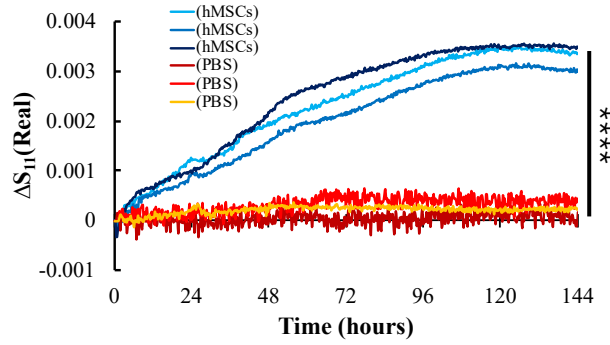


Figure 28. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) and sensor response in PBS over a period of 6 days. ($n = 3$) ****: $p < 0.0001$, unpaired t-test

3.3.4 Gelatin Surface Treatment

Analysis of cell number following gelatin surface treatment indicated that there were no significant differences in the cell density supported by sensors soaked in gelatin for 30 and 60 minutes prior to seeding compared to the control sensor that was not soaked in gelatin. Although the 30 minute treatment appears to support an increased cell density, there were too few samples to support any definitive conclusions regarding the differences in performance. It is well-documented that the stiffness of a substrate can influence adherent cell behavior and proliferation [53-56]. More specifically, Rowlands et. al. concluded that MSCs grown on hydrogels of varying stiffness proliferated more as the stiffness of the hydrogel increased, with the stiffest surfaces having a reverse effect [57]. Variations in stiffness may explain these results, as sensors treated with the 30 min soak supported nearly three times the cell density as the control sensors and twice the density of the sensors that were soaked for 60 min. However, we cannot make any definitive conclusions, as the stiffness of the gelatin on the sensor surface could not be probed with the resources available, and there is not enough sample power to identify significant differences in coating effect on cell density.

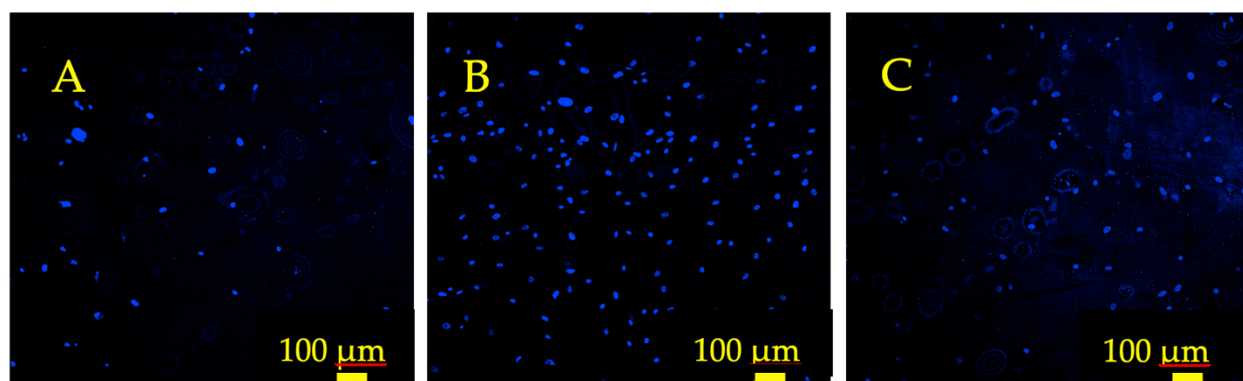


Figure 29. Fluorescence microscope images depicting hMSC nuclei on the sensor surface 24 h after seeding. (A) The control sensor that was not coated with gelatin. (B) A sensor that was soaked in gelatin solution for 30 min. (C) A sensor that was soaked in gelatin solution for 60 min. These sensors were seeded at 12,500 cells/cm², and the cells were stained with Hoescht 33342 and imaged with a DAPI filter [2].

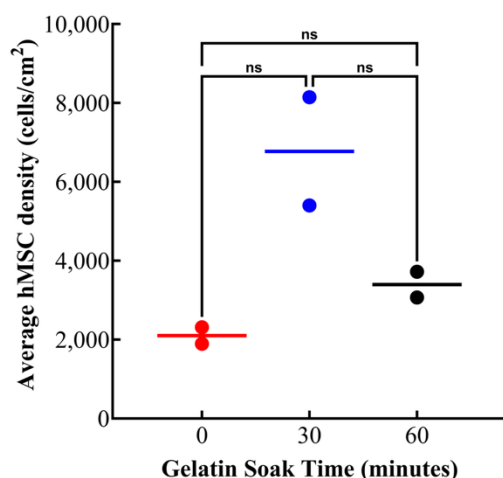


Figure 30. This chart depicts the average density of hMSCs quantified on the surface of sensors soaked in gelatin solution for 0, 30, and 60 min prior to seeding at 12,500 cells/cm² (RoosterBio donor #00175). Each point represents the average cell density quantified across 15 consistent locations on the sensor (Figure 20). ns: $p = 0.5731$ (0 & 60), 0.0557 (0 & 30), 0.1218 (30 & 60), ordinary one-way ANOVA.

3.3.5 Effects of Sensor Activity on Cell Morphology

The results of a quantitative shape factor analysis (Figures 31 and 32) indicate that there were no significant differences in the average shape factor between cells grown on sensors that were subjected to magnetic and mechanical stimulation and cells that were grown on a sensor without stimulation. There is no consensus in the literature regarding the relationship between

hMSC morphology and phenotype, however, advances in microscopy and deep learning techniques have increased our understanding of the relationship between morphology and cell fate. Recently, Mai et. Al. reported convolutional neural network (CNN) models predicting hMSC differentiation to adipocytes and osteoblasts within a day, based on morphological characteristics, with 93% accuracy [58]. **Figure 32** shows regular distribution patterns with similar positive skewness. A qualitative visual inspection of fluorescent microscope images (**Figure 33**) supports that the morphology of the cells are the same or very similar, taking on a predominately spindle-shaped morphology, which is typically associated with undifferentiated hMSCs [58, 59]. The conclusions we can draw from these results are limited – only one cell line was analyzed and the culture period was only 24 hours. These results suggest that the monitoring shows promise as a tool for tracking cell growth without impacting their attachment to the sensor surface.

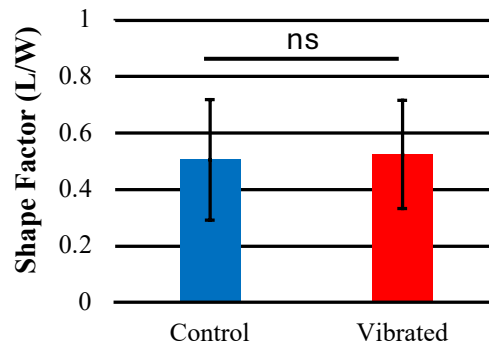


Figure 31. The average shape factor (L/W) of hMSCs imaged on the surface of sensors stained with Cell Tracker Green™ and imaged with FITC filter ($n = 10$). ns: $p = 0.1127$, unpaired t-test [2].

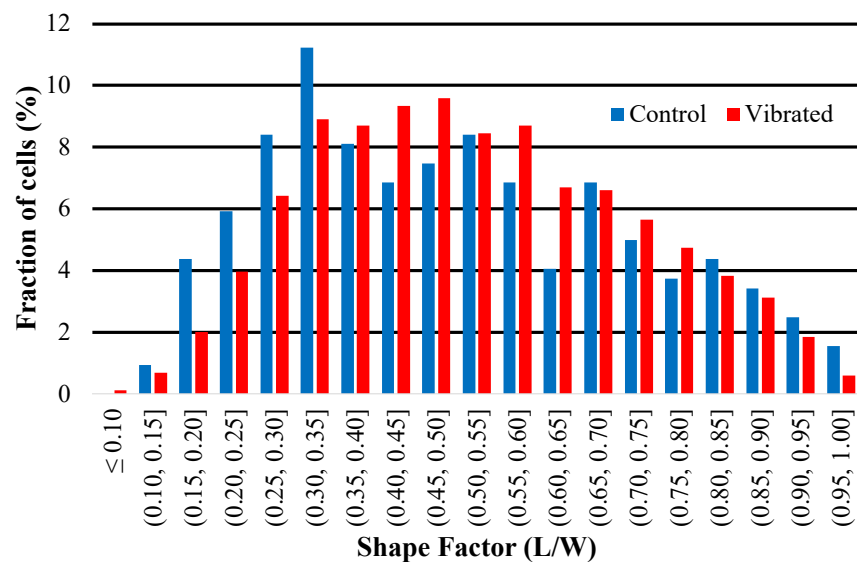


Figure 32. This histogram illustrates the distribution of shape factor (L/W) of hMSCs imaged on the surface of sensors. Skewness (control) = 0.35; skewness (vibrated) = 0.25 ($n = 10$) [2].

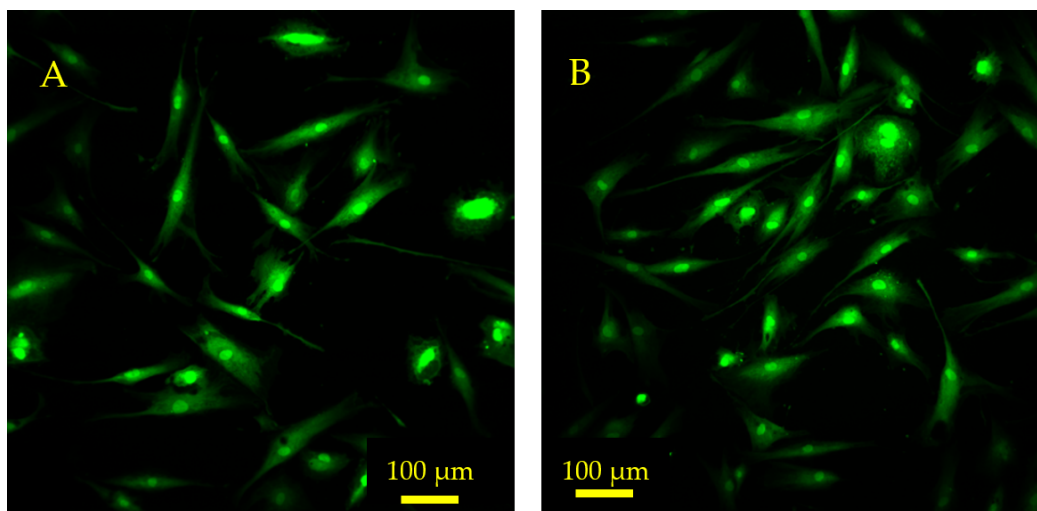


Figure 33. Fluorescence microscope images depict hMSCs growing on the sensor surface 24 h after seeding. (A) The control sensor that was not exposed to magnetic or mechanical stimulation. (B) An experimental sensor that was exposed to magnetic fields and mechanical resonance via the monitoring system. These sensors were seeded at 10,000 cells/cm². The cells were stained with CellTracker™ Green (CMFDA) dye prior to seeding and imaged using a FITC filter [2].

3.4 Conclusions

The results indicate that the magnetoelastic sensors and monitoring system can track adherent cell growth remotely and non-invasively. We demonstrated that the monitoring system is capable of measuring the density of cells at the sensor surface with a high degree of accuracy ($R^2 = 0.9144$) and with a LOD as low as 6,570 cells/cm² in the first 24 hours of culture (**Figure 26**). This is close to the recommended seeding density for the cell line (5,000 cells/cm²) in 2D culture, suggesting that with slight improvements, the sensor could provide reliable data on the attachment and early stages of cell growth. The LOQ over a 7 day culture period was 58,400 cells/cm², which is above the supplier's specified density for confluence for the cell line on TCP (50,000 cells/cm²), indicating that improvements to the sensitivity of the monitoring system are necessary for reliable quantification of cell density in 2D culture systems.

A key limitation of the monitoring system is that it cannot differentiate between the loading from cell growth and loading from extracellular matrix production or protein adsorption. While sensors in complete media showed no significant difference from sensors in PBS (**Figure 23**), additional experiments involving the decellularization and of the hMSCs and preservation of the ECM following culture must be conducted to understand the contributions of damping from the cells and the ECM individually.

The monitoring system was shown to be capable of longitudinally tracking cell growth, and a decreasing rate of change in sensor response over time could be observed as cell-loading approached confluence. Furthermore, examining the shape factor profiles and density of the vibrated and non-vibrated cells suggests that this technology can be used without significantly affecting the density of cells attached to the sensor surface, which is critical to the accuracy and robustness of the monitoring platform.

CHAPTER 4: MODULATION OF CELL ATTACHMENT ON MAGNETOELASTIC SENSORS VIA MELT ELECTROWRITING

This chapter includes previously published and co-authored material adapted from Skinner W.S., Saiz P.G., Reizabal, A., Plumley J.E., Dalton P.D., and Ong K.G., (2024, RSC Sensors & Diagnostics) [3].

4.1 Introduction

For chemical and biological sensing, the sensor's surface can be modified with coatings that selectively interact with the target analyte, transforming the presence of the analyte into a physical change, such as mass. Functional coatings have been applied to magnetoelastic sensors, facilitating the monitoring of various physical, chemical, and biological parameters and processes. Examples include humidity monitoring with titanium dioxide nanotubes [10, 60-63], toluene detection with metal-organic chemical frameworks [64], heavy metal sensing with graphene oxide [65], and even viral and bacterial detection with bioreceptors[16, 18, 20, 27-29, 48, 65-67]. Recent studies have suggested the possibility of enhancing the sensor's sensitivity by adjusting the degree of modification in different regions of the sensor[33], [68]. Given that the sensitivity of the sensor is not uniform across its surface, controlled load attachment throughout the sensor surface could enhance the robustness and reliability of the sensing platform. This is particularly important for certain applications such as adherent cell monitoring, where cells can attach to the surface in a non-uniform distribution.

The wireless nature of magnetoelastic sensors make them excellent candidates for use in controlled biological environments such as viral, bacterial, or cellular cultures [30], [69]. Previous work has demonstrated the use of these sensors to monitor adherent cell growth and modulate cell adhesion [2, 25, 36, 49, 70, 71]. However, these studies have noted a non-uniform distribution of

cells across the sensor and reduction in cell attachment associated with the sensor activity. Consequently, there is a need to develop surface modification to control cell adhesion across the sensor surface [49],[71, 72]. Some common methods for integrating homogeneous coatings onto the surface of magnetoelastic sensors include spin coating, dip coating, and vapor deposition. Additionally, electrospinning has recently been explored for increasing the active surface area of magnetoelastic sensors and thereby improving their overall response [63], [73]. Electrospun coatings are highly functionalizable, making them especially promising in this application [74, 75]. Despite the variety and versatility of these techniques, previous studies have focused on functionalization of the entire sensor surface and lack the capability to introduce user-defined microstructures onto the magnetoelastic sensor surface. Controlled surface functionalization at the local level could improve sensor performance by defining specific surface roughness or promoting higher mass gain in the most sensitive regions of the sensor.

In this context, we explored the application and effect of melt electrowriting (MEW) [76] to define different tailored microstructures onto the magnetoelastic sensor surface, aiming to control the attachment of cells across the sensor surface to reduce variability in sensor response due to non-uniform cell distribution. MEW is a high-resolution 3D-printing technique that leverages electrohydrodynamic phenomena to yield exceptional control over microfiber deposition [77, 78]. Furthermore, MEW has the capacity to process various materials, including functionalizable polymers and material composites, making it a versatile technique [79]. While MEW has been primarily used for fabricating scaffolds with custom microstructures and mechanical properties that mimic the extracellular matrix [80-82], this work focused on integrating defined MEW structures onto the magnetoelastic sensor surface to enhance the sensor's performance in monitoring cell growth. Cells have been previously reported to attach and grow

with a non-uniform density across magnetoelastic sensor surfaces [2]. Therefore, precise control over cell attachment density at different regions of sensitivity on the sensor surface could result in a more reliable and robust monitoring system.

This chapter describes a feasible method for integrating various MEW microstructures onto the surface of magnetoelastic sensors. The stability of these microstructures under different conditions is evaluated, demonstrating the good adherence of the deposited layers. Subsequently, the sensor performance is characterized by examining its response when loaded with growing human mesenchymal stromal cells (hMSCs). Specifically, the effect of the MEW microstructures on the attachment and distribution of hMSCs on both resonating and non-resonating modes is investigated. The capability to define MEW microstructures as the active surface of magnetoelastic sensors results in a highly tunable and versatile sensing platform that allows for tuning cell density across the sensor surface, holding potential for various applications. This chapter includes content adapted from Skinner W.S., Saiz P.G., Reizabal, A., Plumley J.E., Dalton P.D., and Ong K.G., (2024, RSC Sensors & Diagnostics) [3].

