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BioMeld

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“A MODULAR FRAMEWORK FOR DESIGNING AND PRODUCING BIOHYBRID MACHINES”

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D4.2: EXPERIMENTAL PERFORMANCE TESTING

DELIVERABLE FACTSHEET

Project title | Acronym | Number		A Modular Framework for Designing and Producing Biohybrid Machines | BioMeld | 101070328	
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Deliverable leader (Name|Organisation)	Samuel Sánchez | IBEC	**Editor(s)**	Judith Fuentes, Florencia Lezcano, Maria Crespo
Contribution of partners	UniCA developed the current-based electrical stimulation set up and protocols. SSSA developed the flexible catheter. IBEC developed the bioactuator. The three partners contributed to perform the experiments and data analysis.		
Final review and approval	IBEC, UniCA, SSSA		
Keywords	Current-based electrical stimulation, Tetanic force modulation, Catheter bending		
Abstract	Task 4.2 involved evaluating the response of bioactuators to electrical pulse stimulation (EPS) and assessing the functional performance of the biohybrid machine (BHM) in		

	terms of bending capacity, response to various EPS conditions, and contraction profile. Various stimulation parameters, such as current amplitude, pulse width and frequency, were modulated to study their effects on both twitch and tetanic contraction, needed for the motion and bending of the catheter, respectively. Square biphasic symmetric waveforms proved most effective for inducing muscle contractions, while wider pulses resulted in stronger contractions. Assembly of the bioactuator into the catheter was successfully achieved due to the already integrated two-pillar system in the catheter. The effects of bioactuator positioning on bending was also evaluated, suggesting that having the bioactuator in a tilted position improved the catheter bending. Moreover, tetanic contraction induced by pulse width and amplitude modulation, in a linear and stepwise manner, were also explored aiming to generate a controlled bending, being the linear pulse width modulation the best stimulation protocol to induce a gradual tetanic contraction and bending. Future optimization will focus on exploring stimulation protocols to mitigate tissue fatigue and therefore maintain the tetanic contractions for longer periods. Strategies to integrate the bioreactor chamber into the catheter-bioactuator subsystem are under investigation for improved functionality.

Document change history

Date	Authors	Description
03/04/2024	Judith Fuentes, Florencia Lezcano, Maria Crespo	In this first draft we report the preliminary results of i) the response of the bioactuator to different electrical stimulation protocols, ii) the integration of the bioactuator into the catheter and iii) the bending capacity of the catheter under different electrical stimulation conditions.

CONSORTIUM

	Name	Short Name	Country
1.	UNIVERZITET U NOVOM SADU, POLJOPRIVREDNI FAKULTET NOVI SAD	UNSPF	Serbia
2.	SCUOLA SUPERIORE DI STUDI UNIVERSITARI E DI PERFEZIONAMENTO S ANNA	SSSA	Italy
3.	FUNDACIO INSTITUT DE BIOENGINYERIA DE CATALUNYA	IBEC-CERCA	Spain
4.	SMART SENSING S.R.L.	SMART SENSING	Italy
5.	UNIVERSITA DEGLI STUDI DI CAGLIARI	UNICA	Italy
6.	LEVERETTE LANCE	Lance Leverette	Belgium
7.	The University of the West of England	UWE Bristol	United Kingdom

EXECUTIVE SUMMARY

Task 4.2 consisted in the assessment of the bioactuator contraction in response to electrical pulse stimulation (EPS) and the analysis of the biohybrid machine (BHM) functional performance in terms of bending capacity, response to different

