Development of an advanced culture system to establish a physiologically relevant 3D vascular model

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***Abstract* — Vascular tissue engineering aims to regenerate vessels "at the target site" using cell-free synthetic scaffolds that support endogenous regeneration. A critical challenge to the success of this process is maintaining the delicate balance between functional neo-tissue formation, hemodynamic stimuli, and scaffold degradation.**

To explore the biomechanisms underlying the interaction between these factors, we have developed an advanced in vitro culture system capable of replicating in vivo-like physiological wall shear stress and pulsatile pressure, as well as performing automatic cell seeding. In this study, we conducted biological experiments to validate the platform functionality, specifically performing cell seeding with different cell lines in both monoculture and coculture conditions within a vascular graft.

This novel culture system will provide valuable insights into cell-graft interactions, synthetic scaffold colonization, and their response to hemodynamic cues.

***Keywords* — advanced culture system, 3D *in vitro* model, vascular tissue engineering.**

I. Introduction

Vascular tissue engineering exploits the inherent regenerative capabilities of organisms to create complex tissue structures using cells, biomaterials, and chemical or physical signals. An essential factor hindering the success of endogenous regeneration is the delicate balance between functional neo-tissue formation, hemodynamic stimuli, and scaffold degradation [1-2]. Despite promising *in vivo* proof-of-concept studies, complications like intimal hyperplasia, early stenosis, and aneurysm formation remain prevalent in vascular graft implantation [3-4].

3D *in vitro* models are increasingly developed and used to provide a more physiologically relevant environment to study vascular cellular behavior instead of standard 2D *in vitro* models. These culture systems enable important features such as: housing tubular samples, multiple cell coculture, reproducing controllable hemodynamic conditions, monitoring the chemical environment, and tuning single chemo-mechanical parameters that are not possible in animal models [5-7]. Moreover, the use of these platforms has the potential to reduce costs in both preclinical and clinical research phases, in accordance with the principles of the three Rs: Replacement, Reduction, and Refinement.

To establish a relevant vascular *in vitro* model, it is necessary to use a versatile perfusion culture system that allows to reproduce flow-induced stimuli that simulate blood flow in native vessels. Moreover, since the tunica intima is a key element in vascular mechanobiology, it is essential to obtain a homogenous cellular layer on the luminal surface of a tubular scaffold in a controllable and repeatable way. The predominant state-of-the-art seeding technique is defined as static or passive seeding and involves pipetting a cell suspension directly into the lumen or outside of the vascular graft [8]. Despite being the simplest method, manual procedures are strongly operator dependent and fail to achieve uniformity, reproducibility, and high seeding efficiency [9].

In the present work we present a custom-designed advanced cell culture system that allows *in vivo*-like flow-induced stimuli application and automatic rotational seeding to establish a physiologically relevant vascular *in vitro* model.

To validate the novel features of the developed vascular model, the device was subjected to rigorous bench tests and was employed in a biological campaign in which human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were seeded onto the luminal surface of an electrospun vascular graft both in mono-culture and in co-culture conditions. To achieve this last objective, an automatic rotational seeding procedure was set up. Given the lack of unanimous consensus at the state-of-the-art regarding the optimal rotational seeding protocol to be performed, prior to the cell seeding experiments, computational simulations were performed to understand the physical mechanisms governing cell-graft interactions during the seeding procedures. Moreover, the *in silico* model was employed as a useful interpretative tool to correlate the experimentally performed seeding protocol and the obtained cellular distribution.

This multidisciplinary approach, integrating computational modelling with biological experiments, aims to enhance the efficiency of the seeding process, ultimately contributing to the effectiveness of the advanced culture system.

II. Methods

A. Advanced culture system

The system was designed aiming at ensuring versatility and automation of the culture system through the integration of closed-loop control functions to guarantee its effectiveness and reliability during different cell culture experiments. In particular, the system control is totally wireless; in this way the entire system can be placed inside an incubator after the assembly and filling procedures under laminar hood and only the supply cable needs to be connected from outside.

The main system components are: a culture chamber placed on a rotating mixer for semi-automatic cell seeding, an electronic control unit, two hydraulic circuits – one for the

luminal compartment and one for the extraluminal compartment – each one actuated by a roller pump, and composed by silicon tubes, a reservoir and an air chamber to dampen pump pulsatility (Figure 1(A-B)).