4.2 Methods

4.2.1 Materials

Metglas® 2826MB amorphous ferromagnetic alloy employed as the sensor material was obtained commercially in the form of a rolled ribbon of 29 µm thick and 12.7 mm wide (Metglas® Inc., Conway SC, USA). Metglas® 2826MB was used for its exceptional magnetic and magnetoelastic properties (saturation magnetization = 0.39 T, coercive force <50 A/m), low cost, and high corrosion resistance [83]. Medical grade poly(ε-caprolactone) (PCL) (PURASORB® PC 12, 2007001461, Corbion, Amsterdam, Netherlands) was used to create the MEW scaffolds. Granular dichloro-p-cyclophane (DPX-C, Specialty Coating Systems Inc., Indianapolis, IN, USA)

was used in combination with a PDS 2010 Labcoter® (Specialty Coating Systems Inc., Indianapolis, IN, USA) to deposit a conformal layer of Parylene-C onto the surface of sensors with and without MEW microstructures. Finally, xeno-free media (RoosterBasal-MSC-CC, RoosterBio, Frederick, MD, USA), penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), xeno-free supplement (RoosterBooster-MSC-CC, RoosterBio, Frederick, MD, USA), 0.25% trypsin-EDTA (Gibco, Invitrogen, Waltham, MA, USA), phosphate-buffered saline (PBS) (Endotoxin-Free Dulbecco's PBS 1X w/o Ca⁺⁺ & Mg⁺⁺, MilliporeSigma, Burlington, MA, USA), 32% paraformaldehyde (PFA) aqueous solution (Electron Microscopy Sciences, Hatfield, PA, USA) and Hoechst nuclei stain solution (Hoechst 33342, Trihydrochloride, Trihydrate, ThermoFisher, Waltham, MA, USA) were obtained from their respective suppliers and used as recommended for the culturing and quantification of cells.

4.2.2 Sensor Fabrication

Free-standing rectangular sensors of 5 mm × 12.7 mm were cut from the Metglas® 2826MB ribbon roll by using a high-power pulse laser (Coherent ExactCut 430, Coherent, Santa Clara, CA, USA) and subsequently cleaned with ethanol and annealed in an oven (Model 10 Lab Oven, Quincy Lab, Inc., Burr Ridge, IL, USA) at 125 °C for 2 hours. Following the annealing of the sensors, they were characterized by measuring the real part of the S₁₁ parameter using a network analyzer (Keysight ENA E50618, Keysight Technologies, Santa Rosa, CA, USA) with a custom-wound interrogation solenoid (see Section 2.5 for details). Sensors were then sorted into groups, so that the response magnitudes of sensors in each group were within 5% of their expected full-scale output, defined as the difference in response magnitude between the start of the cell experiment and after the final sensor signal was recorded. Afterward, PCL scaffolds were printed

directly onto a set of sensors using MEW. To accomplish this, a magnet was placed on the printing collector beneath a glass slide to keep the sensors in place during the printing process. Sensors were placed on the glass slide and MEW fibers were deposited onto the sensor surface, past the edges of the sensor (see Section 4.2.3 for details of the MEW process). The excess scaffold material over the edges of the sensor was trimmed with a VLS 2.30 laser cutting machine (Universal Laser Systems, USA), which also improved the adhesion of the scaffold fibers to the sensor by partially melting the scaffold around the edges of the sensor. Next, these sensors were coated with a 10 μm -thick conformal Parylene-C layer (PDS 2010 Labcoter system, SCS, USA) to improve corrosion resistance and facilitate biocompatibility [25]. Afterwards, sensors were treated with oxygen plasma in a reactive ion etching system (March Jupiter II RIE, USA) for 30 s at 100 W to promote cell adhesion. Finally, fabricated sensors were cleaned with 70% ethanol, allowed to dry, placed in sterilization pouches, and sterilized with ethylene oxide gas before using them in cell culture experiments. **Figure 34** illustrates the sensor preparation and cell growth detection process. In addition to these sensors, a set of sensors without MEW scaffolds were also prepared using the same protocol (except the MEW printing step) to serve as controls for the experiment.

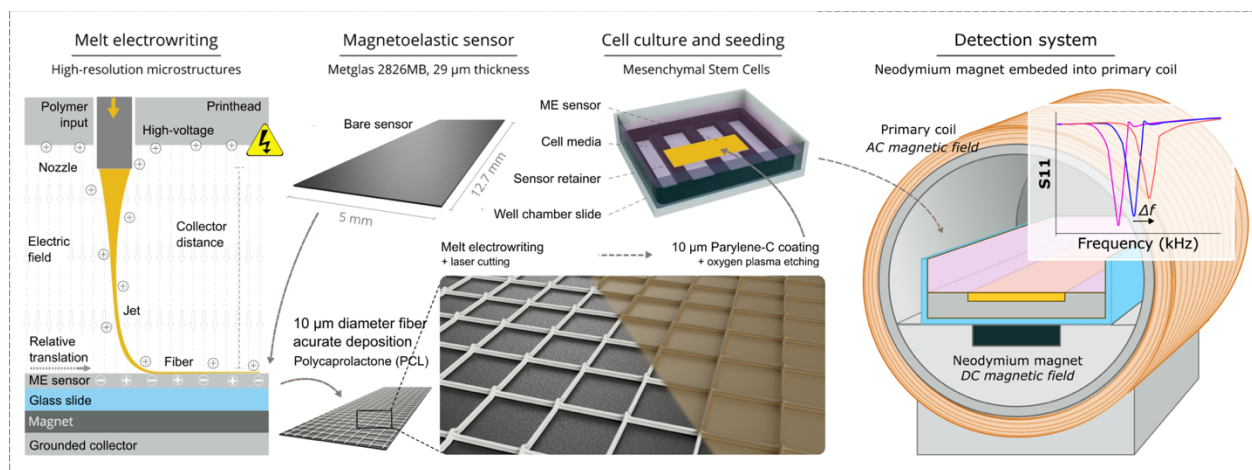


Figure 34. Schematic diagram highlighting key details of sensor preparation and the magnetoelastic monitoring system *Figure generated collaboratively with Saiz, P.G.[3].*

4.2.3 Melt Electrowriting Process

PCL microstructures were electrowritten onto the surface of magnetoelastic sensors using a custom-built MEW device [84]. Briefly, the device consists of a moving collector that is controlled by two linear stages (X/Y) and a heated print head containing a 27-G nozzle plastic syringe (Nordson EFD) connected to an air pressure controller (SMC, USA). A high-voltage source is used to create an electrical potential difference between the positively charged nozzle and the grounded collector. For the electrowriting process, the distance between the collector and the nozzle was set to 3 mm while the voltage, temperature, and pressure were set to 4 kV, 85°C, and 260 kPa, respectively. The jet speed at these conditions, determined by measuring the critical translation speed, was 350 mm/min [85]. This allowed PCL microstructures to be fabricated at a speed of 480 mm/min from 10 μ m thick fibers without excessive jet stretching.

4.2.4 hMSCs Culture

Human bone marrow-derived Mesenchymal Stem Cells (hMSCs) were used for cell culture experiments. Cells were commercially obtained (donor #000175, female, 28 years old, RoosterBio, Frederick, MD, USA), expanded according to manufacturer's protocol, and cryogenically stored in liquid nitrogen. Prior to use, cells were recovered from liquid nitrogen, thawed, seeded at 5,000 cells/cm², and cultured until confluent. These cells were cultured in xeno-free media and supplemented with 1% penicillin-streptomycin and xeno-free supplement. Once confluent (~72 hours, confirmed via light microscopy), cells were lifted with 0.25% trypsin-EDTA, counted using an automatic cell counter (NucleoCounter NC-200, Chemometec, USA), and then resuspended in media to the target seeding density. Cells were incubated at 37.0 °C, 93% relative humidity, and

under 5% CO₂ in a microbiological incubator (HERAcell VIOS 160i, Thermo Fisher Scientific, USA).

Each sensor was placed inside a 2-well chamber slide (Nunc Lab-Tek II Chamber Slide system, Thermo Fisher Scientific) on top of a 3D-printed grated stage. The stage, 3D-printed with an autoclavable and biocompatible resin (High Temp v2, FormLabs, Somerville, MA, USA), has a recessed retainment area designed to allow the sensor to vibrate freely while remaining at the same position. All stages were cleaned with 70% ethanol in an ultrasonic cleaner (CO-Z supplies, Lake Forest, USA) for 5 minutes, placed inside sterilization pouches, and then autoclaved (Beta Star Small Sterilizer Autoclave, Beta Star, Honey Brook, USA). After placing the sterilized sensors on the grated stage in the 2-well chamber slide (see **Figure 34**), cells were seeded at varying densities ranging from 10,000 cells/cm² to 40,000 cells/cm². Culture volume was seeded at 2 mL to ensure the sensor and cells remained submerged for a period of at least 6 days. Finally, the chamber slide was placed inside the interrogation solenoid to wirelessly activate and capture the resonance spectrum of the sensor in real time.

4.2.5 Sensor Characterization

The sensor signals were remotely captured using an experimental set-up that was modified from our previous work [2]. The detection system included a neodymium magnet (B4201, K&J Magnetics, Pipersville, PA, USA) to maximize the magnetostriction of the sensor and increase its resonance amplitude. The magnet was embedded inside an interrogation solenoid (24 AWG, 135 turns, 2 layers, 39 mm in diameter, 39 mm in length) which applied an AC magnetic field that caused the sensor to resonate, while also captured the sensor signal by measuring the S₁₁ parameter of the solenoid. The S₁₁ parameter of the interrogation solenoid was measured with a tabletop

network analyzer (Keysight ENA E50618, Keysight Technologies, Santa Rosa, CA, USA) or a portable network analyzer (NanoVNA-F, Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China). The network analyzer and the transmission cables for connecting the interrogation solenoids were calibrated together with a manual open-short-load calibration kit (Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China). The network analyzer captured and saved the resonance spectrum of the sensor into data files, which were later analyzed and plotted on a computer. A background measurement, generated by collecting the resonance spectrum of the solenoid without a sensor, is subtracted from all sensor measurements to eliminate the effect of the solenoid's impedance on the sensor resonance measurement. To monitor the attachment and growth of cells, sensors seeded with cells were positioned in the interrogation solenoid such that the maximum possible signal was observed on the network analyzer display. The baseline resonance spectrum for each sensor was measured immediately after the addition of cell suspension, marked as the first measurement (0 h). Subsequent resonance spectra were captured every 15 minutes following the 0 h measurement, creating a profile of cell growth over 6 days.

Immediately after collecting the endpoint resonance spectrum, quantification of cell nuclei was conducted to investigate the relationship between changes in the sensor signal and the attachment, growth, and distribution of cells on the sensor surface. The process involved aspirating culture media from the well containing the sensor, rinsing the well with PBS, and aspirating the rinse. Subsequently, cells were fixed by incubating the sensors for 10 minutes in a solution of 4% PFA diluted in PBS. After aspirating the PFA solution, the nuclei of the cells were stained by incubating them for 10 minutes in a solution of Hoechst 33342 nuclei stain, diluted to 50 $\mu\text{g/mL}$ in PBS. Following this incubation period, the staining solution was aspirated, and the sensor was

carefully inverted into the empty, unused well of the chamber slide. The sensor was covered with PBS, and the chamber slide was wrapped in aluminum foil to protect it from exposure to ambient light. Fluorescent microscopic imaging was performed using a confocal fluorescence microscope (CSU-W1 SoRa, Nikon, Japan) with a DAPI (4',6-diamidino-2-phenylindole) filter to show the stained nuclei. To quantify the number of cells attached to the sensor surface, representative images of $1\text{ mm} \times 1\text{ mm}$ were taken for each sensor in 12 specific locations that were kept consistent for each sensor. Cell nuclei were quantified through the analysis of fluorescence microscope images using custom MATLAB analysis software.

Finite Element Analysis (FEM) simulations of the first resonance frequency mode of the sensors were performed using COMSOL Multiphysics® software (COMSOL AB, Stockholm, Sweden) to determine local extremes in sensitivity along the sensor surface and hence define a microstructure according to those results. The microstructure and morphology of the PCL scaffold layers were visualized with a digital microscope (VHX-7000, Keyence, USA), as well as by a scanning electron microscope (SEM) (Apreo 2, Thermo Fisher, USA) to characterize the scaffold geometry and confirm adhesion and stability of the microstructure defined above the sensor surface.

4.3 Results and Discussion

4.3.1 MEW Printability on the Sensor Surface Effect on the Sensor Signal

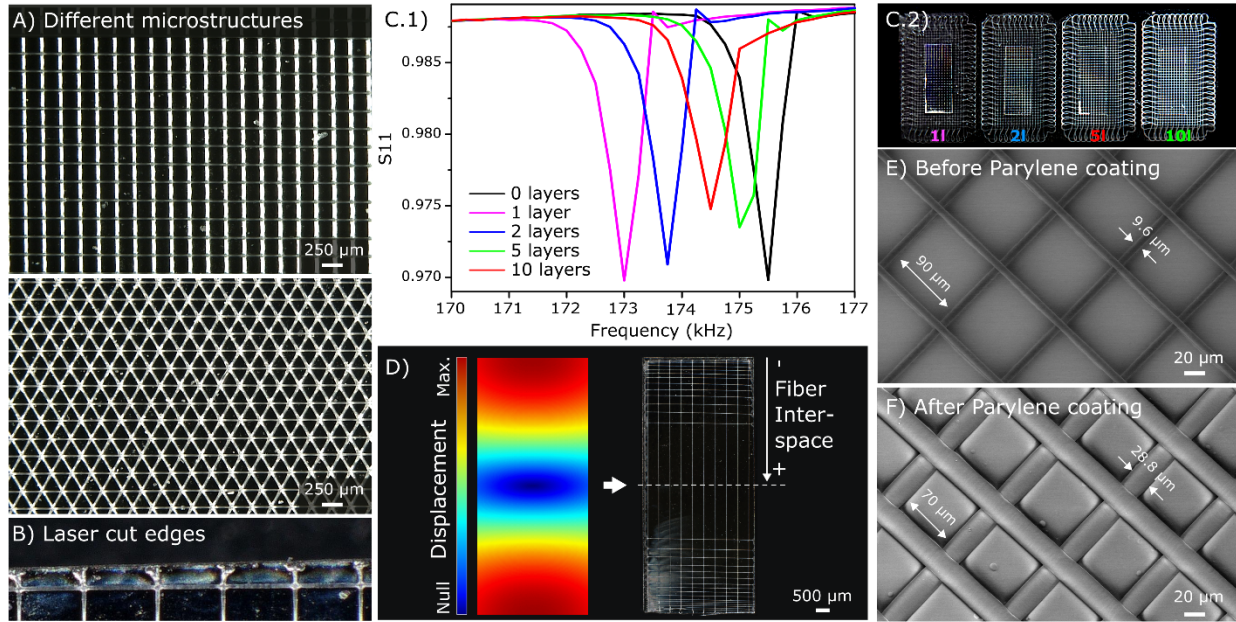


Figure 35. (A) Different PCL microstructures directly printed onto the magnetoelastic sensor surface. (B) detail of the laser cut edge following PCL deposition, where the fibers at the sensor's edges melted, enhancing scaffold adhesion to the sensor, (C) magnetoelastic resonance measurements (C.1) showcasing the impact of increasing number of layers in the MEW microstructure deposited on the sensor (C.2), (D) COMSOL simulation of the resonance displacement and sensitivity across the sensor surface and gradual MEW microstructure designed following the sensitivity profile along the longitudinal direction, (E, F) SEM images of the MEW microstructure before and after the Parylene-C coating. Figure generated collaboratively with Saiz, P.G. [3]

First, the quality of the printing onto the magnetoelastic sensor surface was optically investigated, showing that it was comparable to that achieved when printing directly onto the collector [86]. Under the conditions described previously, fibers of around $9.6 \pm 0.2 \mu\text{m}$ in diameter with a minimum interspace of around $90 \mu\text{m}$ onto different microstructures were achieved (**Figure 35A**). The placement of a magnet beneath the glass slide to hold the magnetoelastic sensors during the printing process was essential, as movement of the collector during printing could otherwise displace the sensors and disrupt fiber deposition. The MEW microstructures were printed beyond the sensor edges, with the excess material later trimmed off with laser. This process further melted

the fibers near the sensor edges (**Figure 35B**), causing them to adhere better onto the sensor surface.

