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Engineering of poly(urethane)-based porous constructs for cardiac tissue model design.

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***Abstract*—Humans are continuously exposed to a wide variety of chemicals. Animal and bi-dimensional (2D) *in vitro* models currently represent the gold standard for chemical toxicity assessment, although they do not adequately reproduce the real human scenario, thus resulting in poorly predictive results. Conversely, three-dimensional (3D) bioengineered *in vitro* models hold the potential to closely represent the human physio-pathological milieu, enabling a more reliable evaluation of chemical toxicity. Among human tissues/organs, the myocardium is a target organ for chemical- and drug-induced toxicity. In this work, a custom-made poly(ester urethane) was synthesized and used as raw material to fabricate a platform of multi-layered structures with different geometrical features through melt extrusion additive manufacturing. The designed matrices were also surface functionalized with proteins to improve their biomimesis of the native cardiac extracellular matrix. The physico-chemical properties of the constructs proved their suitability for the development of *in vitro* bioengineered cardiac tissue models, enabling the investigation of physio-pathological processes and cardiotoxicity testing.**

***Keywords*—poly(urethane)s, *in vitro* cardiac tissue models, additive manufacturing, surface functionalization.**

I. INTRODUCTION

Drug development, chemical toxicity assessment, and disease modeling heavily rely on the use of animal models and two-dimensional *in vitro* models [1]. However, these models exhibit several drawbacks that make their outcomes poorly predictive of the real human scenario. Two-dimensional models are extremely simple and do not allow to replicate the complexity of human tissue structure [2]. Conversely, animal models present high management costs and ethical issues. Furthermore, due to interspecies differences, animal models are unlikely to faithfully replicate the human physio-pathological behavior. The concordance between toxicity levels evaluated in humans and laboratory animals is around 71% when both rodent (mice and rats) and non-rodent (dogs and monkeys) models are used. This concordance is reduced to 43% using only rodents as animal models [3].

The consequences of these issues are diverse and heterogeneous. The selection effectiveness of preclinical trials is reduced: only 11.8% of drugs entering clinical trials reaches approval. In addition, there are considerable cases of approved drugs showing undesirable toxic effects. For instance, during the last decade eight non-cardiovascular drugs have been reported to induce ventricular arrhythmia and sudden death, and thus they have been withdrawn from the market [4]. Furthermore, the proper and reliable assessment of the toxicity of chemicals and chemical mixtures is another key target to achieve, with important health, social and economic repercussions. Until 2008, only 5,000 chemicals out of 100,000 present on the western market had been subjected to an approved analysis process due to the limitations of two-dimensional and animal models [3].

In this complex scenario, the development of reliable 3D bioengineered *in vitro* models is of central importance since they can be very useful tools for overcoming the limitations of previously adopted approaches [5] [6]. In particular, the design of reliable 3D *in vitro* models of the cardiac tissue in gaining increasing interest in the research community. On the PubMed database the number of publications related to "cardiac tissue models" is exponentially increasing over time, with around 4000 publications in 2022. This trend can be correlated to the very high social and economic impact of heart diseases, that are responsible for around 17.5 million deaths worldwide each year [7]. Secondly, the high complexity of the cardiac tissue and the very limited possibility of modelling it with two-dimensional or animal models clearly prove the urgent need for reliable 3D bioengineered *in vitro* models of the cardiac tissue in different physio-pathological conditions and at different degrees of ageing [8].

Based on these premises, in this work, an *ad-hoc* synthesized poly(ester urethane) (PU) was microfabricated into 3D porous constructs by melt extrusion additive manufacturing and superficially modified with vacuum plasma treatment followed by immobilization of an extracellular matrix (ECM) protein (e.g., fibronectin, laminin) using the carbodiimide chemistry. Complete physico-chemical characterization of the developed constructs was performed, demonstrating the suitability of the designed matrices for the establishment of bioengineered cardiac tissue models.