EPS modes, and contraction profile. Additionally, the task included testing the performance of various modular BHM prototypes to evaluate the connectivity of individual units. All bioacuators were fabricated by mold casting using circular molds. Twitch and tetanic contractions were induced by in vitro current-based electrical pulse stimulation. Different stimuli parameters, such as waveform (i.e., squared or triangular), symmetry, frequency, and pulse width were manipulated (Section 2.1) in order to study their effect on the bioactuator contraction. The bioactuator was then integrated into the catheter by means of a two-pillar system protruding from the catheter wall (Section 2.2). The bending of the catheter by electrical induced contraction of the bioactuator was also tested in vitro (Section 2.3). Different bioactuator positions and varied electrical stimulation parameters were explored. Squared biphasic symmetric waveforms resulted to be the more efficient inducing twitch contractions. Wider pulses induce stronger twitch contractions. Strong tetanic contractions were induced by applying high frequency at maximum pulse width. The position of the bioactuator in the pillars of the catheter has a significant impact in the bending of the whole system. Having the bioactuator tilted, that is, the proximal side higher than the distal side, improves the bending in terms of amplitude. Increasing and decreasing the pulse width over time seems to generate a gradual bending of the catheter (induced by a tetanic contraction of the bioactuator) than modulating the current amplitude. Linear modulation not only provides higher bending degree but also increases contraction in a smoother way than non-continuous modulation by steps. The best condition so far to induce controlled tetanic contraction and therefore controlled bending is by modulating pulse width in a linear manner. The performed work is slightly different from the workplan (Section 3), while the contribution of partners is described in Section 4. In Section 5 we outline the main findings and the next steps. The design of the pillar system will be optimized in the future to secure the bioactuator in a fixed position. Further stimulation protocols will be assessed to explore the possibility of inducing tetanic contraction, mitigating tissue fatigue and maintaining the tetanus for prolonged time periods. Different strategies to integrate the bioreactor chamber into the already established catheter-bioactuator sub system are being currently studied. These strategies will be carefully analyzed in order to select the most suitable one.

LEGAL NOTICE
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LIST OF ABBREVIATIONS

Abbreviation	Description
BHM	Biohybrid Machine
EPS	Electrical Pulse Stimulation

1 DESCRIPTION OF TASK 4.2: EXPERIMENTAL PERFORMANCE TESTING

First, a basic characterization of the cells grown on the BHM will be performed in terms of the expression of protein markers of muscle tissue such as alpha-SMA and MHC and the formation of myotubes. Time-lapse microscopy experiments will be performed on swimming BHMs in a bioreactor chamber under static flow conditions and external electrode stimulation, following procedures previously established at IBEC (Valls-Margarit et al., 2019). Videorecording during at least 6 h will be performed to produce enough data to assess (i) the contractility response of the BHMs as a function of the electrical pulse stimulation (EPS) and working time, and (ii) the directional pattern of contraction, the swimming velocity, and net displacement according to the modeling outputs. The time-lapse videos will be analyzed by Image J software and Matlab custom-made algorithms. Once the BHM is mounted in the bioreactor and upon EPS, functional performance in terms of bending capacity, frequency response, contraction and relaxation lag-times, and number of functioning cycles without compromising the functional properties will be evaluated. The performance of several modular BHM for a single task (catheter actuation) will also be tested here in terms of the efficient connectivity between the individual units. The data obtained will be used to feed simulations and improve the predictability of the model constructed.

BHM prototypes will also be tested in terms of their ability to deliver a drug, when desired. This feature will be tested both with the catheter kept straight and undergoing different curves (based on a realistic scenario of catheter navigation in the vascular tree). First saline solution and then drug models (e.g., doxorubicin suspended in an aqueous solution) will be used. The performance parameters to be verified during these tests will be: (1) the force needed to inject the drugs in the different conditions (using manual syringe-based injection or using a motorized system, if the force will result high), (2) the ability of the catheter wall to resist the pressure required to inject the drugs, and (3) the ability to prevent undesired leakages of the drug during catheter navigation (self-delivery to the bloodstream). This may be evaluated by performing spectroscopic measurements on the fluid surrounding the catheter at different time-point (e.g., analyzing absorbance with a plate reader, by setting an excitation wavelength of 470 nm and an emission wavelength of 585 nm, to quantify doxorubicin release in the medium).

2 DESCRIPTION OF WORK AND MAIN ACHIEVEMENTS

The basic characterization of muscle cells grown on the BHM was reported in D2.2. The rest of the work and main achievements are reported in the following sub sections.