Various microstructures were designed and printed onto the sensor surface to investigate its performance. Specifically, square microstructures with a fixed interspace of 500 μm and a varied number of layers (1, 2, 5 and 10) were printed onto the magnetoelastic sensors to determine the effect of these coatings on the resonance frequencies and magnitudes. The results indicate that the sensor magnitude decreased as the number of deposited layers increased due to the increased mass loading from additional MEW fibers (**Figure 35C**). Despite the dampening of the sensor resonance by the microstructures, the quality factor of the resonance profile was only decreased by 25% for 10 layers of microstructure and 8% for 2 layers, supporting the use of MEW microstructures on magnetoelastic sensors.

In addition, a microstructure with a gradual pore size going from very small rectangular pores of $100 \times 500 \mu\text{m}$ on the sensor tips to bigger pores that increase progressively was designed following the tendency of the sensitivity along the longitudinal length of the sensor simulated in COMSOL (**Figure 35D**). This demonstrates the versatility of MEW technique to define specific microstructures above magnetoelastic sensors, including the possibility to deposit different amounts of material in zones [87] with different sensitivities for a more effective sensing process, which can find applications in different areas.

4.3.2 Evaluation of the MEW Coating

After deposition MEW fibers onto the magnetoelastic sensors, the stability of these microstructures was assessed using SEM after exposure to air and liquid media. The same evaluation was repeated following Parylene-C coating and plasma etching of the sensors. This

assessment was conducted for samples with different numbers of layers and fiber sizes, and the results indicated that the microstructure morphology and adhesion were maintained after Parylene-C coating and plasma etching. SEM images revealed that the median diameter of the microfibers increased from $9.6 \pm 0.2 \mu\text{m}$ for the bare PCL fibers (**Figure 35E**) to around $28.8 \pm 0.4 \mu\text{m}$ after the Parylene-C coating (**Figure 35F**). This resulted in a decrease in the median pore size from around $90 \times 90 \mu\text{m}$ to $70 \times 70 \mu\text{m}$, confirming the $10 \mu\text{m}$ thickness of the Parylene-C coating. A similar morphology was observed after the plasma etching, affirming that this process did not affect the defined microstructure.

Sensors coated with MEW microstructures were also placed in water and activated with an interrogation solenoid to investigate the stability of the MEW layer in liquid media. No detachment of the microstructure from the sensor surface was observed, indicating the microstructures were physically stable even through the sensor vibration and in liquid media. Similar experiments were conducted for sensors with the Parylene-C coating and plasma etching, and no differences were observed in terms of stability.

4.3.3 In-situ Monitoring of Cell Growth

While magnetoelastic sensors have been utilized as a sensing platform for non-invasive real-time monitoring of cell growth, certain challenges have been identified with this approach. Notably, resonance activity of the sensors at relatively high magnitudes has been linked to the detachment of cells [49, 71], potentially compromising the accuracy of measurements. Additionally, in the early stages of cell growth and attachment (<24 hours), a non-uniform distribution of cell density along the sensor surface was observed which could affect the reliability of measurements due to the variance of sensitivity across the sensor surface [34, 68]. In addressing

these challenges, the incorporation of a MEW microstructure on the sensor surface holds promises as it can increase the available surface area and introduce 3D structures for cells to attach to, potentially alleviating these issues.

MEW has been commonly employed to develop scaffolds for 3D cell culture [79]. Several studies have highlighted the significance of the scaffold geometry, including pore size, shape, fiber diameter, and stiffness, as a key factor that can influence cellular behavior [88, 89]. A recent study investigating osteogenesis in hMSCs demonstrated the benefits of using 100 μm square PCL scaffolds fabricated via MEW. Compared to other pore sizes, 100 μm square scaffolds facilitated a higher seeding efficiency while maintaining a spread cellular morphology [90]. Based on these results, a microstructure with 100 μm size square pores was selected to investigate the effects of the microstructure on cell adherence and growth at the sensor surface. MEW microstructures with 100 μm square pores and a varied number of layers (1, 2 and 5) were printed onto magnetoelastic sensors and investigated in terms of magnetoelastic signal and cell attachment and distribution. First, magnetoelastic measurements indicated that the 5-layer microstructures severely dampened the sensor signal, leading to its exclusion from further investigation. In contrast, the 2-layer microstructures reduced the Q factor by only 8% compared to the uncoated sensor. The dampening of the signal reduced the sensors' sensitivity to cell loading. In the selection of the coatings for further investigation, it was important to consider the balance between the control over cell adhesion and the preservation of sensor signal magnitude and quality. Therefore, the 1-and 2-layer sensors were further investigated in terms of cell growth. Sensors with and without the MEW microstructures were seeded with human mesenchymal stromal cells (hMSCs) at a density of 40,000 cells/cm² and incubated for 24 hours to investigate the cells' attachment and distribution across the sensors. Results indicated that the average cell density was slightly higher on the sensors

without microstructures. However, the sensors with microstructures showed improvement in the uniformity of cell density as the number of layers increased, as shown in **Figure 36**. Observation of the MEW structure on the fluorescent images demonstrated the stability of the defined microstructure even after prolonged exposure to cell media, which is an important consideration in the application of the sensors with MEW microstructures for monitoring cell growth and other parameters in liquid media.

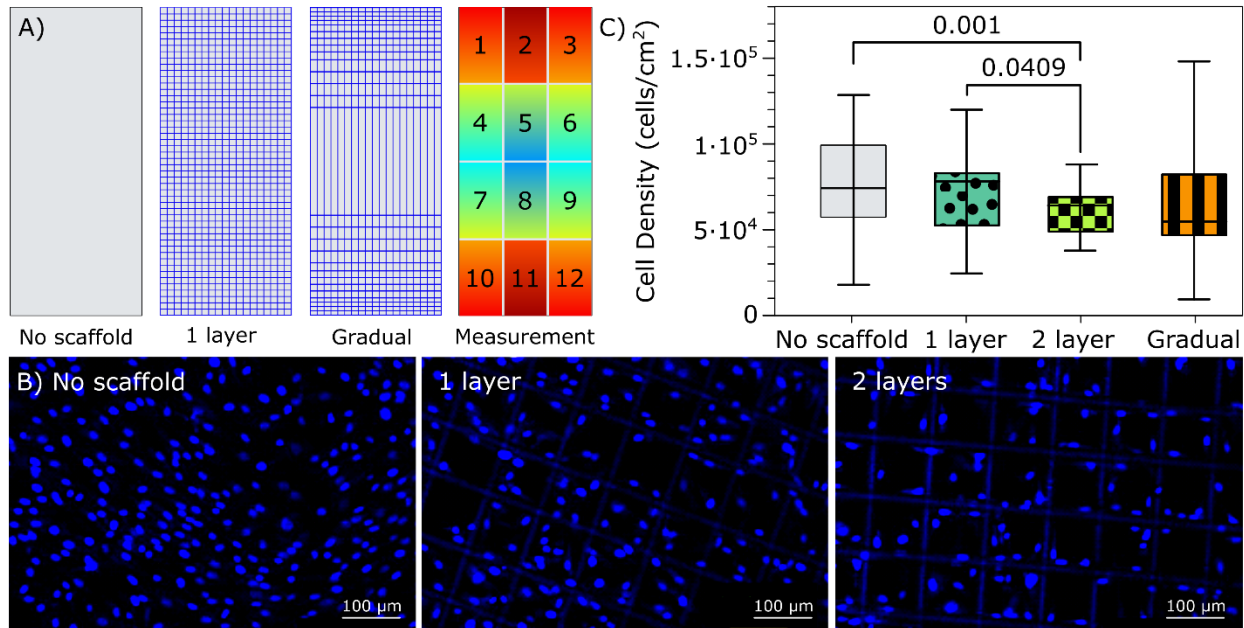


Figure 36. (A) Scheme of the different sensors investigated for cells attachment and of the different key zones analyzed, (B) Images and (C) quantification for cells attachment on the sensors with and without MEW microstructures 24 hours after seeding. Cells were counted from fluorescence microscope images taken in key areas of the sensor and the average cell density per unit area was calculated. The error bars are indicative of the variance in cell density across the sensor surface. One-way ANOVA with Tukey's multiple comparisons test. Figure generated collaboratively with Saiz, P.G. [3]

Microstructures with a gradual pore design (**Figure 35D**) were employed to further investigate the possibilities of MEW microstructural design in tuning cell distribution along the sensor surface. The density of cells along this geometry was explored, revealing that this design allowed for further tuning of cell density across the sensor, increasing the variance in cell density across the sensor surface. Specifically, fluorescence microscopy showed that for these gradual pore

size scaffolds, there were fewer cells attached in the regions with smaller pore sizes. This highlights a key advantage of using MEW fibers on top of magnetoelastic sensors since the pore size along the structure could be precisely tuned to partially avoid cell attachment or implement other functionalities on specific regions of the sensor.

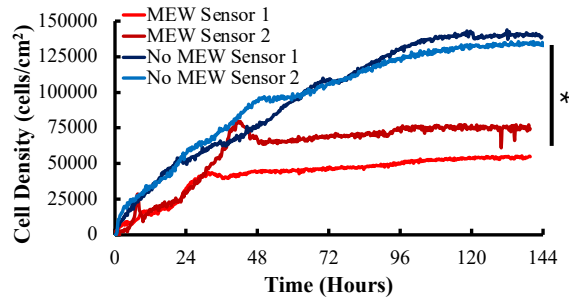


Figure 37. Cell growth profiles resulted from plotting the measured change in S11 parameter at the resonance frequency normalized to the final cell density against the elapsed time. *: $p = 0.0205$, unpaired t-test

After confirming cell attachment and proliferation on the sensors with the MEW microstructures, cell growth was continuously tracked over a 6-day period by measuring the resonance profile of the sensors. For this purpose, the 2-layer sensors with a 100 μm pore size were selected, along with the bare sensors for reference. The prepared sensors were placed in a chamber slide, seeded with cells at a density of 12,000 cells/cm² and the resonance spectrum was captured immediately and then once every 15 minutes for 6 days to generate growth profiles of hMSCs with and without the MEW microstructures. The change in the real portion of the S11 scattering parameter, a measurement directly related to the magnitude of the sensor resonance, was recorded as a function of time (**Figure 37**). This indicates the capacity of those sensors for continuous and wireless monitoring of cell growth during culture. The overall change in signal magnitude was normalized to the average cell density quantified on the sensor surface and plotted against the elapsed time. Comparison of the generated growth profiles suggests that the presence of MEW microstructure may inhibit the growth and proliferation of hMSCs compared to sensors without

the microstructure. Cell growth plateaus after nearly two days for sensors with the MEW structure, while it continues until around day 5 for sensors without it.

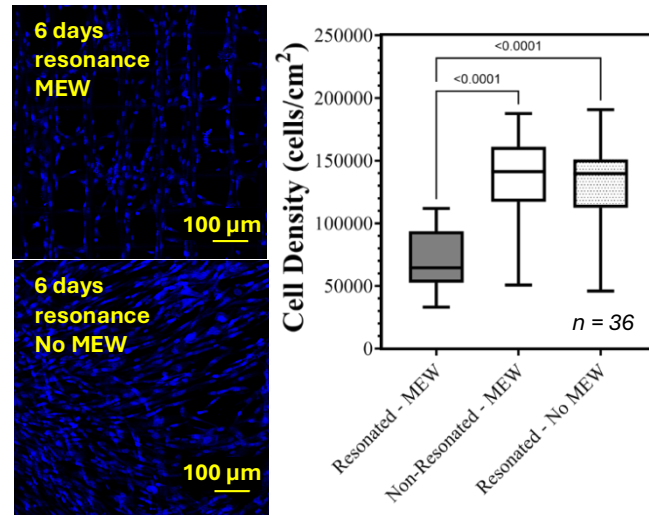


Figure 38. Differences in attachment and retention of cells on the different sensors in the presence and absence of sensor resonance. Each group consists of 3 sensors, with 12 specific locations imaged for each sensor ($n = 36$). The error bars are indicative of the variance in cell density across the sensor surface. Two-way ANOVA with Tukey's multiple comparisons test was performed [3].

In conclusion, cell adhesion under sensor resonance was investigated to determine if the MEW microstructures could improve the attachment or retention of the cells during resonance. Similar to previous experiments, the presence of the MEW microstructures improved the uniformity in cell density across the active sensor surface compared to sensor without the MEW layer. However, the reduction in average cell density under resonance for the sensors with the MEW microstructure was similar to that measured for the sensors without the MEW microstructure, which indicates that the MEW microstructure does not improve the retention of cells under resonance (**Figure 38**).

This study demonstrated that cell adhesion and proliferation on the surface of magnetoelastic sensors can be controlled by the incorporation of MEW microstructures on the sensor surface. Furthermore, specific microstructural designs, such as the gradual pore one shown here, can further facilitate the control of cell density at local regions along the sensor surface,

taking advantage of regions with differences in sensitivity. Overall, the capacity to define complex microstructures on the surface of magnetoelastic sensors introduces numerous possibilities for different applications beyond monitoring cell growth.

4.4 Conclusions

The feasibility of using MEW to define different microstructures on magnetoelastic sensors has been demonstrated. Direct writing through MEW, followed by laser cutting, was found to be a good approach for integrating various MEW microstructures onto magnetoelastic sensors. This method resulted in good adhesion to the sensor surface, precise control over the microstructure, and excellent repeatability. Furthermore, the stability of the MEW microstructure in liquid media, as well as after Parylene-C coating and plasma etching has been confirmed. Magnetoelastic resonance magnitude and quality were found to vary with the microstructure properties, including the number of layers, the pore size, and the scaffold geometry.

An evaluation of the performance of sensors with and without MEW microstructures as platforms for non-invasive and continuous monitoring of cell growth was conducted. This included an analysis of the change in resonance amplitude with cell density and time, as well as an analysis of the cells' distribution and attachment across the surface of sensors with and without the MEW microstructures. Results demonstrated that sensors with MEW microstructures can be used for monitoring cell growth while maintaining sufficient signal quality. The incorporation of MEW microstructures on the sensor surface can improve the uniformity of cell density across its surface. Analysis of the normalized growth profiles measured by monitoring the magnetoelastic resonance frequency suggests that the presence of the microstructure on the sensor inhibited the growth and proliferation of cells across its surface relative to sensors without the microstructure. Future work

could explore additional functionalization of the microstructure sensor surface by altering the geometrical parameters and using additional cell-affinity coatings such as heparin and collagen to improve the attachment and uniformity of cells on the sensors. Overall, the incorporation of melt-electrowritten microstructures as a coating on magnetoelastic sensors resulted in a versatile sensing platform with highly tunable surface characteristics that can be employed in a broad range of sensing applications in addition to monitoring cell growth.

CHAPTER 5: EVALUATION OF THE EFFECT OF FUNCTIONAL COATINGS ON THE GROWTH OF HUMAN MESENCHYMAL STROMAL CELLS

The functional coating described in this chapter was developed by Mara Domenech's lab at the University of Puerto Rico Mayaguez and was applied to the sensors by Said Cifuentes and Carolina Rivera-Crespo. I was the primary executor of the remaining experimental tasks including sensor fabrication, cell culture, and preparation of the figures and written materials.

5.1 Introduction

As cell therapeutics become increasingly successful and popular, the need and desire to improve the efficiency and robustness of culture methods also increase. Expansion of cells to therapeutic yields while maintaining therapeutic potency is still a challenging and costly endeavor [91]. While sensor and reactor designs can improve the yield and quality of cells cultured, the improvement and advancement of the culture substrate, especially for anchorage dependent cells, is also important. Previous work by Cifuentes et al. at the University of Puerto Rico Mayaguez (UPRM) has demonstrated that the treatment of tissue culture surfaces with a coating consisting of alternating layers of collagen I (COL) and heparin (HEP) (referred to by the group as “layer-by-layer” or “LBL” coating) can improve their growth in low-serum conditions (yielding similar performance in 2% serum media as compared to 10% serum), improve the secretion of immunosuppressive factors by hMSCs, and also delay senescence [92]. The success of these HEP/COL LBL coatings spurred Cifuentes et al. to develop additional coatings using collagen I and recombinant heparan sulfate (rHS). Compared to HEP, rHS, a synthetic glycosaminoglycan (GAG), exhibits strong binding affinity extracellular matrix components and multiple growth factors and lacks the xenobiotic health risk and anti-coagulant effects of HEP. The group found that the LBL coatings supported culture in low-serum (2%) and serum-free conditions comparable

to standard culture conditions [93]. The degree of sulfation of GAGs has an effect on the GAGs' interaction with collagen as well as the attachment and proliferation of hMSCs to the culture surface [94, 95].

