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Designing 3D bioengineered *in vitro* cardiac tissue models as reliable tools for the evaluation of chemical cardiotoxicity

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Humans are continuously exposed to a huge amount and a variety of chemicals. Animal tests are the gold standard for toxicity testing. However, they often fail in finely replicating the real physio-pathological scenario, and their use is associated with ethical issues. Thus, there is an urgent need for Novel Approach Methodologies providing more reliable and robust methods for toxicity assessment. In this regard, 3D *in vitro* tissue models more efficiently mimic the native human environment and bring clear ethical advantages [1]. To design a 3D bioengineered tissue model, the 3D matrix used to guide cell behavior, extracellular matrix (ECM) production, and new tissue formation should replicate the architecture and composition of the native tissue. In this context, the proper selection of the biomaterial, the fabrication method, and the functionalization protocol is pivotal. In cardiac tissue engineering, elastomeric polymers are required as constituents of porous structs replicating *in vitro* myocardium architecture and mechanical properties. Moreover, surface functionalization with cardiac ECM proteins can be exploited to replicate *in vitro* the biochemical cues present in the native tissue. In this work, we exploited the versatility of poly(urethane) (PU) chemistry to design a plethora of polymers with a wide range of physico-chemical properties with the final aim to identify the most suitable one to be used to fabricate a 3D framework for *in vitro* cardiac tissue model development. PUs were synthesized using poly(ε-caprolactone) diol (2000 Da), an aliphatic diisocyanate, and different chain extenders (e.g., 1,4-butanediol, 1,8-octanediol, L-lysine ethyl ester, N-Boc serinol) [2]. PU physico-chemical properties were thoroughly characterized, with particular attention to their mechanical performances. In detail, 1,8-octanediol gave a PU with higher elongation at break (ε%, ≈ 30-40%), while 1,4-butanediol resulted in a more brittle and stiffer PU. Conversely, N-Boc serinol gave a PU with around 150 MPa Young's Modulus (E) and ε% of approx. 150%. L-lysine ethyl ester instead provided the resulting PU with an elastomeric behavior (E and ε% of around 10 MPa and 700%, respectively) that made it the optimal one to fabricate constructs replicating the cardiac ECM. This PU was then microfabricated by thermally induced phase separation and melt extrusion additive manufacturing, resulting in porous structs with aligned pores along a preferred direction and multi-layered matrices with a 0°/90° lay-down pattern, respectively. The structs were surface plasma treated in the presence of acrylic acid to expose -COOH groups and then grafted with ECM proteins (laminin, LN, fibronectin, FN) through carbodiimide chemistry [3,4]. Colorimetric assays and spectroscopic analyses proved the successful functionalization of the constructs. Lastly, the developed *in vitro* replicas of the cardiac ECM were seeded with cardiac cells (rat neonatal cardiomyocytes, CMs, cardiac progenitor cells, CPCs) to establish cardiac tissue models. LN promoted CPC proliferation and expression of differentiation markers for CMs, endothelial, and smooth muscle cells. CMs exhibited a high survival rate and stable beating. RT-PCR evidenced a relevant modulation of cardiac muscle, hypertrophy-specific, and metabolism-related genes at 14 days of cell culture. Our results proved the potential of the developed structs as cardiac tissue models with tunable structural, mechanical, and biochemical features. Such models will allow the investigation of physio-pathological processes and cardiotoxicity testing.

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