2.1 RESPONSE OF THE BIOACTUATOR TO ELECTRICAL STIMULATION

All BHM bioactuators used in this stage were fabricated by mold casting using circular molds with an inner perimeter of 22.6 mm, size optimized to hold the resulting bioactuators between two pillars with a distance of 9 mm between them (more details about this design were reported in deliverable D3.2). Before the bioactuator integration into the catheter, we evaluated its response to different electrical stimuli aiming to induce a muscle contraction strong enough to move and bend the catheter.

For this purpose, and in collaboration with UniCA, we measured the contraction force changing different parameters for the current-based electrical pulse stimulation, such as the waveform and the pulse width (i.e. the duration of the electrical pulse). The waveforms tested were either squared or triangular; the squared ones were either biphasic symmetric, biphasic asymmetric or monophasic (Figure 1A). For this test, we maintained the frequency fixed at 1Hz, to induce twitch contractions, the ones needed for the catheter motion, and a current amplitude of 18 mA.

Figure 1B shows the maximum force obtained during the twitch contraction of the bioactuators under the different protocols of stimulation. We observed that higher forces were obtained when biphasic asymmetric and symmetric waveforms were applied, which are the most physiological type of waveforms as they mimic the action potential generated by the motor neurons and muscle cells in vivo (Someck et al., 2023). The action potential generated by electrical stimulation opens the reservoirs of calcium ions which are the ones that induce muscle contraction. When a biphasic waveform is applied, the action potential is generated in the cathodic and anodic phase, producing a higher calcium flux (i.e. more release and more uptake) and therefore, a stronger contraction. On the other hand, monophasic waveforms, which only induces action potential in the positive/cathodic phase, have the disadvantage of inducing the accumulation of electric charge in the tissue which could result in tissue damage (Milosevic et al., 2020), being this the possible reason why reduced forces were obtained when stimulating with monophasic waveforms. For this reason, biphasic electrical field stimulation is commonly used in tissue engineering as less current is needed to excite the cells in comparison with monophasic stimulations, making the former a more secure and efficient form of stimulation (Chiu et al., 2011). The triangular waveform, although biphasic, seems not to induce the action potential as effectively as the squared ones. From this test, we also observed that the longer the pulse width, the stronger the contraction. This could be expected because by increasing the duration of the current passing through the tissue, more cells are being stimulated, leading to a stronger contraction. All these results demonstrate that the twitch contraction force can be modulated by changing the waveform and the pulse width of the electrical stimulation.