In this chapter, we collaborated with Domenech's lab at UPRM to apply LBL coatings of HEP/rHS and COL to the surface of the sensor. The goal of this work was to evaluate the effect of each coating on the growth kinetics of the hMSCs. HEP and rHS used in combination with COL to form the LBL coatings contain varied levels and locations of sulfate groups and therefore are expected to have slight differences in binding affinity for biomolecules, which could result in differences in attachment and growth of cells grown on the LBL surfaces. Because the sensor is sensitive to the attachment and loading of hMSCs, we hypothesized that we could elucidate differences in the performances of the coatings by applying these coatings to the sensor surface and monitoring the growth of cells seeded onto the sensors.

5.2 Methods

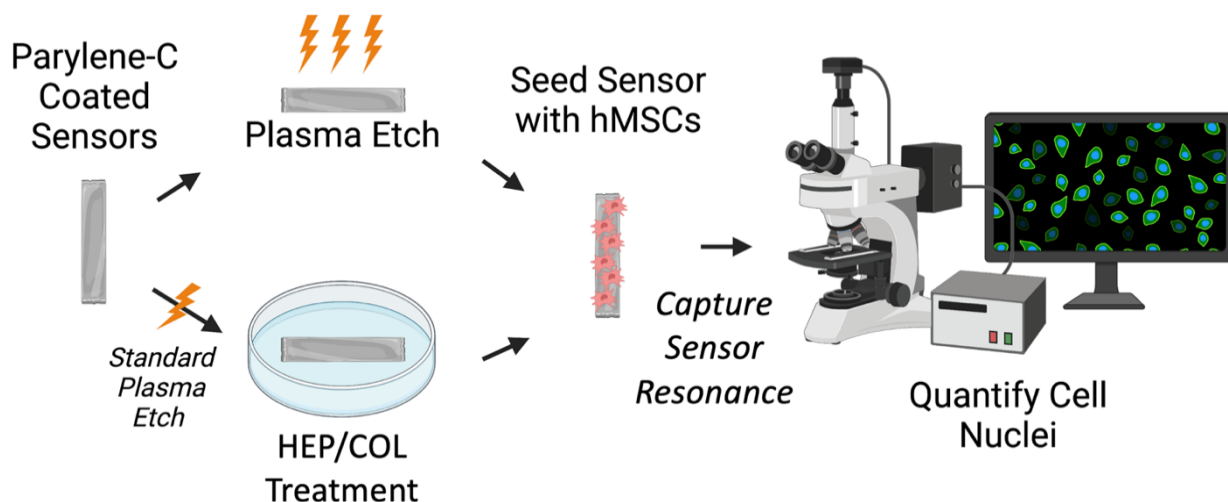


Figure 39. Overview of the basic methodology and flow of experimentation in this work. Sensors were coated with Parylene-C and plasma-etched to promote cell adhesion. A selection of sensors were coated with heparin or heparan sulfate and collagen I in alternating layers. Sensors were seeded with cells, and the sensor resonance was tracked during culture. Following culture, samples were fixed and stained for fluorescent microscopic analysis.

5.2.1 Sensor Fabrication

Sensors were made from a commercially available magnetoelastic material, Metglas 2826 MB (Metglas Inc., Conway, SC, USA) [35]. Metglas 2826 MB was used because of its good magnetization (saturation magnetization = 0.39 T) and low coercivity (coercive force < 50 A/m) that result in a strong signal as well as large magnetostriction (saturation magnetostriction = 12 ppm) that makes them sensitive to mechanical loading. The material was purchased as a rolled, thick-film strip of 12.7 mm wide and 29 μm thick. A stack of 4 magnetoelastic strips of about 15 cm long was placed on top of a 0.5 mm thick steel sheet (McMaster-Carr, Elmhurst, IL, USA) that was covered in a layer of aluminum foil (Thermo Fisher Scientific, Waltham, MA, USA). Free-standing rectangular sensors of 5 mm $\times$ 12.7 mm were cut from the prepared stack of magnetoelastic strips by using a high-power pulse laser (Coherent ExactCut 430, Coherent, Santa Clara, CA, USA) and subsequently cleaned with ethanol and annealed in an oven (Model 10 Lab Oven, Quincy Lab, Inc., Burr Ridge, IL, USA) at 125 $^{\circ}\text{C}$ for 2 hours. Following the annealing of the sensors, they were characterized by measuring the real part of the S_{11} parameter using a network analyzer (Keysight ENA E50618, Keysight Technologies, Santa Rosa, CA, USA) with a custom-wound interrogation solenoid. 24 sensors were then sorted into a group, so that the response magnitudes of each sensor were within 5% of each other. Next, these sensors were coated with a 10 μm -thick conformal Parylene-C layer (PDS 2010 Labcoter system, SCS, USA) to improve corrosion resistance and facilitate biocompatibility. Afterwards, sensors were treated with oxygen plasma in a reactive ion etching system (March Jupiter II RIE, USA) for 30 s at 100 W on each side of the sensor to promote cell adhesion, improve wettability, and mitigate the formation of bubbles at the sensor surface. Finally, fabricated sensors were cleaned with 95% ethanol,

allowed to dry, placed in sterile conicals, packaged, and shipped to UPRM for the application of LBL coatings.

5.2.2 LBL Coatings

At UPRM, 6 of the sensors were treated with heparin (HEP) and collagen I (COL) in alternating layers via polyanionic (HEP) and polycationic (COL) solutions to form a 9-layer coating beginning and ending with HEP. Lyophilized collagen I from bovine tendon (Integra Lifesciences Holdings Corporation, Añasco, PR) and heparin sodium (PH3005, Celsus Laboratories, Cincinnati, OH, USA) were utilized to deposit multilayers on the surface of magnetoelastic sensors, following the procedure as described by Castilla et al. [96]. Polymers were dissolved at a concentration of 1.0 mg/mL in acetate buffer pH 5 (0.1 M sodium acetate anhydrous and 0.1 M glacial acetic acid dissolved in ultrapure water 18 M Ω ·cm from RiOs™ and Milli-Q® water system). LBL technique was employed to construct nine consecutive polymeric multilayers of HEP/COL (ending in HEP). The prepared sensor surface was charged by immersion in 1 mL of polyethyleneimine (PEI) at 1.0 mg/mL in acetate buffer (P3143, Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. PEI created a charged surface for the subsequent deposition of alternate layers of HEP and COL. Each polymer layer deposition was incubated for 5 minutes followed by a 3-minute wash with acetate buffer (pH 5) until the desired number of alternating HEP and COL layers were formed. A final wash was performed using sterile Dulbecco's phosphate-buffered solution (DPBS), followed by UV sterilization in the biosafety cabinet for 10 minutes. 12 more sensors were treated following the same protocol, except that the heparin was replaced with a recombinant heparan sulfate (rHS01, rHS29, TEGA Therapeutics) for a total of 3 experimental groups and 1 control group (no experimental coating, standard surface treatment) of 6 sensors

each. Samples were then shipped back to the University of Oregon (UO) for the cell growth monitoring experiment (**Figure 39**).

5.2.3 Retention Stage Fabrication

Sensors were suspended in wells by a grated stage with a recessed retainment area (**Figure 4**). Retention stages were designed with computer-aided design (CAD) (Autodesk Fusion 360 v2.0) and 3D printed using a stereolithography (SLA) printer (Form 4B, Formlabs, Somerville, MA, USA) with biocompatible and autoclavable resin (High Temp V2 Photopolymer Resin, Formlabs, Somerville, MA, USA). 3D printed stages were cleaned with 95% ethanol in a sonicator and then cured (Form Cure, Formlabs, Somerville, MA, USA) according to the manufacturer's protocol. Following the cure, retention stages were placed in sterilization pouches (Beta Star Small Sterilizer Autoclave, Beta Star, Honey Brook, PA, USA) and autoclaved. Autoclaved retention stages were coated with Parylene-C and etched on the top and bottom surface alongside the sensors to improve wettability and mitigate the formation and retention of bubbles.

5.2.4 hMSCs Culture

Human bone marrow-derived Mesenchymal Stem Cells (hMSCs) were used for cell culture experiments. Cells were commercially obtained (donor #000277, RoosterBio, Frederick, MD, USA), expanded according to manufacturer's protocol, and cryogenically stored in liquid nitrogen. Prior to use, cells were recovered from liquid nitrogen, thawed, seeded at 5,000 cells/cm², and cultured until confluent. These cells were cultured in xeno-free media and supplemented with 1% penicillin-streptomycin and xeno-free supplement. Once confluent (~72 hours, confirmed via light microscopy), cells were lifted with 0.25% trypsin-EDTA, counted using an automatic cell counter

(NucleoCounter NC-200, Chemometec, USA), and then resuspended in media to the target seeding density. Cells were incubated at 37.0 °C, 93% relative humidity, and under 5% CO₂ in a microbiological incubator (HERAcell VIOS 160i, Thermo Fisher Scientific, USA).

Each sensor was placed inside a 2-well chamber slide (Nunc Lab-Tek II Chamber Slide system, Thermo Fisher Scientific) on top of a 3D-printed grated stage. Because our experimental set-up is limited to 6 samples, the experiment was conducted over the course of 4 weeks, where each week contained at least 1 but no more than 2 of each experimental and control group. After placing the sensors on the grated stage in the 2-well chamber slide, sensors were seeded with human mesenchymal stromal cells (hMSCs) suspended in xeno-free media at 12,000 cells/cm² (27,000 cells/mL) and monitored for 6 days. Following capture of the final resonance profile, sensors were immediately fixed with 4% paraformaldehyde, stained with Hoescht 33342, and imaged with a fluorescent microscope using a DAPI filter to capture cell nuclei on the sensor surface. 12 1×1 mm images were taken across the sensors in specific locations: the 4 corners, the center of the 4 edges, and 4 images from the center of the sensor and analyzed using custom MATLAB software to quantify the cell nuclei in each image.

5.3 Results and Discussion

Heparin and heparan sulfate are glycosaminoglycans (GAGs) that can primarily be found on the surface of cells or in the extra cellular matrix (ECM). The amount and position of sulfate groups in these polysaccharides can affect the binding and activity of signal molecules and growth factors, which can impact the way the cells adhere and proliferate on a surface. Heparin, rHS01, and rHS29 present different levels and positions of sulfate groups within the molecule. Analysis of the results revealed that cells grown on each surface presented similar growth profiles, with no significant differences in the cell density supported by the different surfaces (**Figure 44**). Most of

the profiles appear more similar following normalization of the resonance data to the cell density on the sensor, with the exception of the rHS01/COL coated sensors (**Figures. 40-43**). One reason the profiles may appear very similar is that the culture conditions used in this experiment were recommended by the supplier of the cells (10% serum) and the experimental coatings are designed to support enhanced growth and proliferation in low-serum conditions (2% serum).

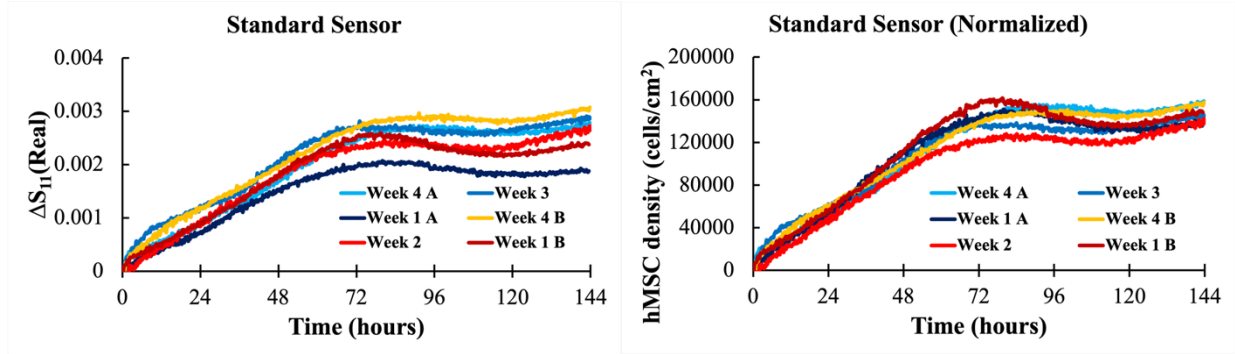


Figure 40. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) on magnetoelastic sensors (**left**) and the response normalized to the final cell density counted on the sensor surface (**right**).

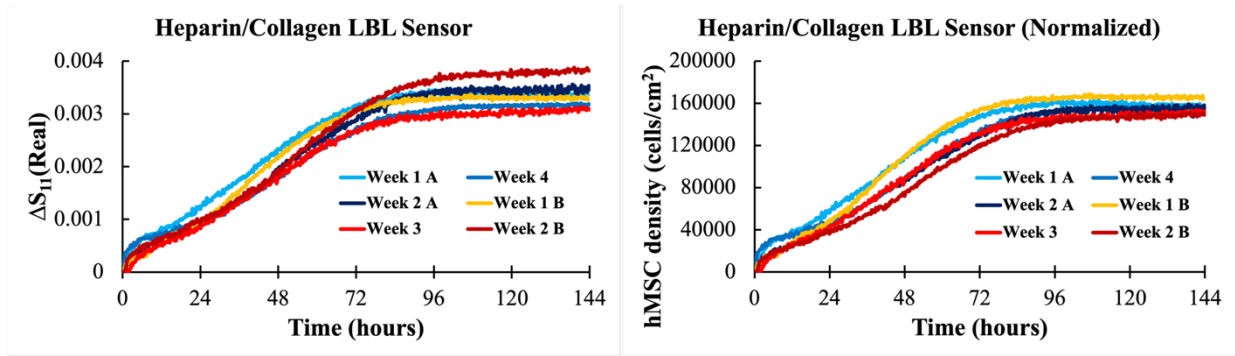


Figure 41. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) on magnetoelastic sensors that have been coated with a layer-by-layer coating of heparin and collagen I (**left**) and the response normalized to the final cell density counted on the sensor surface (**right**).

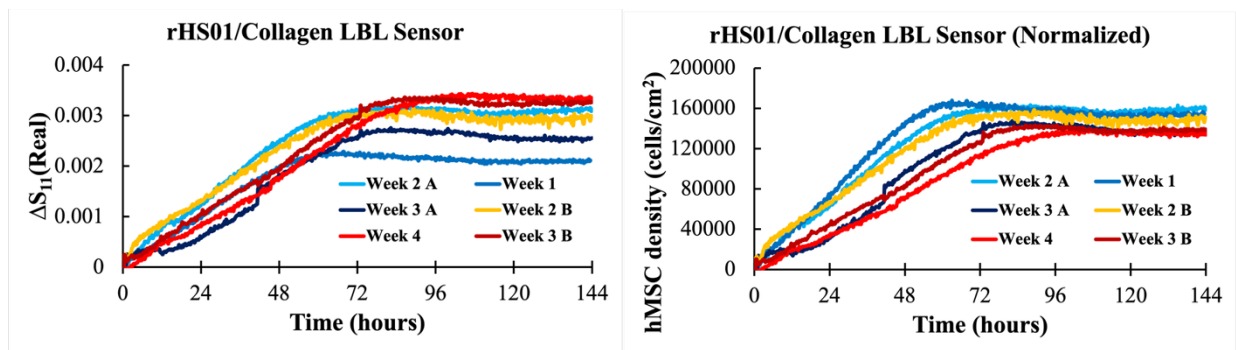


Figure 42. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) on magnetoelastic sensors that have been coated with a layer-by-layer coating of rHS01 and collagen I (**left**) and the response normalized to the final cell density counted on the sensor surface (**right**).