The culture chamber consists of a polymethylmethacrylate (PMMA) tube ($Ø_{in}$ 50 mm, $Ø_{ext}$ 60 mm, length 200 mm) in which a sample support system is inserted. This component consists of a fixed element and a movable one, which slides using a belt-pulley mechanism that operates two worm screws. This feature allows samples of different lengths (10 ÷ 120 mm). To house samples of different diameters (1 ÷ 8 mm), threaded barb connectors can be changed in the housing structure. The sample support is directly connected to the luminal and extraluminal hydraulic circuit.

The electromechanical peripheral devices of the culture system (two peristaltic pumps, a stepper motor and a pinch-valve) and the custom printed circuit board (PCB), are placed inside an IP68 box. Four ESP32® microcontrollers (mounted on the PCB) manage the entire culture system functions. The control unit constantly monitors the system status showing pressure values, bioreactor rotation, and temperature and humidity inside the control unit box. When measured values exceed preset thresholds, the execution of each peripheral device is halted, ensuring a high robustness level and functioning reliability during culture experiments.

Through the graphic user interface (GUI) it is possible to control each peripheral device in manual control or set an experiment choosing between different stimuli applications: stationary mode (constant flow rate and pressure), ramp mode (initial and final flow rates are set for gradual flow-induced stimuli application) or pulsatile mode (flow rate or pressure feedback loop control). Once the parameters are set, the connection between the GUI and the culture system is no longer necessary for its autonomous running.

B. In silico evaluation of different cell seeding strategies

Two main automatic rotational seeding methods were investigated: continuous rotation and discrete rotation (where cells settle at specific angular positions).