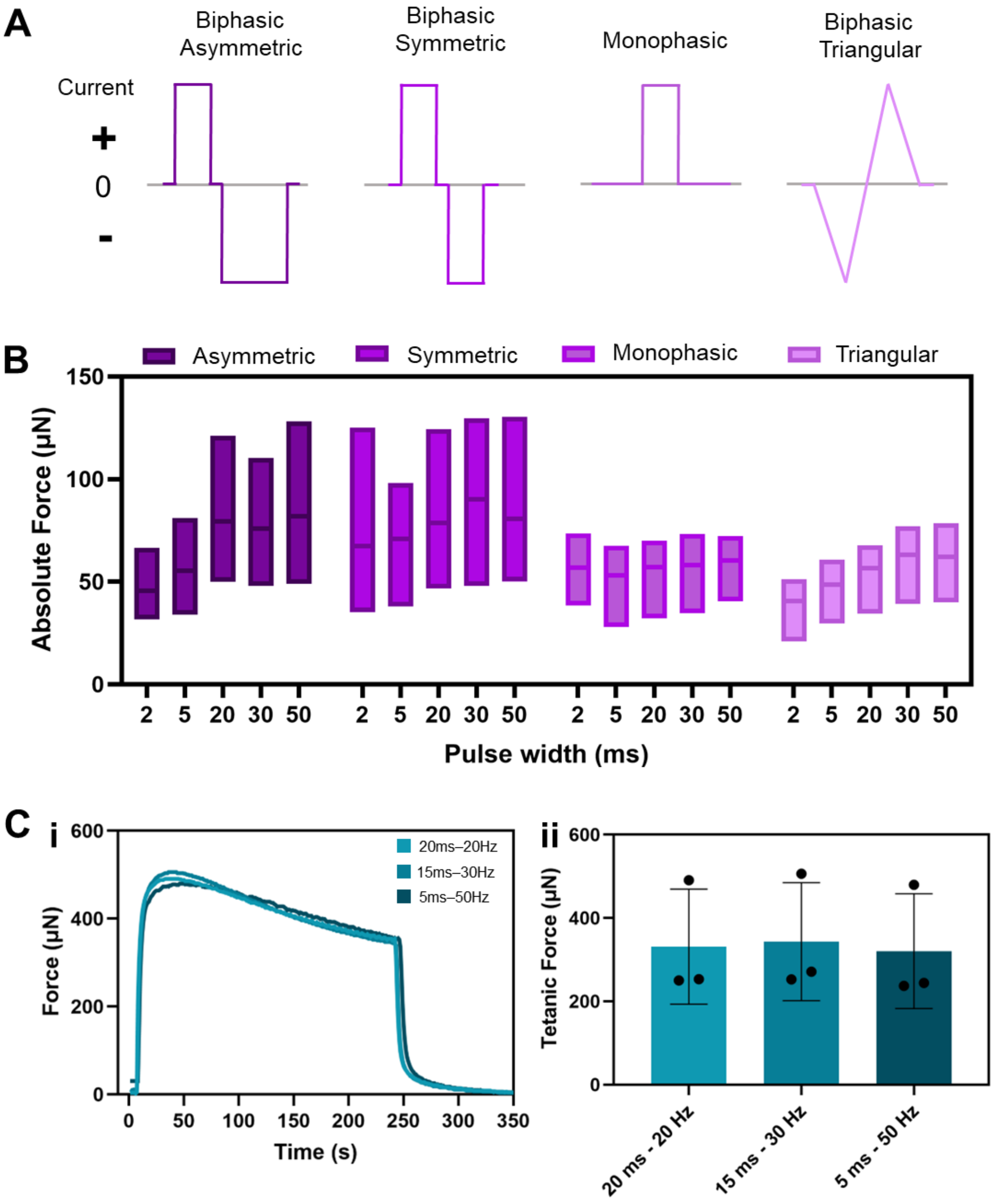

Figure 1. Characterization of the contraction force achieved by muscle bioactuators under different electrical pulse stimulation protocols. A) Schematic of the waveforms used for the current-based electrical stimulation. B) Floating bars (min to max) graph showing the maximum force values obtained during twitch contractions under the different stimulation protocols. N=3. Middle line corresponds to the mean value. C-i) Tetanic contraction of a bioactuator stimulated with biphasic waveform, applying different frequencies at adjusted pulse width for each frequency. C-ii) Average tetanic force (i.e. max value of force during tetanic contraction) obtained by applying different stimulation conditions. N=3. No statistical difference was obtained after performing a one-way ANOVA ($p<0.05$).

Tetanic contractions, which are needed for the bending of the catheter, can be induced with electrical stimulation at high frequency. To stablish the best protocol to generate this type of contraction, we evaluated different protocols using a symmetric waveform (as it was the one that produced stronger twitch contractions), at high frequencies, 20, 30 and 50 Hz, and applying an adjusted pulse width for each frequency, 20, 15 and 5 ms, respectively. Figure 1C-i shows the tetanic contraction pattern of a sample stimulated with the different conditions previously mentioned. In this graph, we can observe a peak of contraction followed by a smooth relaxation due to the little fatigue of the tissue, until it reaches a total relaxation once the stimulation is over. Very similar patterns were obtained with all stimulation conditions which suggests that changing the frequency at maximum pulse width may not have a big impact in modulating the tetanic contraction force. We also need to optimize the stimulation protocol to avoid causing muscle fatigue, in this way the tissue could be able to maintain the tetanized state as much as needed. Regarding the maximum tetanic force generated (i.e. peak of contraction), although no significant differences were obtained when comparing the different protocols (Figure 1C-ii), the high average tetanic force obtained (around 330 µN) was enough to induce the bending of the catheter, as will be shown in the next section 2.3 “Analysis of catheter bending under electrical stimulation”.

As next steps, we will evaluate how different current amplitudes affect force output, also we will explore other frequencies and pulse widths aiming to not only induce the tetanic contraction but maintain it over time without damaging the muscle bioactuator.