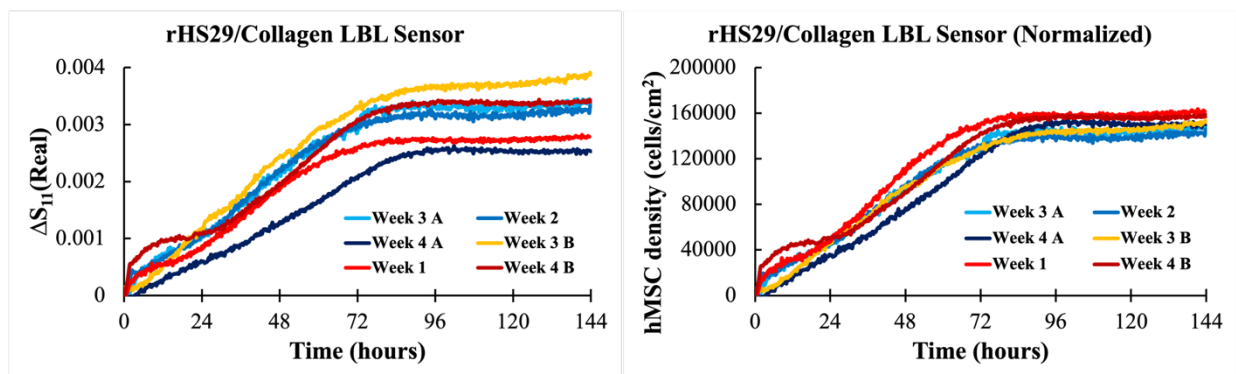


Figure 43. Sensor response with hMSCs seeded at 12,000 cells/cm² (27,000 cells/mL) on magnetoelastic sensors that have been coated with a layer-by-layer coating of rHS29 and collagen I (**left**) and the response normalized to the final cell density counted on the sensor surface (**right**).

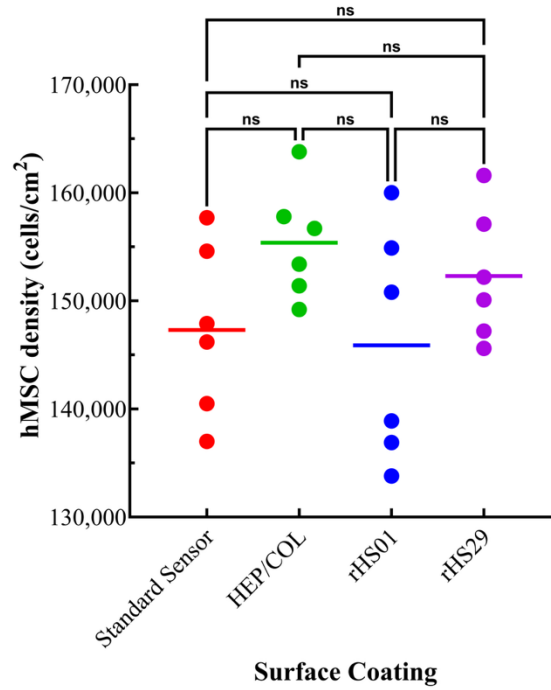


Figure 44. Plot of the average cell density captured across each coating surface. There were no significant differences in the average cell densities between the different culture surfaces. ns: $p > 0.05$, ordinary one way ANOVA

Normalization of the resonance profile to the cell density on the sensor surface is essentially a post-process calibration and can allow us to make more confident comparisons between profiles of individual sensors as well as give some indication to the consistency and robustness of our fabrication and characterization process for the sensors and monitoring system. Prior to experimentation, sensors were sorted for matching signal magnitude within 5% of each other. The standard deviation of the final signal magnitude of the standard sensors (**Figure 40**) was 16%. If sensor Week 1 A is omitted from the group, this dropped to 9%. The standard deviation of the average cell density across the standard sensors was 5%. This comparison indicates that tighter fabrication and sorting specifications can be implemented to push the sensor performance and manufacturing consistency further. Furthermore, the sensors were sorted for maximum signal magnitude prior to the Parylene-C coating. Sorting the sensors after this coating could reduce the effects of potential coating variability and improve the consistency of sensor

performance. This will be critical for the application of this technology outside of a research setting, as the sensors cannot be calibrated during the culture process and their accuracy and precision as sensors for indicating cell density in real-time will rely on strict fabrication and characterization requirements related back to robust calibration curves.

5.4 Conclusions

This collaboration was a good opportunity to assess the performance of our improved surface treatment against the heparin/collagen I coating that demonstrated superior performance on the sensor in previous experiments. The sensors with the standard surface treatment supported an average cell density of 147,000 cells/cm² with a standard deviation of 5.0% across the 12 specific zones. The sensors with the heparin/collagen I coating supported an average cell density of 155,000 cells/cm² and a standard deviation of 7.2% across the 12 specific zones. Comparison of the performance of these coatings shows a vast improvement over previous results (using a different line of hMSCs) where the uncoated sensors supported an average cell density of 127,500 cells/cm² with a standard deviation of 33% and the heparin/collagen I coated sensors supported an average cell density of 188,400 cells/cm² and standard deviation of 7.6%. Considering this comparison, we feel that we have enhanced the sensor surface treatment (Parylene-C coating and plasma etching) for the attachment of hMSCs within the context of our system, and have shown that functional coatings and surface modification can be further applied without substantial changes to sensor performance, design, or materials used.

CHAPTER 6: TRACKING HUMAN MESENCHYMAL CELL GROWTH IN A HOLLOW FIBER BIOREACTOR BY AN IN-LINE MAGNETOELASTIC CELL MONITORING SYSTEM

6.1 Introduction

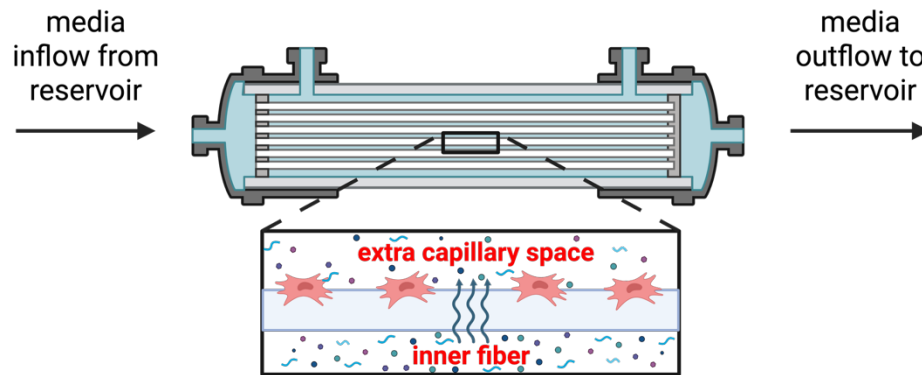


Figure 45. Illustration of a hollow fiber bioreactor cartridge. The hollow fiber cartridge is composed of hollow fibers of porous polysulfone bundled together and sealed on the outside of the fibers at the ends. Culture medium is pumped through the interior of the fibers and perfuses through the porous polysulfone. Cells are inoculated into the space outside the fibers, known as the extra capillary space (ECS).

There is no technology currently available for directly monitoring adherent cell density in real-time for hollow fiber bioreactors or small-scale (<5L) single-use bioreactors used in the production of autologous cells and products. Hollow fiber bioreactors are being adopted at an increasing rate (CAGR 7.4%) due to their high surface area, low culture volume, and membrane perfusion capabilities, resulting in highly efficient production of cells and cell products [97]. The reactors are comprised of a bundle of porous fibers and media is circulated through the intracapillary space, allowing oxygen and nutrients perfuse through the fibers directly to the cells growing on the fiber surface in the extra capillary space (ECS), resulting in a bioreactor that offers a more *in-vivo*-like environment for tissue culture compared to other typical commercial bioreactor designs (**Figure 45**). These reactors are also one of the most popular choices for producing and isolating extra-cellular vesicles, monoclonal antibodies, and other therapeutic biologics from cells.

Autologous therapies will be more favorable from regulatory and risk perspective, however, the scale at which the cells and biological products can be produced are limited by regulation. Therefore, a robust product that is scalable from the smallest to largest reactors is likely to be well-received by the market. With current technologies, manufacturers of adherent cells must grow more cells than necessary because a considerable number of cells die in the process of monitoring and harvesting the cells. As well, the invasive and labor-intensive nature of industry standard methods for tracking cell density mean that cell manufacturers can lose entire batches by missing important events in-between monitoring time points. The development of an integrated and continuous monitoring system could reduce waste and streamline production to save resources for manufacturers. This chapter focuses on the integration of the magnetoelastic monitoring system into a hollow fiber bioreactor and assessing the correlation between the generated profile of cell growth and the cell growth activity inferred from sampling the glucose concentration of the media (using the method and equipment recommended by the manufacturer of the bioreactor).

6.2 Methods

6.2.1 Sensor Fabrication

Sensors were made from a commercially available magnetoelastic material, Metglas 2826 MB (Metglas Inc., Conway, SC, USA) [35]. Metglas 2826 MB was used because of its good magnetization (saturation magnetization = 0.39 T) and low coercivity (coercive force < 50 A/m) that result in a strong signal as well as large magnetostriction (saturation magnetostriction = 12 ppm) that makes them sensitive to mechanical loading. The material was purchased as a rolled, thick-film strip of 12.7 mm wide and 29 μm thick. A stack of 4 magnetoelastic strips of about 15 cm long was placed on top of a 0.5 mm thick steel sheet (McMaster-Carr, Elmhurst, IL, USA) that

was covered in a layer of aluminum foil (Thermo Fisher Scientific, Waltham, MA, USA). Free-standing rectangular sensors of 5 mm $\times$ 12.7 mm were cut from the prepared stack of magnetoelastic strips by using a high-power pulse laser (Coherent ExactCut 430, Coherent, Santa Clara, CA, USA) and subsequently cleaned with ethanol and annealed in an oven (Model 10 Lab Oven, Quincy Lab, Inc., Burr Ridge, IL, USA) at 125 °C for 2 hours. Following the annealing of the sensors, they were characterized by measuring the real part of the S11 parameter using a network analyzer (Keysight ENA E50618, Keysight Technologies, Santa Rosa, CA, USA) with a custom-wound interrogation solenoid. A selection of sensors were then sorted into a group, so that the response magnitudes of each sensor were within 5% of each other. Next, these sensors were coated with a 10 μ m-thick conformal Parylene-C layer (PDS 2010 Labcoter system, SCS, USA) to improve corrosion resistance and facilitate biocompatibility. Afterwards, sensors were treated with oxygen plasma in a reactive ion etching system (March Jupiter II RIE, USA) for 30 s at 100 W on each side of the sensor to promote cell adhesion, improve wettability, and mitigate the formation of bubbles at the sensor surface. The plasma-etched sensors were then again sorted into groups, so that the response magnitudes of each sensor were within 5% of each other. Finally, fabricated sensor groups were cleaned with 95% ethanol, allowed to dry, and stored in sterile conical tubes prior to flow cell assembly.

6.2.2 Flow Cell Fabrication and Assembly

Sensors are integrated in-line with the hollow fiber bioreactor via a custom-designed flow cell with flanged port connections and a retention area for the sensor (**Figure 46**). The flow cells were designed with computer-aided design (CAD) (Autodesk Fusion 360 v2.0) and 3D printed using a stereolithography (SLA) printer (Form 4B, Formlabs, Somerville, MA, USA) with

biocompatible and autoclavable resin (High Temp V2 Photopolymer Resin, Formlabs, Somerville, MA, USA). 3D printed flow cells were cleaned with 95% ethanol in a sonicator and then cured (Form Cure, Formlabs, Somerville, MA, USA) according to the manufacturer's protocol. Following the cure, flow cells were placed in sterilization pouches (Beta Star Small Sterilizer Autoclave, Beta Star, Honey Brook, PA, USA) and autoclaved.

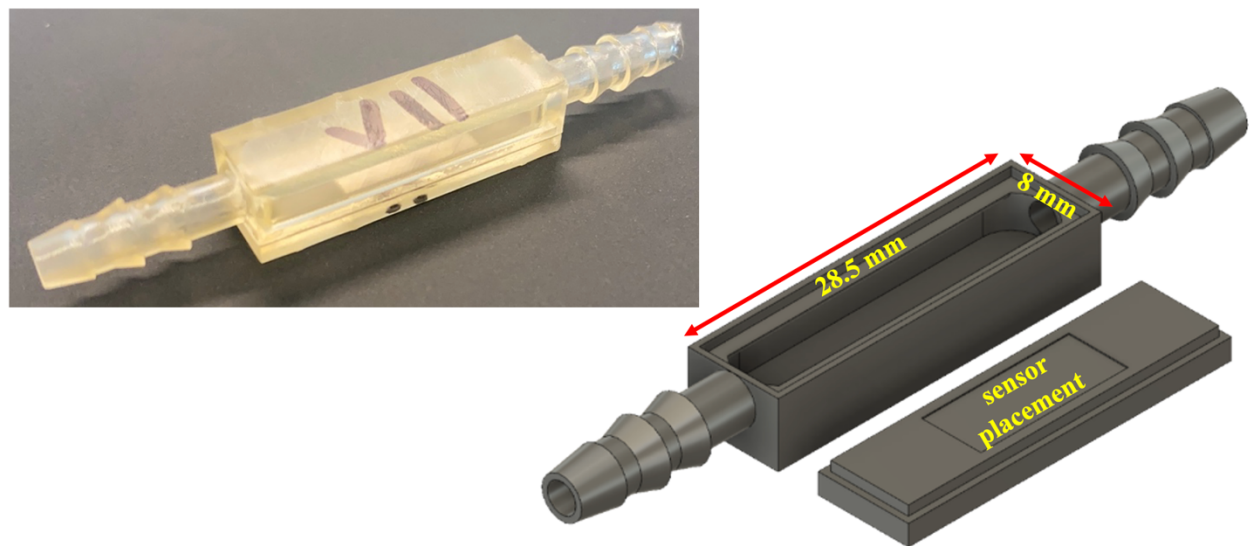


Figure 46. Photograph of an assembled prototype flow cell for the magnetoelastic monitoring system (**left**) and 3D CAD rendering of the flow cell (3D). The prepared sensor is placed in a retention area – the flow cell is designed and printed with tight tolerances to allow the sensor to vibrate freely but remain in place within the flow cell.

Autoclaved flow cells were fitted with a sensor and then clamped together between two 6 mm thick pieces of acrylic. Medical grade epoxy (EP30MED, MasterBond, Inc., Hackensack, NJ, USA) was mixed using a pair of sterile luer-lock syringes according to the manufacturer's protocol. The epoxy mixture was pushed into one syringe. The exposed luer-lock connection was covered with parafilm, and the syringe plunger was removed. The syringe was placed into a vacuum chamber, and the vacuum was cycled twice at the minimum pressure (maximum vacuum) to remove bubbles from the mixture. The epoxy was then placed into a 100 °C oven for 3.5 minutes

to initiate the curing process and increase the viscosity of the mixture. Finally, the plunger was carefully replaced into the syringe, the parafilm covering was removed, and a blunt 22-gauge luer-lock needle was attached the syringe to facilitate precise dispensing of the epoxy mixture. The epoxy mixture was carefully dispensed along the seam of the flow cell assembly, and the assembly was left overnight in the clamps to cure (**Figure 47**).

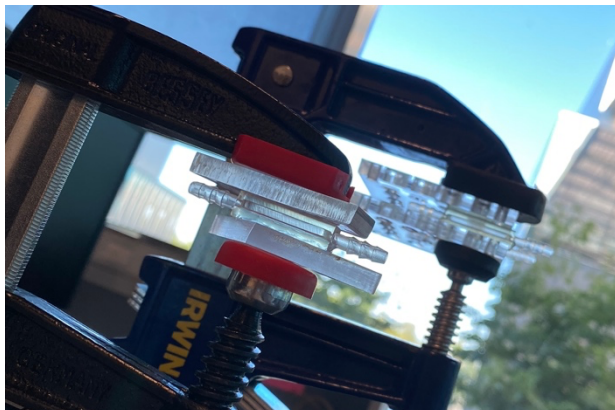


Figure 47. Photograph of the clamping jigs for the flow cells. The acrylic calls ensure even clamping pressure across the surface of the flow cell. This is critical for the flow cell assembly – as the parts are slightly warped from the printing and curing process, and glue can seep in and bind the sensor if the clamping is insufficient.

