Abstract of the thesis

Currently, two-dimensional (2D) cultures and animal models are the gold standard for evaluating chemical toxicity and the efficacy of newly developed therapeutic approaches. However, their limited biomimetic relevance and lack of methodological standardization pose substantial challenges to obtaining reliable and predictive data on human responses. Moreover, these systems fail to accurately reproduce aging-related effects on physiological processes. In this context, three-dimensional (3D) bioengineered *in vitro* models offer enhanced potential to replicate human physio-pathological environments, thereby enabling more accurate and human-relevant toxicity assessments. Among human tissues, the myocardium is highly influenced by aging and chemically induced cardiotoxicity.

This PhD project was developed within this framework and aimed to design 3D bioengineered *in vitro* models of young and aged cardiac tissues for cardiotoxicity evaluation. The research was conducted as part of the H2020 ALTERNATIVE project. Specifically, the *in vitro* models developed within this PhD project were designed in the form of biphasic constructs, including a 3D printed polymeric framework and a hydrogel counterpart. In detail, the first component consisted of a polymeric scaffold based on an optimized poly(urethane urea) (PUR) and fabricated via melt additive manufacturing (AM), intended to provide mechanical support and confer anisotropic properties to the model. The second component was a methacryloyl gelatin (GelMA) hydrogel used as a cell carrier for human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and human coronary artery endothelial cells (HCAECs). The properties of the hydrogel were modulated by adjusting its concentration to replicate different cardiac tissue conditions. The two components were combined to form the final cardiac model, which was subsequently placed in a bioreactor with electrical stimulation and dynamic perfusion.

In the first part of the work, a series of PURs was engineered by a two-step synthesis using poly(caprolactone) (PCL), 1,4-butane diisocyanate (BDI), and L-lysine ethyl ester (Lys) as chain extender. The PURs synthesis procedure was modulated with the aim of synthesizing a polymer best matching the mechanical and technological demands for soft tissue applications and processability through melt extrusion AM. To this aim, the amount of catalyst (dibutyltin dilaurate, DBTDL) added in the first synthesis step, and the quantity of tertiary amine (triethylamine, TEA) added as neutralizing agent/catalyst during the chain extension phase were finely tuned. DBTDL catalyst was added at 0.1% or 0.3% w/w with respect to PCL diol, while TEA was added at 1:1, 1.5:1 or 2:1 molar ratios with respect to Lys. The formation of urethane and urea bonds was confirmed by proton nuclear magnetic resonance (^{1}H NMR) and attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopies. In the ^{1}H NMR spectra, the intensity of the signals associated with urea bonds showed an increase with increasing the amount of TEA added during the chain extension step (i.e., at TEA:Lys molar ratio of 2:1). For these polymers, ATR-FTIR spectroscopy revealed an increase in hydrogen bonding, while size exclusion chromatography evidenced a significant increase in number average molecular weight. Conversely, in PURs with the same TEA concentration, increasing the DBTDL amount led to slight molecular weight increase (around 5-10 kDa). The increase in the amount of TEA also caused a progressive reduction in PCL crystallinity. Atomic force microscopy evidenced that higher TEA content led to smaller PCL crystals, whereas increasing DBTDL concentration promoted crystal growth. At low TEA contents, increasing DBTDL concentration shifted the material from brittle to tough, whereas high TEA contents caused a gradual decrease in Young's modulus making the PURs elastomeric. Consistently, the Young's modulus (E) estimated through force spectroscopy analyses decreased with increasing TEA content, ranging from about 13 MPa at a 1:1 TEA:Lys molar ratio to approximately 3 MPa at 2:1 molar ratio. Rheological analyses indicated that PUR viscosity increased with increasing molecular weight and hydrogen bonding. At 120 °C, low-TEA containing polymers showed complex viscosities of 20–30 Pa·s, while viscosity increased to about 200 Pa·s when the TEA:Lys molar ratio was set at 1.5:1. The processability of the synthesized PURs was then evaluated using a melt extrusion AM system. PURs prepared at a