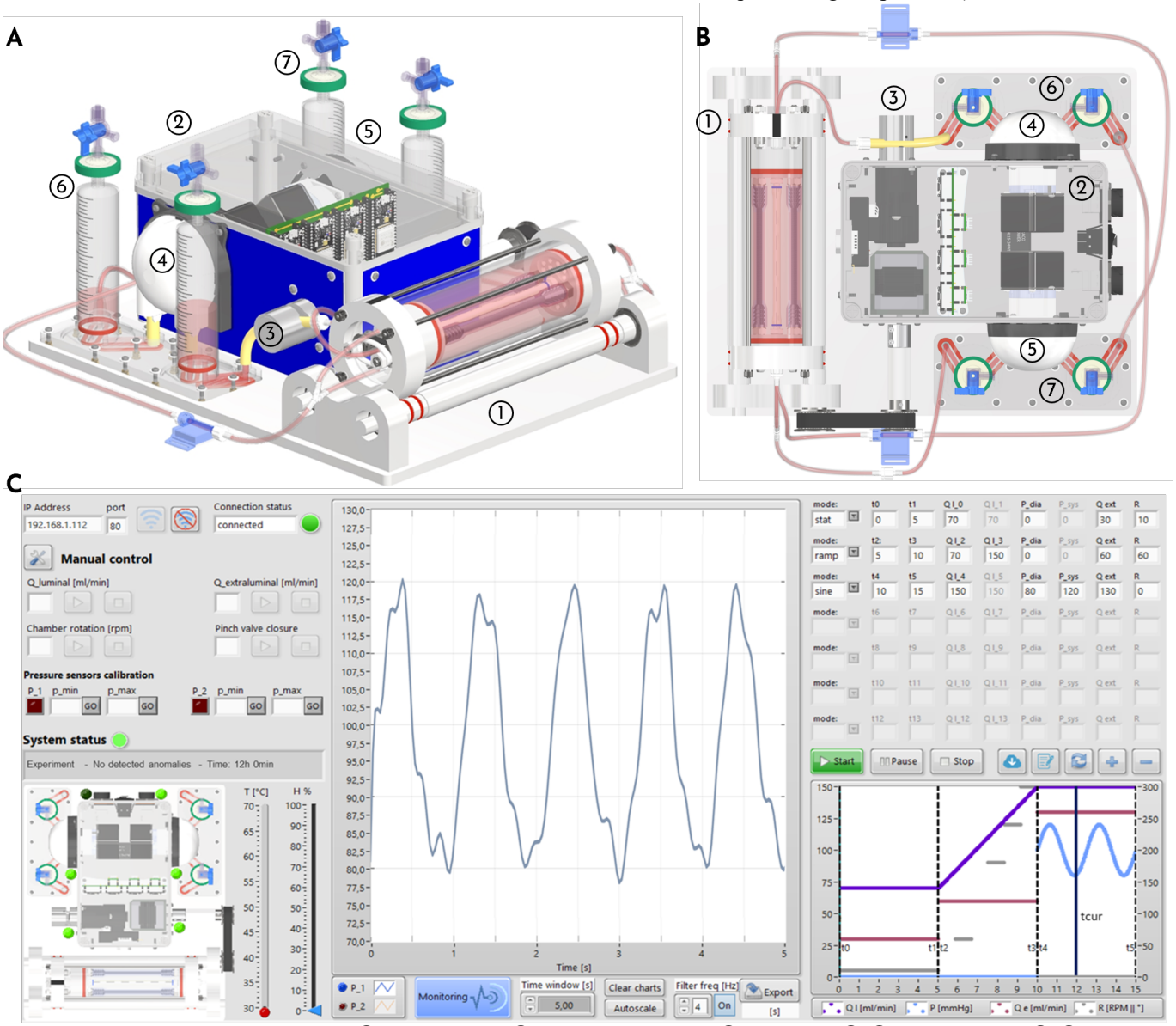

Fig. 1 (A-B) Culture system assembly CAD: ① culture chamber, ② electronic control unit, ③ pinch-valve, ④-⑤ peristaltic pumps, ⑥-⑦hydraulic circuits. (C) wireless graphic user interface developed in Labview®: left side for direct system use ("Manual control", "Pressure sensors calibration", "System Status"), central panel for pressure monitoring and data export, right side for setting protocol parameters and experiment management.

A 2D circular graft slice of radius 2.75 mm was employed as the fluid domain representative of the phenomena occurring inside the sample. The 2D geometrical simplification was adopted after preliminary verifying that the fluid velocity contributions along the sample longitudinal axis were negligible. The simulations were conducted using COMSOL Multiphysics®. The culture medium was modelled as Newtonian fluid at temperature of 37°C, with viscosity μ of 10^{-3} Pa*s, and density ρ of 1000 kg/m^3. The cells were modelled as spherical particles with a diameter of 15 μm and particle density ρ equal to 1050 kg/m^3 [10-11].

At the initial instant of the simulation, 10^4 particles uniformly random distributed inside the fluid domain were released. Adhesion between the cells and the graft wall was modelled through a "stick condition" set on the circumference.

For continuous rotation, three simulations were performed with graft revolution speeds of 1, 5, and 10 RPM.

For discrete rotation, static sedimentation periods and transition phases, during which the domain rotates, were simulated separately to significantly reduce computational cost. Different protocols were tested to determine which provided the most uniform coverage of the graft wall by the particles representing cells injected into the vascular graft.

C. Cell seeding experimental protocols and analyses

Three-layered electrospun grafts (dry condition characteristics: $Ø_{in}$ 5.5 mm, wall thickness 355 μm, length 60 mm), composed of a nanometric mesh of silk fibroin (SF) and polyurethane (PU) enclosed within SF layers, were manufactured and used for these tests [12].

For both mono-culture and co-culture experiments, the same discrete rotation seeding protocol (selected based on *in silico* analysis) was applied: 0° for 12 minutes, 180° for 15 minutes, 90° for 18 minutes, and 270° for 20 minutes. In mono-culture experiments, HUVECs or HASMCs were injected into the graft, seeded using the discrete rotation protocol, and after 24 hours, small ring samples were taken from the tubular sample. In co-culture experiments, HAMSCs were seeded first, and after 48 hours under static conditions, HUVECs were luminal injected and seeded; 24 hours later (72 hours from the start of the experiment), small ring samples were retrieved.

The effectiveness of the seeding protocol along the entire length and circumference of the tubular scaffold was evaluated by analyzing cell morphology through immunostaining of ring-shaped samples, followed by imaging with a Zeiss Axio Imager fluorescence widefield microscope equipped with the Apotome module (structured illumination). For staining, we used DAPI (nuclear marker) and phalloidin (actin cytoskeleton marker). To distinguish endothelial and smooth muscle cells in the co-culture samples, von Willebrand Factor (vWF) and alpha-smooth muscle actin (αSMA) were included as additional markers.

III. Results and Discussion

A. Advanced culture system bench tests

The system underwent several bench tests to evaluate its suitability and reliability. Initially, all components in direct contact with the sample and culture medium were tested for cytocompatibility, yielding successful results. The system was then assembled, filled with culture medium under a laminar flow hood, and placed in an incubator for 21 days to assess its ability to maintain sterility. No contamination was observed at the end of this period.

The system's functions were thoroughly examined to ensure their robustness and consistency. A key feature is the automatic generation of the user-defined pulsatile stimuli (both flow rate and pressure): numerous conditions were successfully obtained using a fluid with a viscosity of 3.54 mPa·s and a silicone phantom tube.