2.2 INTEGRATION OF MUSCLE BIOACTUATOR INTO THE CATHETER

After the fully maturation of the muscle tissue (after at least 7 days in differentiation media), it was placed between two posts on top of the catheter designed and fabricated by SSSA. The catheter was about 20 mm long, with an external diameter of 2 mm, and two circular posts protruding from its surface. The two pillars were 3 mm high and 2 mm diameter with 9 mm between them. To fix the catheter in the petri dish where the performance tests were done, SSSA designed the blue structure showed in Figure 2A. This structure was glued in the wall of the petri dish and the catheter was assembled on it. To induce muscle contraction different protocols of electrical stimulation were tested using a home-made electrode based on graphite was integrated also in the lid of the petri dish as shown in Figure 2B.

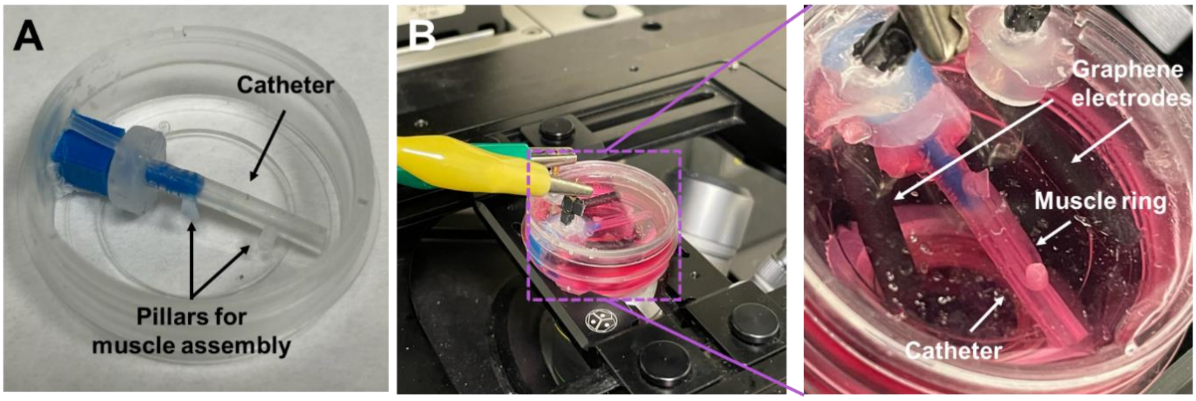

Figure 2. Images of the set up used to test the bending capacity of the catheter with the integrated muscle bioactuator. A) Petri dish containing the glued anchoring structure (blue) with the catheter fixed, which contains the pillars needed for muscle assembly. B) Set up for the electrical stimulation. Home-made graphene electrodes are integrated in the petri dish, containing the catheter and the muscle tissue assembled on the pillars. The bending is videorecorded with a microscope.

2.3 ANALYSIS OF CATHETER BENDING UNDER ELECTRICAL STIMULATION

2.3.1 EFFECT OF MUSCLE POSITION ON CATHETER BENDING

The petri dish containing the catheter with the muscle assembled on pillars, the electrodes and cell media was imaged using the Thunder microscope obtaining the videos and images shown in Figure 3.

Firstly, we evaluated how the position of the tissue in the pillars could affect the bending of the catheter. We tested two configurations: a) both sides of the tissue ring were at the bottom of the pillar, touching the catheter wall (Figure 3A-i), and b) the proximal side is in a higher position while the distal side remained at the bottom (Figure 3B-i). We stimulated the samples by applying a biphasic symmetric waveform at 50 Hz and 2 ms pulse width. Images ii and iii correspond to the position of the catheter when tissue is relaxed (i.e. not electrical stimulation is applied) and when tissue reached tetanic contraction, respectively. We can conclude from comparing the angle of bending, 1.6° for configuration A and 3.6° for configuration B, that having the muscle tissue in a tilted position improved the catheter bending probably due to a more effective force transmission since in this way there is a perpendicular force component in addition to the axial component. Therefore, we used this configuration for the following experiments where we tested how the different parameters of electrical stimulation affect the bending.