After the epoxy cured, flow cells were tested with the detection system to confirm the sensor response was not affected by the flow cell assembly or glue. Following this check, the flow cell assemblies were tested for leaks by attaching sterile, platinum-cured silicone tubing (Nalgene Pharma Grade Silicone Tubing, Thermo Fisher Scientific, Waltham, MA, USA) to each end of the flow cell. One end of the tubing was clamped, and a disposable transfer pipette was fitted to the other open end. The assembly and tubing were submerged in a bath of 95% ethanol, and the pipette was squeezed. If the pipette bulb provided resistance and no bubbles were observed from the flow cell assembly, it was considered to have passed the leak test. Assembled and tested flow cell assemblies were placed into sterilization pouches and sterilized with ethylene oxide gas.

6.2.3 Magnetoelastic Monitoring System



Figure 48. Photograph of the detection solenoids designed specifically for the flow cell. The interrogation solenoid slides into a housing that keeps the coil in the same orientation and allows it to sit flat. The neodymium magnet that provides the DC bias field is embedded inside front of the housing.

The flow cell detection assembly included a neodymium magnet (K&J Magnetics, Pipersville, PA, USA) embedded inside a housing for the interrogation solenoid (24 AWG, 106 turns, 2 layers, 16 mm spool diameter, 30 mm spool length) which applied an AC magnetic field that caused the sensor to resonate. The interrogation solenoid was also used to capture the sensor signal by facilitating measurement of the S_{11} parameter of the solenoid. The interrogation solenoid spool was designed with a rectangular port and a lip at one end to ensure the flow cell would remain in a constant position within the flow cell detection assembly. Compared with the chamber slides used in most of our experiments, the flow cell was smaller, which allowed us to design an improved detection system (**Figure 48**). The copper windings of the solenoid were closer to the sensor, and there was more freedom of placement and selection of the neodymium magnet, which resulted in a greater sensor resonance magnitude than previous experiments.

The S_{11} parameter of the interrogation solenoid was measured with a portable network analyzer (NanoVNA-F, Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China). The network analyzer and the transmission cables for connecting the interrogation solenoids were calibrated together with a manual open-short-load calibration kit (Hangzhou Minghong Electronic Technology Co., Ltd., Hangzhou, China). The network analyzer captured and saved the resonance spectrum of the sensor into data files, which were later analyzed and plotted on a computer. Three impedance-matched detection coils, network analyzers, and computers with custom software were used to monitor sensors embedded in three flow cells simultaneously. Custom MATLAB software calculated and subtracted a running background during analysis to eliminate the effects of the solenoid's impedance and electromagnetic interference on the sensor resonance measurement.

To monitor the attachment and growth of cells, flow cells were integrated in series into an auxiliary flow circuit adapted into the extra capillary space (ECS) of a hollow fiber bioreactor system (**Figures 49 and 50**) and were seeded with cells simultaneously with the hollow fiber cartridge. The baseline resonance spectrum for each sensor was measured 1 hour after the seeding, the soonest allowed by the hollow fiber seeding protocol, and marked as the first measurement (0 h). Subsequent resonance spectra were captured every 5 minutes following the 0 h measurement, creating a profile of cell growth over 13 days.

6.2.4 Hollow Fiber Bioreactor Assembly and Monitoring System Integration

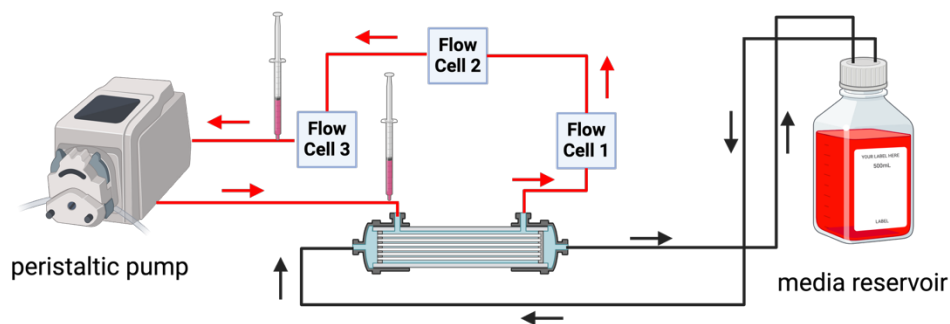


Figure 49. Illustration of the integration of the MMS with the hollow fiber bioreactor system. The traditional flow path for the hollowfiber bioreactor is shown with black arrows and is driven by a pump integrated with the bioreactor stand. The flow path of the auxiliary sensing line is shown in red and is driven by an additional peristaltic pump.

A small-scale hollow fiber bioreactor system (C2025D, Fibercell Systems, Inc, New Market, MD, USA) was acquired and prepared according to the manufacturers protocol. Briefly, a reservoir cap assembly was fitted with 2 luer-lock connectors on silicone tubing provided with the bioreactor system. These connectors were covered with aluminum foil which was secured with autoclave tape. The cap was placed inside a sterilization pouch and autoclaved at 125 °C for 45 minutes. The flow cells and necessary adaptive connectors and silicone tubing were placed in sterilization pouches and sterilized with ethylene oxide gas. Flow cell detection assemblies were cleaned with 70% ethanol and 10% bleach. The bioreactor pump and platform (Duet Pump, Fibercell Systems, Inc, New Market, MD, USA) was also cleaned with 70% ethanol and 10% bleach. All prepared materials and the bioreactor system were placed into the biosafety cabinet (BSC). The pump was positioned and set so that the auxiliary flow path was as follows: right ECS port → flow cell 1 → flow cell 2 → flow cell 3 → T-port 1 → auxiliary pump tubing → T-port 2 → left ECS port (**Figure 49**). The entire auxiliary sensing circuit was connected with platinum-cured silicone tubing. The flow cells and detection assemblies were designed and assembled such that they were locked from moving in one direction after assembly and then secured with tape to

the pump platform to prevent movement in the opposite direction. Care was taken to ensure there were no kinks or other obstructions or obstacles to flow in the tubing's placement.

Following assembly of the auxiliary sensing circuit, the bioreactor system was removed from its sterile packaging and the luer-lock connections for syringe manipulations at the right and left ECS ports were removed and replaced with the terminal connections of the auxiliary sensing circuit. The luer-lock connections were fitted onto the perpendicular port of the two T-ports. Clamps were positioned such that the flow cells, the auxiliary pump (tubing), the hollow fiber cartridge (ECS and inner fibers), and luer-lock connections for syringe manipulations could be individually isolated from the flow circuit.

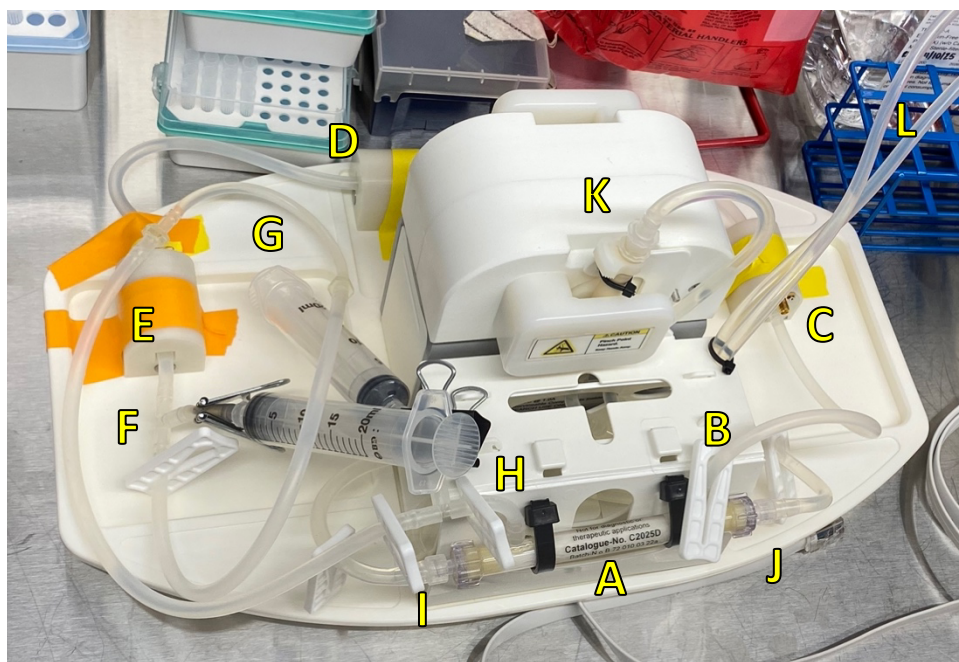


Figure 50. Photograph of the magnetoelastic monitoring system (MMS) integrated with the hollow fiber bioreactor via an auxiliary flow circuit. (A) hollow fiber cartridge (B) right ECS port (C) sensor assembly 1 (D) sensor assembly 2 (E) sensor assembly 3 (F) T-port 1 (G) auxiliary pump tubing (H) T-port 2 and left ECS port (I) left end port, fiber intake from media reservoir (J) right end port, fiber outflow to media reservoir (K) duet pump (L) media reservoir lines

6.2.5 Hollow Fiber Bioreactor Pre-Culture, Seeding, and Culture

Following bioreactor assembly and auxiliary flow circuit integration, the system was ready for pre-culture. All reagents used in the pre-culture, culture, and analysis of the bioreactor are warmed in a 37 °C water bath. Briefly, PBS was aliquoted from a fresh 1L bottle. The PBS bottle cap was removed and replaced with the reservoir cap. The reservoir lines from the hollow fiber cartridge were connected to the reservoir cap. Each time a new connection was made, or a syringe was connected, the luer-lock connections were carefully sprayed with 70% ethanol and the excess wiped away. All clamps begin in the closed position. Left and right end port clamps are opened, and PBS was perfused through the flow path by squeezing the cartridge's pump tubing (hidden inside the duet pump in **Figure 50**) manually until the circuit was filled and no bubbles come from the stainless steel tubing in the reservoir. The cartridge was tilted with the right side upward to aid in the removal of bubbles. Afterward, the right and left end port clamps were closed again.

A sterile syringe was connected to the luer-lock connector at T-Port 1 (**Figure 50F**), and another sterile syringe was fitted with an 18 gauge needle and 20 mL of PBS was drawn into the syringe. All manipulations were done with fresh syringes and needles. Syringes were left on the luer-lock connectors after manipulation to guard against contamination. The PBS filled syringe was connected to the luer-lock connector at T-port 2. The clamps were opened such that the flow path through the ECS and auxiliary sensing circuit was open and isolated from the rest of the system. The cartridge was tilted with the right side facing upward and PBS was injected into the circuit and the empty syringe filled with displaced air. This step was repeated until there were no more air or bubbles being displaced. The clamps to the luer-lock connections for the 2 syringes were shut and the left and right end port clamps to the reservoir were opened. The system was placed onto the duet pump, and the auxiliary pump tubing was fitted into the auxiliary pump

(Peristaltic Pump P-1, Cytiva Life Sciences, Marlborough, MA, USA). The duet pump was set to 10 and the auxiliary pump was set to 1 and the system was left in the incubator for 24 hours.

A fibronectin solution was prepared by mixing 1 mg of fibronectin in 20 mL of PBS. The fibronectin solution was drawn into a syringe. The fibronectin filled syringe was connected to the luer-lock connector at T-port 2. The clamps were opened such that the flow path from T-port 2, through the ECS and auxiliary sensing circuit to T-port 1 was open and isolated from the rest of the system. Fibronectin was swished back and forth between the syringes 10 times. Following the final swish, half the solution was left in each syringe, the reservoir cap was cracked ¼ turn, and the fibronectin solution was ultrafiltered through the fibers and into the reservoir by isolating one syringe and depressing the other, repeating with the other syringe. The cap was tightened, and the cartridge pump tubing was squeezed manually until no bubbles were seen coming into the reservoir. The bioreactor system was left in the BSC overnight to allow the fibronectin to adhere to the culture substrates.

The next day, xeno-free media (RoosterBasal™ 2.0-CC, RoosterBio, Frederick, MD, USA) without supplements or antibiotics was aliquoted from a fresh 500 mL bottle. The reservoir cap was removed from the PBS and placed onto the bottle of media without supplements or antibiotics. Following the same technique used to fill the ECS and auxiliary sensing circuit with PBS, the system was filled with the aliquoted media. All clamps were opened on the system, and it was moved into the incubator and placed on the duet pump at a setting of 10 and the auxiliary pump at a setting of 1 for 24 hours.

The following day, the bottle of media without supplements or antibiotics was replaced with a fresh bottle of xeno-free media supplemented with 10% xeno-free serum (RoosterBooster™-MSC-XF, Rooster Bio, Frederick, MD, USA) and 1% penicillin-

streptomycin using the same technique. The system was placed in the incubator and allowed to run using the same pump settings for another 24 hours. Following the 24 hour period, the system was ready for inoculation with cells.

6.2.6 Hollow Fiber Bioreactor Inoculation and Culture

Human bone marrow-derived Mesenchymal Stem Cells (hMSCs) were commercially obtained (donor #000277, RoosterBio, Frederick, MD, USA), expanded according to manufacturer's protocol, and cryogenically stored in liquid nitrogen. Prior to use, cells were recovered from liquid nitrogen, thawed, seeded at 5,000 cells/cm², and cultured until confluent. These cells were cultured in xeno-free media and supplemented with 1% penicillin-streptomycin and 10% xeno-free serum. Once confluent (~72 hours, confirmed via light microscopy), cells were lifted with 0.25% trypsin-EDTA, counted using an automatic cell counter (NucleoCounter NC-200, Chemometec, USA), and then ~4.5 million cells were resuspended in 20 mL media to prepare for inoculation of the bioreactor and auxiliary sensing circuit. Cells expanded in the t-flask and grown in the bioreactor system were incubated at 37.0 °C, 93% relative humidity, and under 5% CO₂ in a microbiological incubator (HERAcell VIOS 160i, Thermo Fisher Scientific, USA).

The bottle of media was replaced with a fresh 500 mL bottle of xeno-free media containing the same supplements. The cell solution was drawn into a syringe. The cell filled syringe was connected to the luer-lock connector at T-port 2. The clamps were opened such that the flow path from T-port 2, through the ECS and auxiliary sensing circuit to T-port 1 was open and isolated from the rest of the system. The cell solution was swished back and forth between the syringes 10 times. Following the final swish, half the solution was left in each syringe, the reservoir cap was cracked ¼ turn, and the cell solution was ultrafiltered through the fibers and into the reservoir by

isolating one syringe and depressing the other, repeating with the other syringe. The cap was tightened, and the cartridge pump tubing was squeezed manually until no bubbles were seen coming into the reservoir. The bioreactor system was left in the BSC for 1 hour, rotating the cartridge 180 degrees after 30 minutes, to allow the cells to adhere to the culture substrates. All clamps were opened. The system was moved into the incubator and placed on the duet pump at a setting of 10 and the auxiliary pump at a setting of 1 for 13 days (**Figure 51**).



Figure 51. Photograph of the bioreactor system with the integrated magnetoelastic monitoring system inside of the incubator. The auxiliary pump can be seen behind the duet pump in the incubator.

6.2.7 Monitoring Culture Media Glucose Concentration

The concentration of glucose in the media was measured every 12-24 hours throughout the duration of the culture. The initial glucose concentration was measured by attaching a new 1 mL sterile syringe to the luer-lock connector at T-port 2. The clamp at that port was opened and 1 mL

of media was slowly drawn through into the syringe. The port clamp was closed, and the media-filled syringe was removed and replaced with a new sterile 1 mL syringe. Subsequent samples were collected by opening the clamp, drawing the media into the syringe, closing the clamp, and replacing the media-filled syringe with a new syringe. The sampled media was dispensed into a sterile conical. The sampled media's glucose concentration was measured using a glucose monitoring system (GlucCell® Glucose Monitoring System, Chemglass Life Sciences LLC, Vineland, NJ, USA) designed specifically for concentrations of glucose found in cell culture media. The samples were analyzed according to the manufacturer's protocol. Briefly, a new test strip was inserted into the glucose measurement device. A micro-pipette was used to draw a 1.5 μ L sample of media into a sterile filter tip. The media was expelled, forming a small drop on the tip of the pipette. The drop was carefully and completely wicked into the aperture of a glucose test strip. The glucose reading was recorded. This was repeated twice for each measurement and additional measurements were conducted if an obvious error was made during the procedure, such as incomplete wicking of the drop into the test strip aperture.

