Fused deposition modelling supporting the design of *in vitro* models of diseased muscular tissues – PhD Thesis by Mattia Spedicati

In contemporary biomedical research, the demand for advanced *in vitro* models as preclinical platforms is rising. These models are essential for validating new therapeutic strategies, conducting preclinical drug screening, and understanding pathological progressions, reducing reliance on traditional models and animal testing. Accurate replication of specific physiological or pathological features is crucial. Tissue engineering has made significant progress, using advanced approaches to create three-dimensional supports for tissue replication. Among fabrication techniques, additive manufacturing methods like fused deposition modelling (FDM) have become indispensable due to their versatility and precision in creating complex structures.

This thesis focuses on the *in vitro* modelling of human muscle tissues, specifically cardiac and skeletal muscle. Both tissue types share structural and functional similarities and can be affected by various pathologies that impair their contractility and functionality. Cardiac tissue, for instance, can be damaged by myocardial infarction and cardiomyopathies, while skeletal muscles can be compromised by muscular dystrophies, age-related sarcopenia, and injury-induced hypertrophy. Given the significant impact of these conditions, developing accurate *in vitro* models is crucial for advancing treatments and drugs. The FDM technique has therefore been proposed as a versatile tool for the design and production of *in vitro* models of human muscle tissues. On one hand, it has been used for the production of 3D biomimetic scaffolds employed as support for cell culture and the formation of new tissue. This technology indeed allows for precise architectural and functional design, thereby improving the accuracy of the final models. Furthermore, the same technique has also been employed to produce functional components used to incorporate stimuli into the model that are useful to trigger an appropriate response that mimics the target tissue.

Three-dimensional bioartificial scaffolds were designed and fabricated as platform for *in vitro* model of early-stage post-myocardial infarct fibrotic tissue, characterized by non-aligned and stiffened features. Exploiting the versatility of FDM techniques, polycaprolactone (PCL) 3D scaffold with 150 µm square-meshed architecture was fabricated from PCL. Subsequently, scaffold was then surface-grafted with gelatin by mussel-inspired approach and cultured with human cardiac fibroblasts (HCFs). Following 3 weeks culture scaffold triggered the HCFs activation into myofibroblast phenotype and the deposition of pathological-like ECM.

A step forward in this approach was the addition of the mechanical triggering to the model, then stretchable bioartificial scaffolds with a biomimetic composition and stiffness comparable to human cardiac fibrotic tissues were implemented. PCL scaffolds with a stretchable mesh architecture were initially designed through structural and finite element method (FEM) analyses and subsequently fabricated using FDM. Scaffold pores were then filled with gelatin Methacryloyl (GelMA) hydrogels to support *in vitro* HCFs 3D culture. Scaffolds were surface-functionalized using a mussel-inspired approach to enhance the interaction between PCL and GelMA hydrogels. Bioartificial PCL/GelMA scaffolds exhibit mechanical behaviour, including stretchability and stiffness, similar to that of

pathological cardiac tissue. HCFs were cultured within PCL/GelMA scaffolds under dynamic conditions, undergoing 7 days of cyclic mechanical stimulation after 7 days of static culture. *In vitro* cell tests conducted on these dynamically stretched bioartificial scaffolds confirmed HCF activation, which did not occur in HCF-loaded scaffolds cultured under static conditions. These findings support the hypothesis that applying cyclic mechanical stimulation to cellularized bioartificial scaffolds may trigger HCF activation into myofibroblasts.

The potential of cyclic mechanical stimulation to induce pathological conditions was also explored to model cardiac hypertrophy in healthy cardiac muscle tissue. A custom-made stimulator was developed to stretch engineered heart tissues (EHTs), which consist of a fibrin hydrogel embedded with iPSC-derived cardiomyocytes and cast around two flexible silicon poles to support their spontaneous contractility. The mechanical stimulation system comprised a motor and an actuator that manoeuvres a custom-made PLA stimulation grid, which exerts pressure on the silicon structure housing the two flexible poles. Through this connection, the structure can translate its motion into the stretching of EHTs. FDM proved useful as a rapid prototyping technique, enabling the production of appropriately designed parts for EHTs stimulation. The efficacy and quality of the stimulation device were initially optimized and validated. Subsequently, the device was used to stimulate mature EHTs for seven days. Following stimulation, the EHTs exhibited hypertrophic symptoms, including reduced force production and increased levels of TnI and Nt-ProBNP, commonly recognized as cardiac stress markers.

Similarly to the approach used with early-stage cardiac fibrosis, 3D PCL scaffolds, mimicking proper morphological features, were coated with gelatin and seeded with C2C12 myocytes to reproduce muscle fascicles architecture for *in vitro* modelling of skeletal muscle tissue (SMT). FDM was used to produce a fibrous scaffold with an aligned morphology, creating 3D artificial fascicles through the synergistic combination of FDM and porogen leaching, utilizing PCL as bulk material and poly(ethylene glycol (PEG) as porogen. The resulting 3D PCL aligned fibrous scaffolds were then coated with gelatin, resulting in micrometric fibers with a superficial stiffness similar to that of skeletal muscle tissue. C2C12 cells were subsequently cultured on the scaffold, demonstrating spontaneous maturation and myotube formation induced by the scaffold morphology. The resultant anisotropic scaffold morphology promoted SMT-like cell conformation, establishing a versatile platform for developing *in vitro* models of tissues with anisotropic morphology.

Overall, this thesis highlights the versatility of FDM in producing tools for *in vitro* modelling and the potential of mechanical stimuli, combined with biochemical mimicry, to induce specific pathological conditions in cardiac muscle tissues, such as cardiac fibrosis and cardiac hypertrophy. Additionally, the flexibility provided by FDM techniques and synthetic polymers enabled the production of 3D aligned scaffolds that mimic muscle bundles for SMT modelling. These scaffolds serve as a valid platform to potentially reproduce SMT hypertrophy, demonstrating the utility of FDM in advancing tissue engineering and disease modelling.