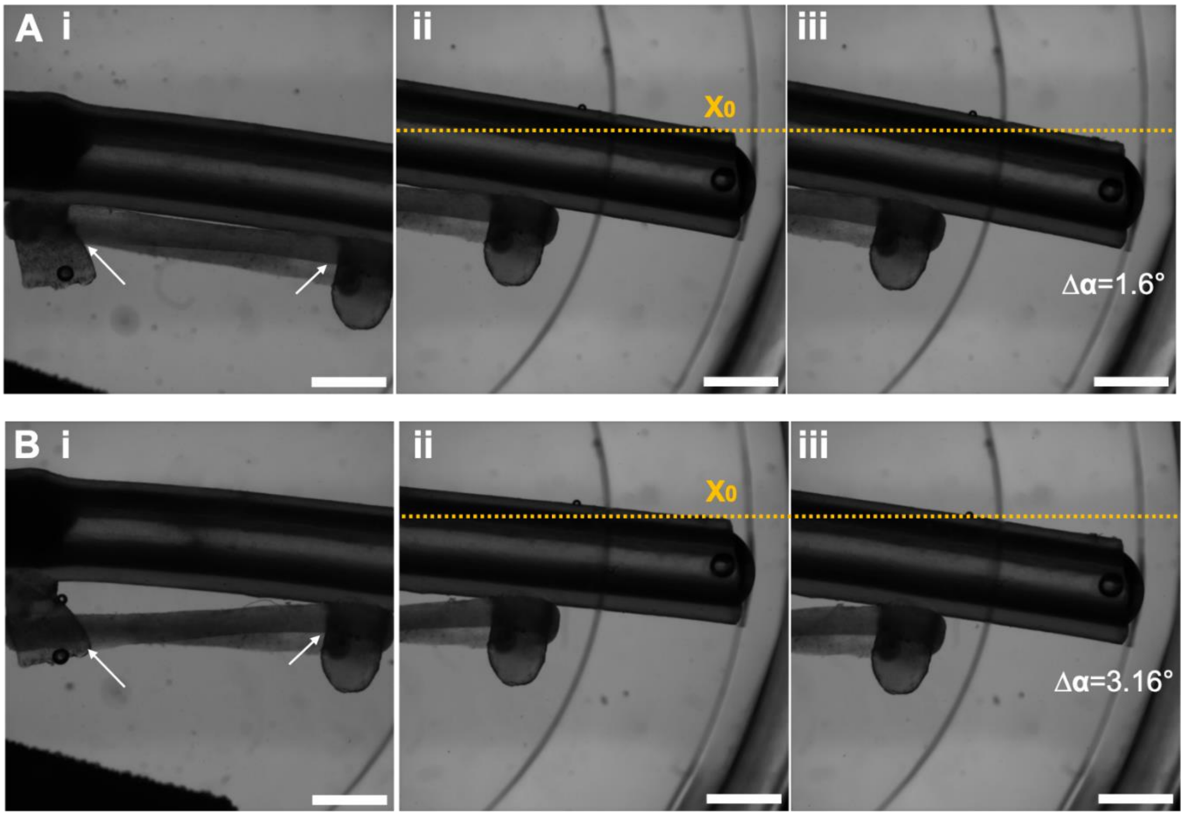

Figure 3. Evaluation of the influence of the position of muscle tissue in the catheter bending. A-i) Microscope image of the muscle tissue assembled on the pillars. This is the configuration a), where both sides of the tissue are at the bottom of the pillar indicated by white arrows, ii) initial position of the catheter tip when electrical stimulation was not applied, indicated by the yellow line and iii) bending of the catheter when electrical stimulation was applied. B) Microscope images like the ones in panel A but with the muscle tissue in the configuration b), that is, one side of the tissue is in a higher position than the other, as indicated by the white arrows. Bending angle difference is indicated in A/B-iii as Δα.

2.3.2. MODULATION OF CATHETER BENDING WITH ELECTRICAL STIMULATION

The bending capacity of the bioactuator assembled in the catheter was evaluated under different electrical stimulation protocols. Our main goal was to induce and modulate the tetanic contraction on the muscle bioactuator to generate a smooth bending of the catheter. For this purpose, two electrical stimulation parameters were evaluated: amplitude and pulse width. Also, we studied how the controlled increment and decrease of those parameters in a linear and stepwise manner affected the bending capacity.