6.2.8 Staining, Imaging, and Counting of Cells

Immediately after collecting the endpoint resonance spectrum and the final glucose sample, quantification of cells on the sensor surface and in the hollow fiber cartridge was performed to investigate the relationship between changes in the sensor signal and the attachment, growth, and distribution of cells in the bioreactor system.

The hollow fiber cartridge was clamped at each port connection, and the sensor line was clamped at each end of the flow cells. The bioreactor system was disassembled and the luer-lock connectors at the T-ports were reconnected to the ECS ports of the hollow fiber cartridge. An empty

sterile 10 mL syringe was connected to the right ECS port. 10 mL of PBS was drawn into another sterile 10 mL syringe. The PBS filled syringe was connected to the left ECS port and the PBS was pushed through the ECS to rinse the cartridge and displace the media into the empty syringe. This process was repeated 2 more times. With an empty syringe on the right ECS port, 10 mL of 0.25% trypsin-EDTA was drawn into a 10 mL sterile syringe that was afterwards connected to the left ECS port. The trypsin solution was swished back and forth between the syringes 20 times. The cartridge was placed in the incubator for 10 minutes. The cell suspension was removed to a sterile conical and 40 mL of media was flushed through the cartridge and removed to the same sterile conical. The conical was centrifuged for 5 minutes at 500g, the supernatant aspirated, and the cell pellet was resuspended in 10 mL of media. The suspension was counted using an automatic cell counter (Via 1 Cassette, NucleoCounter NC-200, Chemometec, La Jolla, CA, USA).

The flow cells were isolated and reassembled into a shorter flow path through a series of clamps, cuts, and reconnections. The flow path was terminated with a luer-lock connector and clamp at each end and a clamp between each flow cell. Syringes were used to rinse the flow path with PBS. Next, the PBS was displaced with a solution of 4% PFA diluted in PBS using syringes and the flow cells were placed in the incubator for 10 minutes. Following this period, the PFA solution was displaced with PBS using syringes, and the flow cells were moved to an adjacent lab space where a rotary tool was used to carefully grind above the glue seam on the flow cell. The flow cells were then submerged in 95% ethanol to clean the dust and debris from the grinding procedure. The flow cells were carefully disassembled using a set of locking pliers and a razor blade. Sensors removed from the flow cells were placed into retention stages (**Figure 4**) in the rear well of a chamber slide. The nuclei of the cells were stained by incubating them for 10 minutes in a solution of Hoechst 33342 nuclei stain, diluted to 50 $\mu\text{g/mL}$ in PBS. Following this incubation

period, the staining solution was aspirated, and the sensors were carefully inverted into the empty, unused well of the chamber slides. The sensors were covered with PBS, and the chamber slides were placed on a tray and covered with aluminum foil to protect it from exposure to ambient light. Fluorescent microscopic imaging was performed using a confocal fluorescence microscope (CSU-W1 SoRa, Nikon, Japan) with a DAPI (4',6-diamidino-2-phenylindole) filter to show the stained nuclei. To quantify the number of cells attached to the sensor surface, representative images of $1\text{ mm} \times 1\text{ mm}$ were taken for each sensor in 12 specific locations that were kept consistent for each sensor. Cell nuclei were quantified through the analysis of fluorescence microscope images using custom MATLAB analysis software.

6.3 Results and Discussion

6.3.1 Culture Media Glucose Concentration

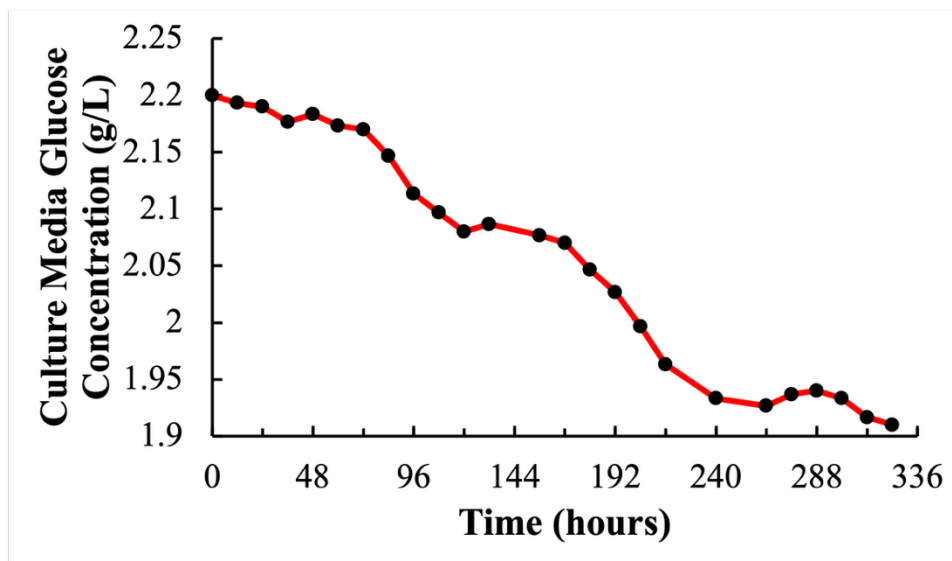


Figure 52. The concentration of glucose throughout the culture period. Glucose measurements were manually performed at every 12-24 hours.

Glucose is the primary nutrient metabolized by hMSCs under normal conditions. Tracking the concentration of glucose in cell culture media provides an indirect method of assessing cell

growth and proliferation to compare against the sensor data. The overall concentration of glucose in the media decreased over the culture period, suggesting that cell growth was taking place in the bioreactor system (**Figure 52**). Extrapolating from technical data provided from the manufacturer of the bioreactor, the ideal glucose uptake for this cartridge would be 0.1 - 0.15 grams per day, and the manufacturer does not recommend letting the consumption to rise above 0.2 grams per day. Glucose consumption as measured by the GlucCell® system averaged 0.01 grams per day. There are multiple factors that contributed to this relatively low consumption compared to the manufacturer's ideal. The cartridge is recommended to be inoculated with 1 to 20 million cells suspended in 3 mL of media. Due to the additional volume added by the auxiliary flow path with the sensor, the volume to be inoculated was roughly 20 mL. Using the minimum recommended inoculation density, this would require roughly 6.7 million cells in total. In our experience with this cell-line, 1 million cells seeded into a T-75 flask will typically expand from cryogenic recovery into about 6 million cells in 3 days. We chose to inoculate with only a single flask of expanded cells with the hopes that the lower cell density would avoid the masking of key growth profile features by attachment and proliferation. Lower seeding densities can also limit cell-to-cell interaction and signaling essential to cell growth and proliferation. The additional shear stress introduced by the auxiliary sensing line and pump could have contributed to a longer adaptation phase than usual, inhibiting and delaying cell attachment and proliferation..

6.3.2 Endpoint Cell Quantification

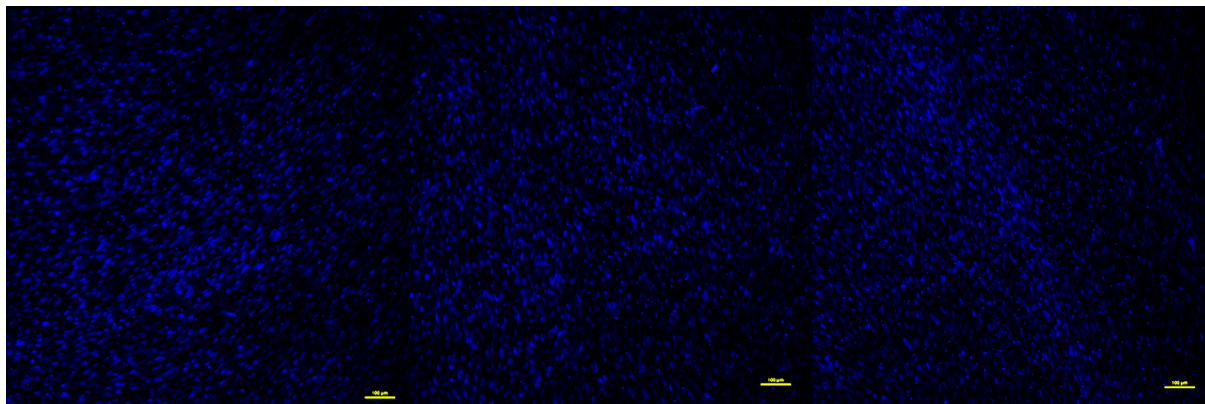


Figure 53. Fluorescence microscope images depicting hMSC nuclei on the sensor surface 13 days after inoculation of the bioreactor. **(left)** Sensor 1 – average cell density of 147,417 cells/cm². **(middle)** Sensor 2 – average cell density of 153,517 cells/cm². **(right)** Sensor 3 – average cell density of 181,250 cells/cm². The cells were stained with Hoescht 33342 and imaged with a DAPI filter. Images are 1 mm × 1 mm and the scale bar is 100 μm.

The average cell density across the surface of Sensor 1 (147,417 cells/cm²) and Sensor 2 (153,517 cells/cm²) were very close in value, while Sensor 3 (181,250 cells/cm²) supported a higher density of cells than Sensor 1 and Sensor 2 (**Figure 53**).

An automated cell counter was used to count cells trypsinized from the hollow fiber bioreactor. The automated counter reported 213,000 cells/mL with 91.7% viability, which translates to about 2,130,000 cells total which were trypsinized from the hollow fiber cartridge. The cartridge had a surface area of 450 cm², meaning that the measured cell density at the fiber surface using this method was roughly 4,730 cells/cm², which is substantially lower than the cell densities quantified on the sensor surfaces by fluorescent microscopy. The manufacturer does not recommend using this method to quantify cells from the hollow fiber bioreactor, as the cells are difficult to trypsinize from the hollow fiber surface, which leads to inaccurate results.

The cells quantified on the sensor surface by microscopy and on the hollow fiber surface by automated cell counter only account for roughly 2.5 million of the 4.5 million cells inoculated into the bioreactor system, leaving a considerable portion of the inoculated cells unaccounted for.

It is possible that the cells were so well-adhered to fiber surface that it inhibited the trypsin's ability to act on the cells. It is also possible that some fraction of detached or dead cells were removed during the media sampling process to measure glucose concentration. Whether the unaccounted cells were unable to be detached from the fiber surface or were already detached and subsequently removed during sampling, there is not enough data to draw any conclusions regarding the sensors' ability to estimate cell density at the hollow fiber surface.

6.3.3 Tracking Cell Growth Kinetics in a Hollow Fiber Bioreactor with Magnetoelastic Sensors

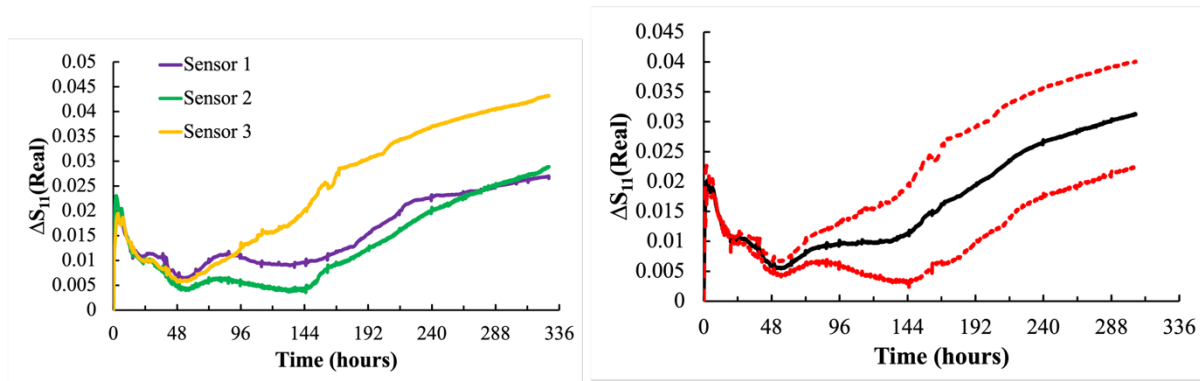


Figure 54. Sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system (**left**) and the average response (**right**). Red-dashed lines indicated the standard deviation of the average sensor response.

Analysis of the magnetoelastic sensor data (**Figure 54**) shows that the cell growth profiles on the sensors follow a similar trend until they start to diverge at 48-72 hours. One explanation for the enhanced growth seen on Sensor 3 is that it was located at the end of the sensor flow path and just before the pump tubing. There was a minimal flow rate (~ 0.1 mL/min) in the auxiliary line to minimize shear forces experienced by the cells. The tubing leading to the pump, out of the exit for flow cell 3, tilts upwards and rotates 180 degrees towards the pump. This means that any floating cells that did not attach to any of the sensors on their initial pass may have a greater chance of accumulating in this region and attaching to Sensor 3 than any other sensor.

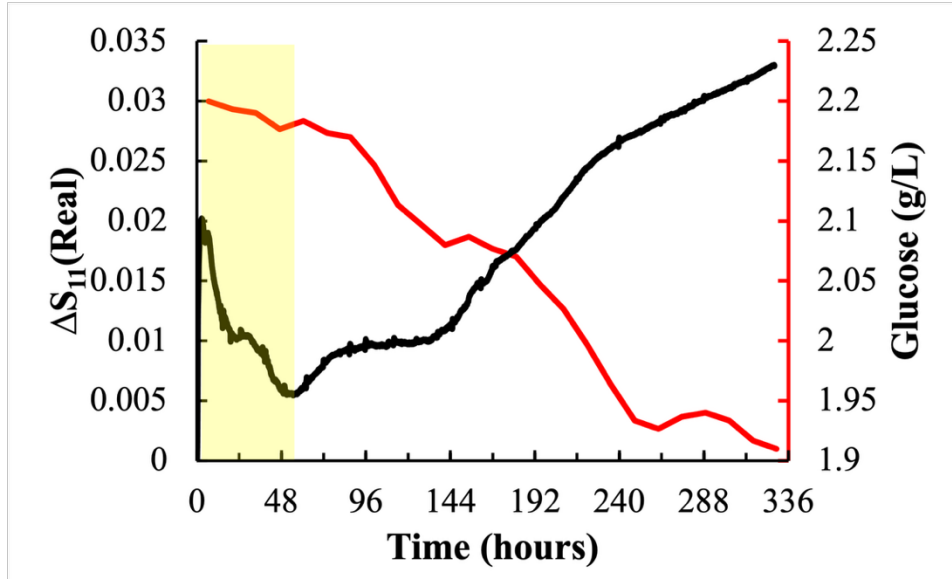


Figure 55. Measured glucose concentration plotted with the average sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system. Highlighted portions show a sharp decrease in sensor loading and a gradual decrease in glucose concentration.

Plotting the measured glucose concentration with the average sensor response provides insight into the relationship between cell metabolic activity and the cell activity at the sensor surface. The yellow highlighted portion of the plots are interpreted to be the portions of data that correspond to different phases of cell growth indicated by changes in sensor resonance and the glucose concentration. The beginning of the sensor signal plot shows a substantial drop in loading at the sensor surface during the first 48 hours of culture (**Figure 55**). This portion of the sensor data does not seem to correlate well with the glucose data. A gradual decrease in glucose concentration at the beginning of the culture suggests the cells may be in an adaptive or lag phase, which is the expected behavior for cells during the early stages of growth. However, the sensor data shows a relatively large and quick increase followed by a sharp decrease in loading, consistent between all 3 sensors. This behavior could be explained by the bioreactor inoculation conditions.

The inoculation density was nearly 10 times the density that we typically seed the sensors with in a 2D experiment. Seeding density is a critical consideration in the culture of adherent cells and particularly hMSCs. Excessive seeding densities lead to overcrowding on the culture substrate, which can inhibit the cells' adaptation and attachment to the culture substrate. It is plausible that the inoculation density combined with the shear stress provided by the auxiliary pump resulted in conditions that made it initially challenging for the cells to form a strong attachment to the sensor surface, resulting in a gradual loss of cells from the sensors during the first 48 hours of culture. This observation highlights a limitation in the application of the monitoring system in culture systems where the ideal seeding density of the culture substrate may greatly exceed that of the sensor.