TEA:Lys molar ratio of 2:1 were not processable. In contrast, PURs with TEA:Lys molar ratios of 1:1 and 1.5:1 were extrudable and thermally stable for up to 3 hours. The PUR synthesized with DBTDL at 0.3% w/w concentration and TEA:Lys molar ratio of 1.5:1 exhibited the best balance between printability and mechanical performance (tensile tests results: E ≈ 47 MPa; strain at break ≈ 1120%), making it the most suitable for soft tissue engineering. However, the selected PUR showed poor cytocompatibility, likely due to residual solvents and DBTDL. To mitigate this, various strategies were tested, including changes in the solvent/non-solvent used for PUR precipitation and purification and washing protocols. The most effective PUR collection method (i.e., precipitation in diethyl ether followed by solubilization in dimethyl sulfoxide and precipitation in MeOH) reduced Tin content from 6.2 to 2.4 mg/kg. Lastly, an additional PUR washing step in EDTA and cell culture medium yielded a biocompatible material. The selected PUR was then used to fabricate multi-layered anisotropic structures via melt extrusion AM, with the potential to better recapitulate *in vitro* the typical anisotropy of the cardiac tissue. The scaffolds possessed smooth surfaces with no observable micro-pores and maintained a relatively uniform strand diameter of approximately 400 μm, although some strands exhibited variability in diameter and minor deformations, attributable to the printing process (warping effect). The scaffolds were also surface functionalized with a fibronectin (FN) coating obtained through plasma polymerization of acrylic acid and covalent bonding via carbodiimide chemistry. X-ray photoelectron spectroscopy confirmed the effective grafting of FN. The amount of grafted FN ranged between 18 and 25 μg/cm^2 and the coating showed prolonged stability in aqueous solution at 37 °C.

In the second part of the study, methacryloyl gelatin (GelMA) was synthesized with a *ca.* 100% degree of methacryloylation and used to prepare hydrogels with varying polymeric concentrations. GelMA hydrogels prepared at 5% and 10% w/v exhibited E values of *ca.* 8.5 kPa and 55 kPa, respectively, aligning with values for healthy young and mildly fibrotic cardiac tissues. GelMA gels at 10% w/v concentration displayed a denser, more fibrous structure, consistent with the increased collagen crosslinking observed in fibrotic cardiac tissue. These GelMA formulations effectively replicated the mechanical and structural features of both healthy and fibrotic or aged cardiac tissue, confirming their suitability to design *in vitro* models of physiological and pathological cardiac conditions.

Lastly, biphasic constructs were prepared by depositing the GelMA aqueous solution (at 5% or 10% w/v) into the surface-functionalized PUR scaffolds, followed by photopolymerization (365 nm, 10 mW/cm², 40s). Compression tests were limited in assessing mechanical anisotropy due to the presence of an external polymeric shell surrounding the internal anisotropic structure of the constructs. Force spectroscopy data evidenced that the hydrogels progressively increased their stiffness from the pore center toward the scaffold filament, highlighting a combined influence of the 3D framework and the GelMA component in shaping the mechanical behavior of the biphasic constructs. Scanning electron microscopy images revealed interconnected porous architectures, likely promoting cell proliferation by improving nutrient, gas and waste transport. In aqueous conditions at 37 °C, GelMA gels showed a burst weight loss within the first 24h of incubation, followed by stability for up to 14 days, with 40–50% dried weight loss. Cell-based models were then constructed utilizing GelMA hydrogels as carriers for hiPSC-CMs and HCAECs (80:20 ratio). The young and aged models (PUR scaffold loaded with GelMA at 5% or 10% w/v, respectively) exhibited distinct cellular behaviors, with the aged model showing a more advanced cell maturation. Multi-omics analyses revealed that the aged model presented cellular features indicative of cardiac injury, mitochondrial dysfunction, proteolysis, lipid degeneration, as well as disruptions in contraction and inflammatory pathways. Lastly, the young tissue model was placed in a bioreactor with electrical stimulation (1 Hz) and perfusion (200 μl/min). Culturing under external stimulation enhanced both cell viability and maturation within the model.

Overall, the developed 3D models can faithfully replicate both young and mature cardiac tissue *in vitro*. Although they were not specifically used for chemical cardiotoxicity testing, these models have great potential as tools to study how drugs affect the heart throughout various aging stages.