The performance of the biohybrid catheter was analyzed by IBEC and SSSA using custom-made algorithms. While IBEC determined the linear displacement of the bending (Figure 4), SSSA obtained the maximum angle and the arc of the circumference from the bending (Table 1).

Both conditions, amplitude and pulse modulation, induced the tetanic contraction of the muscle, obtaining the typical tetanic profile shown in figure 4. From the angular displacement analysis (table 1) we conclude that the level of bending was very similar, 1.52° during amplitude modulation and 1.6° for pulse modulation. However, we observe interesting differences when comparing the displacement profile (Figure 4). While pulse modulation seems to induce the tetanic contraction in a more gradual manner, increasing the amplitude generated a faster tetanic contraction and relaxation.

Focusing on the linear and stepwise modulation, we observed that the level of bending was lower when the increment of amplitude and pulse width was done in a 6-step format, shown by the decrease in maximum angle (table 1) and displacement (figure 4). The displacement graphs from figure 4B and D clearly show the different increasing force steps, making the contraction transition less continuous/smooth.

These results are very encouraging as they demonstrate that we can modulate the level/degree of bending by controlling muscle contraction with electrical stimulation. This approach is very interesting as for the future application of the BHM we do not want an abrupt bending inside the body, but we are aiming for smooth and controlled movements.

As next steps, we will be working on the analysis of the muscle actuation under other electrical stimulation protocols, considering applying higher amplitude values. We are encouraged by these results, and we believe we could analyze the motion of the system soon. However, we are still missing the integration of the chamber, therefore so far, the performance is only done with the catheter and assembled muscle.