We believe that versatility and ease of use are the standout features of the culture system developed and presented here, setting it apart from commercially available systems and state-of-the-art technologies. The system is accurate and reliable, enabling the setup and application of various seeding and stimulation protocols. It allows for precise and controlled cell seeding experiments, offering flexibility to fine-tune experimental procedures, including both rotational seeding and fluid dynamic conditions.

B. Computational results and seeding protocol setting

For continuous rotation at low speeds (1 and 5 RPM), no more than 6% of the particles adhere to the graft wall, while at high-speed rotation (10 RPM), over 80% of the particles adhere. No significant differences were observed in terms of particles' distribution on the graft wall.

Regarding discrete rotation simulations, the protocol with the angular sequence 0° - 180° - 90° - 270° resulted in the best particle wall coverage, reaching 96%.

Computational results should be interpreted biologically, as the simulated particles represent cells, which are more complex than simple rigid spheres. It is important to note that HUVECs and HASMCs are adhesion-dependent cells, so their suspension time must be limited [13-14].

Considering these factors, the high RPM strategy is preferable because it quickly brings cells into contact with the wall, although it also generates undesired wall shear stress (WSS) due to increased fluid velocity. In the discrete rotation protocol cells reach the graft wall in few minutes, allowing them to establish adhesion with the substrate without stress.

Based on these considerations, the discrete rotation seeding protocol with the 0° - 180° - 90° - 270° sequence was chosen and applied in the biological experiments using the developed advanced culture system, as it provided satisfactory computational results.

C. Mono-culture and co-culture seeding results

For both the type of cell tested, cell adhesion occurred rapidly following injection of cell suspension within the graft. The luminal surface of the graft was almost entirely populated, and the morphological analyses are highly promising. We observed that HUVECs and HASMCs almost completely covered the graft (*i.e.,* without leaving empty spaces between them) and established a complex cell-graft and cell-cell interaction network (Figure 2 A, B).

A preliminary experiment using a co-culture of HUVECs and HASMCs was conducted to replicate the layered structure of vascular tissue. First, HASMCs were seeded onto the graft, and after 48 hours of adhesion, HUVECs were seeded on top.

The graft appeared completely covered by healthy cells. Immunostaining for two different markers, α-SMA and vWF, confirmed the presence of both cell types (Figure 2C). However, a sectional analysis of the samples is still required to better visualize the distribution of the two layers within the electrospun graft.

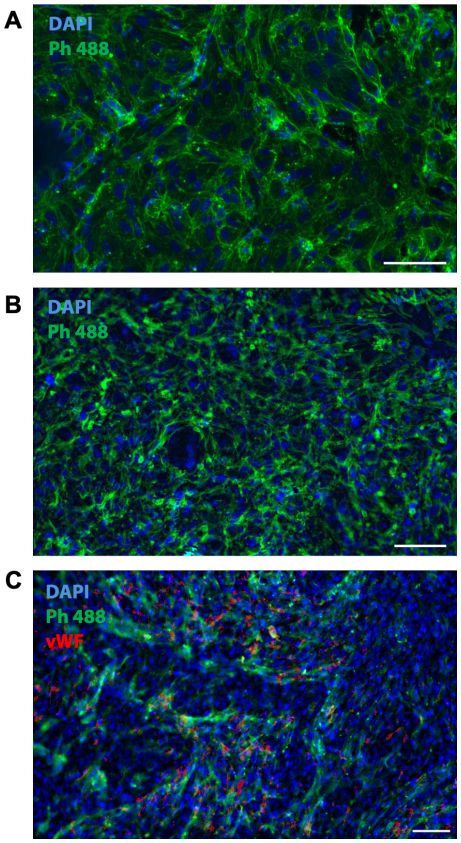

Fig. 2 Immunostaining representative images of (A) HUVECs, (B) HASMCs, and (C) co-culture of HUVECs and HASMCs seeded into the electrospun graft. DAPI (blue), phalloidin (green), vWF (red). Scale bars represent 100 µm.

IV. Conclusion

The developed culture system is a valuable platform for both scientific research and industrial applications, facilitating the study of vascular mechanobiology and the validation and development of new medical devices in the vascular field. The biological results we obtained are a key milestone: achieving a homogeneous cellular coculture is essential for using this custom-made culture system as an effective 3D *in vitro* vascular model. Additionally, the seeding protocol established in this study is highly adaptable to various experimental conditions (such as graft diameter and cell line), enhancing the overall versatility of the system and making it well-suited for investigating vascular mechanobiology as an alternative to animal models.

We are currently applying controlled dynamic wall shear stress and intraluminal pulsatile pressure to investigate cell function in response to different physical stimuli. These mechanobiology studies aim to provide deeper insights into the underlying cellular mechanisms.

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