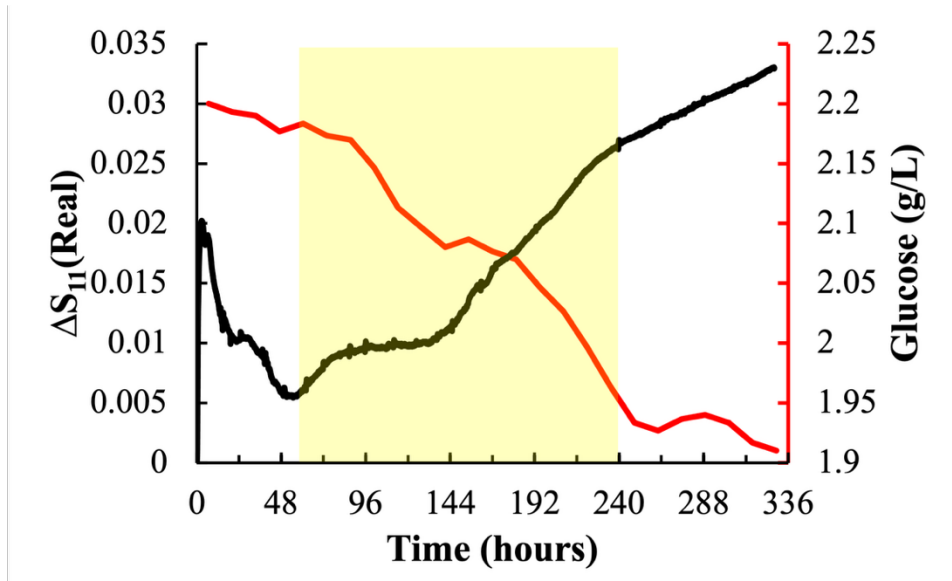


Figure 56. Measured glucose concentration plotted with the average sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system. Highlighted portions show an increase in the consumption of glucose as well as an increase in the rate of loading at the sensor surface.

After the initial 48 hours of cell culture, the change in the rate of loading at the sensor surface compared with the change in the rate of glucose consumption seem to correlate well, with a bit of delay between sensor loading activity and glucose concentration sampled (**Figures 55-57**). One simple explanation for the delay is that the glucose measurements were conducted every 12-24 hours, which corresponds well with the duration of the delay observed in the plots. Both plots show an initial spike in cell growth at 55 hours (sensor) and 60 hours (glucose), indicated by an increase in the rate of loading on the sensor and an increase in the rate of glucose consumption (**Figure 56**); and what could be the beginnings of the plateau or steady phase, indicated by a slight decrease in the rate of sensor loading (225 hours) and a decrease in the rate of glucose consumption (240 hours) (**Figure 57**).

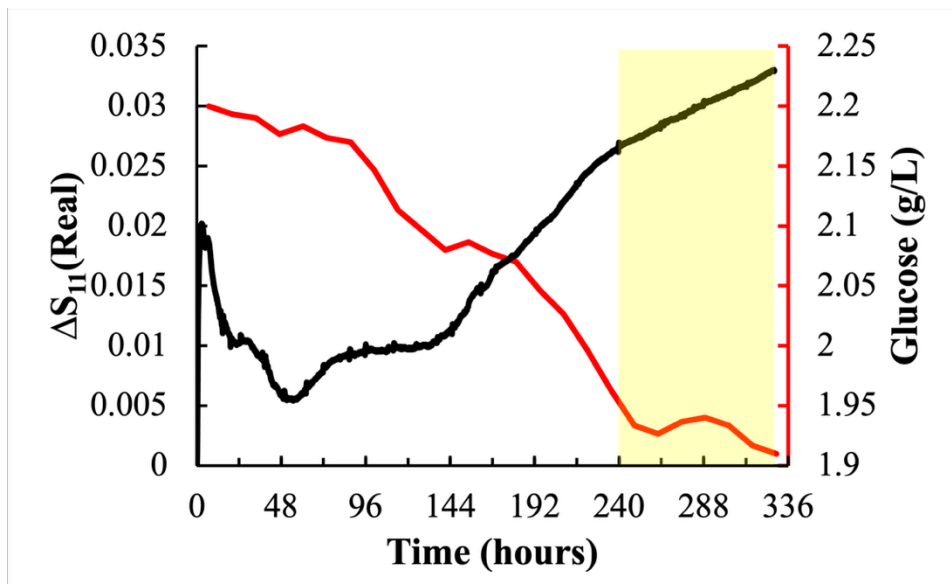


Figure 57. Measured glucose concentration plotted with the average sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system. Highlighted portions show a decrease in glucose consumption with a decrease in the rate of loading at the sensor surface.

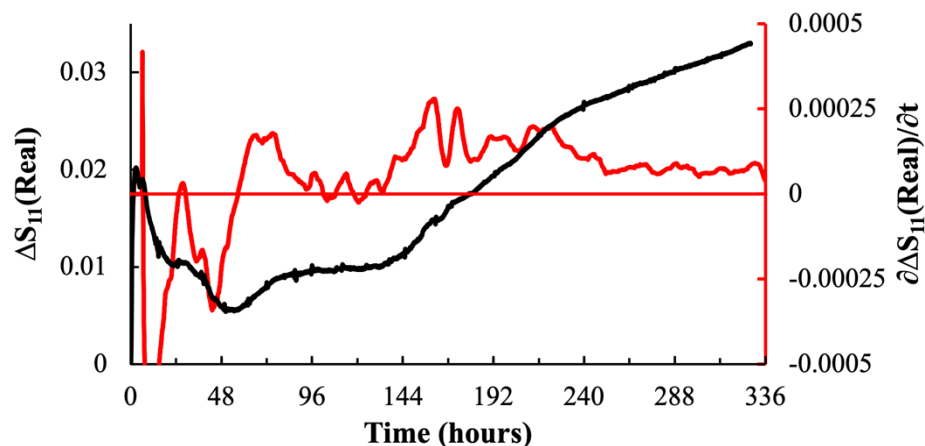


Figure 58. Sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system plotted with the time-derivative of the sensor response (red).

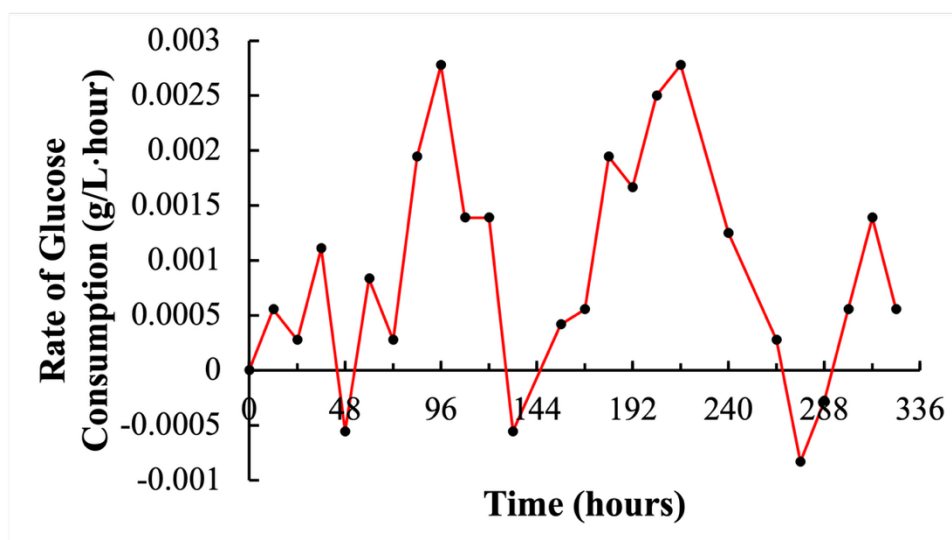


Figure 59. Plot of the rate of glucose consumption throughout the culture period.

The presence of negative slope for the rate of glucose consumption suggests there are limitations in the accuracy of the glucose monitoring system (**Figure 59**). While the manufacturer claims >90% accuracy with >95% precision, the quality of the measurements are reliant upon, and therefore subject to, operator technique. Cell culture media is a complex environment that contains sugars, salts, amino acids, growth factors, and other biomolecules that can interfere with the

enzymatic reaction the glucose monitoring system relies on. It is unlikely that any glucose was produced in the system, and these negative slopes are probably due to a combination of operator technique and limitations of the glucose monitoring system.

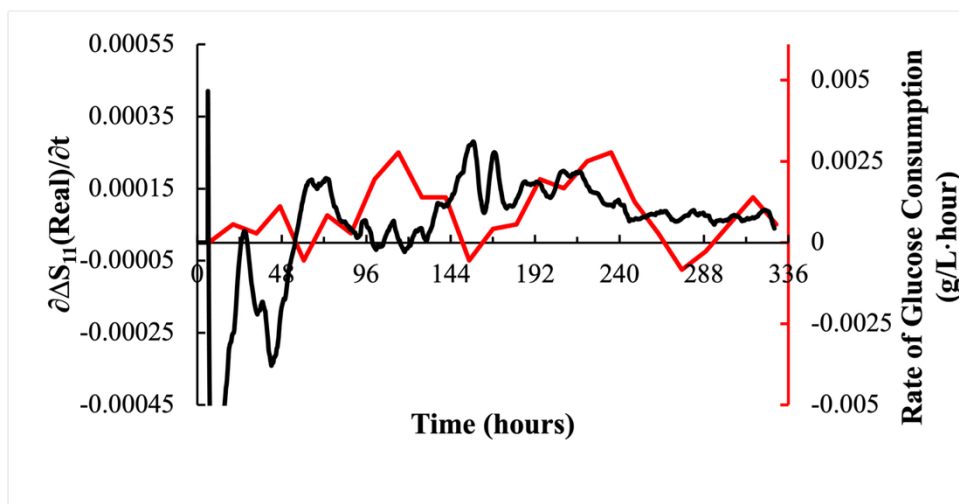


Figure 60. The time-derivative of the average sensor response with hMSCs inoculated at 225,000 cells/mL on magnetoelastic sensors that were embedded inside of a flow cell integrated in-line with a hollow fiber bioreactor system plotted with the rate of glucose consumption (red) throughout the culture period.

Comparing the derivative of the sensor response with the rate of glucose consumption (Figures 58-60) can also provide a point of analysis for the relationship between sensor response and the bulk metabolic activity of the culture, as positive values of the sensor response derivative and the rate of consumption of glucose are both associated with increases in cell activity and proliferation. The derivative of the sensor response begins with a positive slope (~ 0.0004). The initial rate of glucose consumption at $t = 0$ hours was assumed to be 0. The slope of sensor response then becomes negative. The slope approaches 0 and becomes slightly positive around 24 hours before decreasing again. This correlates well with the low rate of glucose consumption during the same period. The first peak in the rate of loading on the sensor occurs around 72 hours; during this same period, the rate of glucose consumption is still relatively low. The first peak in the rate of

glucose consumption occurs around 120 hours; during this same period, the rate of loading at the sensor surface is close to zero. The greatest peak in the rate of loading at the sensor surface occurs around 156 hours. During this same period, the rate of glucose consumption is again at a minimum. The rate of loading on the sensor surface gradually decreases around 216 hours until it approaches a relatively steady value around 240 hours; during this same period, the rate of glucose consumption gradually increases, and peaks around 240 hours. Although the rate of sensor loading and rate of glucose consumption seem well-correlated at the onset of the culture, generally the trends are counterintuitive – periods of peak activity according to the rate of sensor loading were contemporary with periods of minimum activity according to the glucose consumption rate.

Both plots show two larger peaks in activity; the rate of loading on the sensor surface peaks from the periods of 55 – 100 hours (~45 hour period) and 130 – 245 hours (~115 hour period). The rate of glucose consumption peaks from the periods of 91 – 150 hours (~ 59 hour period) and 163 – 264 hours (101 hour period). The sample size of the study is too limited ($n = 1$) to make any definitive conclusions. However, these results suggest that there could be some correlation between the rate of loading at the sensor surface and the rate of glucose consumption as the differences in the periods are 14 hours, which could be explained by the 12-24 hour delay between glucose measurements. The difference in the onset of these periods are similar at ~36 hours and ~33 hours respectively. If these periods are related, the data suggests that increased loading at the sensor surface could be predictive of increased metabolic activity. However, more rigorous controls and additional replicates would be necessary to validate and confirm these observations.

6.4 Conclusions

The experiment incorporating the magnetoelastic monitoring system into a hollow fiber bioreactor system suggests that the monitoring system has potential for further development in this pursuit. The data generated by the sensor regarding cell proliferation activity appeared to correlate with the glucose cell measurements. However, the small sample size of the study ($n = 1$) limits the conclusions that can be drawn from this work regarding the relationship between the sensor response and the cell growth and metabolic activity in bulk of the bioreactor. Directly embedding the sensor into the hollow fiber bioreactor could improve the correlation between the sensor data and metabolic data, however, the hollow fiber systems cannot be readily retrofitted with the sensor. The addition of the sensor would need to be integrated into the manufacturing process of a system like the hollow fiber.

Hollow fiber bioreactors are generally selected to produce extra-cellular vesicles, exosomes, proteins, and aren't usually a good choice for expansion of adherent cells, as it is difficult to lift cells from the surface of the fibers due to the high cell density, high surface area, and porosity. As such, the sensor might provide greater value to users if it was integrated into a bioreactor system more suited to the expansion of cells, such as a wave bag or hyper-stack T-flasks, where it can be more challenging and more critical to sample all the regions of the bioreactor and get an accurate indication of cell density and stages of growth.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

The primary contribution of this work to the field of bioengineering is the development of the magnetoelastic monitoring system for tracking cell growth kinetics and density in real-time. We have shown that the monitoring system can predict cell density in a well-calibrated 2D culture system. We have also observed that the monitoring system can indicate changes in cell growth kinetics that correlate with the changes in metabolic activity of the bulk culture in real-time when integrated in-line with a bioreactor system. Even if the magnetoelastic monitoring system can only provide similar data to glucose, it is a more direct approach to monitoring the attachment and proliferation of cells and could provide another means of monitoring and control in automated-feedback control systems. The magnetoelastic sensor also possesses different limitations than glucose and impedance monitoring systems. For example, magnetoelastic sensor directly measures cell mass unlike glucose which tracks the product of cells, and it is also not sensitive to electrical properties in the medium unlike the impedance approach. RF impedance and glucose monitoring will not be sensitive to changes in temperature, fluid flow, and biomass adsorption like the magnetoelastic sensor. Therefore, combining the monitoring methods may provide a more robust and complimentary analysis than any monitoring system individually.

A potential application of this monitoring system would be integration into bioreactors and automated feedback-controlled processes for cell manufacturing. Continued development of this technology should focus on the adaptation of the monitoring system for bioreactor systems by adjusting the detection system and stimulus conditions to accommodate for the dynamic conditions of a reactor. To accommodate different bioreactor designs, the sensors and detection system can be fabricated into a variety of shapes and sizes. To translate this technology into industrial and clinical spaces, further investigation into the effects of magnetic fields and mechanical stimulation

(generated by the monitoring system) on cell phenotype and quality will be needed. Characterization of the contribution of the ECM on the damping of the sensor will be necessary before the technology is ready for commercial applications. Additional work to enhance the detection system and improve the surface conditions of the sensor for cell adhesion and proliferation will play a critical role in facilitating the integration of this technology into commercial bioreactors of varying scale, design, and application.

The magnetoelastic monitoring system can also be a powerful tool for investigating the effects of different surface treatments and environmental conditions on stages of cell growth and growth kinetics in real-time. Immediate future work includes the continuation of the collaboration with the Domenech Lab at UPRM by utilizing the magnetoelastic monitoring system to investigate the effects of the various LBL coatings on hMSC growth kinetics under low serum (2%) conditions. We are also interested in decellularization of the sensor surface at regular intervals across the culture period, to understand the contribution of the ECM generation on sensor damping. Another area of research is the integration of the magnetoelastic monitoring system into different bioreactor designs, especially the wave bag and the hyper-stack T-flasks. These bioreactor platforms are more popular for the expansion of therapeutic cells at smaller scales and lack commercially available real-time probes for cell density or viability.

I was honored to conduct this research as a trainee member of the National Science Foundation's (NSF) Engineering Research Center (ERC) for Cell Manufacturing Technologies (CMaT). As a CMaT trainee, my PhD work was fully-funded and I had the opportunity to visit, network, and collaborate with state-of-the-art facilities and researchers in the areas of cell and tissue engineering. The experiences and collaborations formed in CMaT helped to shape me as a

researcher and enhanced my understanding of key frontiers and concepts in the cell manufacturing landscape.

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