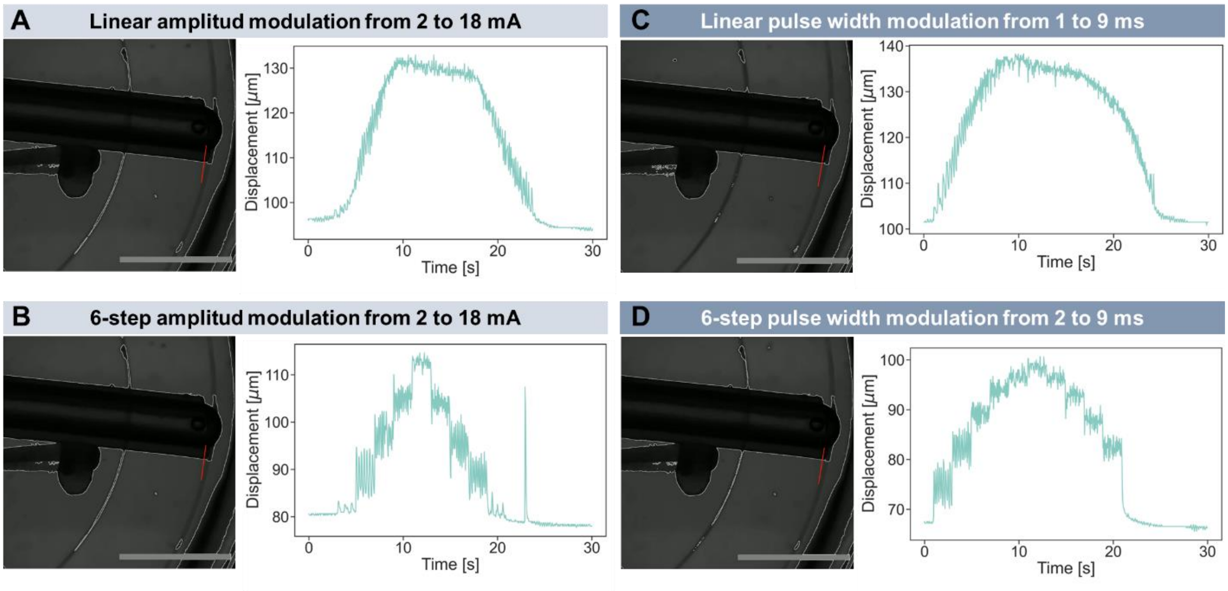

Figure 4. Evaluation of the linear displacement of the catheter when the bioactuator performs a tetanic contraction under different electrical stimulation protocols, in all of them applying a frequency of 50Hz. For all panels, the left image corresponds to a frame of the video recording of the catheter bending being analyzed by a custom-made Python code, providing the graph on the right that shows total displacement (i.e. bending) of the catheter. A) Bending induced by applying a linear increase and decrease of current amplitude from 2 to 18 ms. B) Same as A), but the modulation of the amplitude is done in 6 steps. C) Bending induced by applying a linear increase and decrease of pulse width from 1 to 9 ms. D) Same as C) but the pulse modulation is done in 6 steps.

Table 1. Values of maximum angle and arc of circumference of the catheter bending under different electrical stimulation protocols.

Stimulation conditions	Angle max (°)	Arc of circumference max (mm)
Linear amplitud modulation from 2 to 18 mA	1.52	0.23
6-step amplitud modulation from 2 to 18 mA	1.38	0.21
Linear pulse width modulation from 1 to 9 ms	1.6	0.24
6-step pulse width modulation from 2 to 9 ms	1.46	0.22

3 DEVIATIONS FROM THE WORKPLAN

Until now, the performance of the BHM has been done with only the muscle bioactuator assembled in the catheter. The integration of the chamber could not be done, as its assembly with the catheter is still a work in progress.

Also, only bending could be evaluated, although these encouraging results suggest that the motion of the biohybrid catheter could be done in the near future.

4 PERFORMANCE OF THE PARTNERS

All the work presented here has been done in a collaborative way, from a short stay of UniCA and SSSA in IBEC. All partners contributed to the development of the experimental part and further analysis.

UniCA developed the current based electronic system for the stimulation of the muscle bioactuators. They also designed the experimental plan, determining the electrical stimulation conditions to test.

SSSA designed and fabricated the catheter. Also analyzed the angular displacement of the catheter bending.

5 CONCLUSIONS

<u>Response of the muscle bioactuator to electrical stimulation</u>

- We successfully evaluated the force output of muscle bioactuators under different electrical stimulation protocols reaching the following conclusions:
  - Biphasic waveforms are more efficient in inducing twitch contractions than monophasic ones. Among the biphasic ones, the symmetric waveform is the best one.
  - Higher pulse width seems to induce stronger twitch contractions.
  - Tetanic contraction was induced by applying high frequency at maximum pulse width, inducing strong contraction forces.

<u>Integration of the muscle bioactuator into the catheter</u>

- We successfully assembled the muscle tissue in the catheter through pillars/notches integrated into the catheter wall.
- We studied the catheter bending capacity under electrical stimuli by preparing a set up that included a fixing element to attach the catheter in a petri dish and stimulation electrodes.

<u>Analysis of the catheter bending under electrical stimulation</u>

- The relative position of the muscle in the pillars has a significant impact in the catheter bending. Having the tissue tilted, that is, the proximal side higher than the distal side, improves the bending in terms of amplitude.
- Response to amplitude and pulse width modulation, in a linear (continuous) and a 6-step way, had been evaluated:
  - A very similar bending degree is observed in both stimulation conditions.
  - Increasing and decreasing the pulse width over time seems to generate a more gradual bending (induced by a tetanic contraction of the bioactuator) than modulating the current amplitude.

  - Linear modulation not only provides higher bending degree but also increases contraction in a smoother way than non-continuous modulation by steps.
  - The best condition so far to induce controlled tetanic contraction and therefore controlled bending is by modulating pulse width in a linear manner.

Future work

- The design of the pillar system will be optimized in order to secure the bioactuator in a fixed position, with the proximal side higher than the distal side.
- Further stimulation protocols will be assessed to explore the possibility of inducing tetanic contraction, mitigating tissue fatigue and maintaining the tetanus for prolonged time periods.
- Different strategies to integrate the bioreactor chamber into the already established catheter-bioactuator sub system are being currently studied. These strategies will be analyzed in terms of compatibility, stability, functionality, among other factors in order to select the most suitable one.
- In addition to catheter bending analysis, its motion will be analyzed in the near future.

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