II. MATERIALS AND METHODS

The PU used in this work to fabricate the 3D structures was synthesized using poly(ε-caprolactone) diol (2000 Da) as macrodiol, 1,4-butanediisocyanate as diisocyanate and L-lysine ethyl ester as chain extender [9]. The synthesis was performed under inert atmosphere through a two-step procedure according to an already optimized protocol [9]. The physico-chemical properties of the synthesized material were studied using infrared (IR) spectroscopy, size exclusion chromatography (SEC), and tensile tests. Rheological (dynamic temperature ramp tests, frequency sweep tests) characterization, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were also conducted to investigate the thermal and thermo-mechanical properties of the PU. A melt extrusion additive manufacturing (AM) system was employed to realize three-dimensional multi-layered structures with different patterns (e.g., square and rhomboid grid). A vacuum plasma treatment in the presence of acrylic acid vapor was then employed to superficially modify the

structures and expose carboxyl groups subsequently used to covalently decorate the surface of the scaffolds with ECM proteins (laminin, LN, fibronectin, FN) through the carbodiimide chemistry [13] [14]. Optical microscopy was employed to analyze the morphology of the structures. IR spectroscopy, X-ray photoelectron spectroscopy (XPS), contact angle measurements and colorimetric assays (e.g., toluidine blue assay, bicinchoninic acid assay) were used to characterize the surface chemistry of the scaffolds.

III. RESULTS

The synthesis procedure of the PU was successfully carried out. IR spectroscopy evidenced the appearance of the typical peaks of the newly formed urethane and urea bonds: at *ca.* 1630 cm^{-1} the stretching vibration of carbonyl groups, at 1535 cm^{-1} the concurrent C-N stretching and N-H bending vibrations, and at 3340 cm^{-1} N-H stretching vibration. SEC analyses revealed a number average molecular weight of around 70 kDa, with a polydispersity index of 1.4. The PU showed an elastic behavior (Young's Modulus around 10 MPa, strain at break of hundreds %) suitable for the engineering of *in vitro* models replicating the cardiac tissue. Rheological characterization coupled with DSC and TGA results demonstrated the suitability of the material for processing via melt extrusion additive manufacturing. Dynamic temperature ramp tests evidenced a transition from a rubbery towards a viscous behavior around 135 °C while DSC thermogram evidenced a complete melting of both soft and hard domains at *ca.* 140 °C. Frequency sweep tests evidenced a shear thinning behavior at these temperatures, which is useful for PU processing through melt extrusion AM. Furthermore, TGA highlighted that PU thermal degradation started at 230 °C, thus enabling polymer microfabrication within a wide range of temperature.

Multi-layered structures with different patterns were successfully fabricated (Fig. 1) with high resolution and good fidelity to the CAD models.

Fig. 1: Multi-layered structures with different patterns fabricated by melt extrusion additive manufacturing.

The scaffold fibers had a diameter of about 500 μm, Plasma surface modification and subsequent surface binding of LN or FN was validated with contact angle measurements (decrease in static contact angle from around 90° to 60°), spectroscopic analyses and colorimetric assays. The bicinchoninic acid assay confirmed the presence of a protein coating of 10-15 μg/scaffold. Tests with human induced pluripotent stem cell-derived cardiomyocytes are currently in progress. Preliminary data have showed good cell viability accompanied by cell adhesion to the polymeric structures.

IV. DISCUSSION

The search for adequate biomaterials and processing techniques is a fundamental step towards the development of three-dimensional cardiac models. Biomaterials not only perform a passive function of structural support for the cellular counterpart of the model, but directly influence cellular development with their chemical and mechanical properties [8]. Biomaterials can be of synthetic or natural origin. The main synthetic polymers studied in the cardiac tissue engineering field are poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL). Particularly interesting polymers of natural origin are, for example, collagen, gelatin, fibronectin, alginate, fibrin, chitosan and hyaluronic acid [10]. Synthetic materials are cheap, easily processed and tunable in terms of mechanical and chemical properties, but they do not exhibit the biochemical features of the native ECM. Natural materials more faithfully reproduce the biochemical properties of the extracellular matrix, but they show high production costs, poor physico-chemical stability, inadequate mechanical properties, limited processability, and higher batch-to-batch variability. To integrate the characteristics and overcome the limits of both categories of materials, numerous bioartificial systems and functionalization procedures have been studied in the literature [11]. The manufacturing techniques of polymer scaffolds are numerous. The conventionally used techniques are difficult to control and do not allow to obtain structures with controlled properties. Examples of these techniques are induced phase separation, emulsion freeze-drying, particle leaching and solvent casting [10] [12]. In order to produce structures with more precise geometries, spinning and additive manufacturing techniques can be used. In particular, additive manufacturing allows the fabrication of complex three-dimensional structures with controlled geometry [7]. The main AM techniques are inkjet, laser and extrusion-assisted bioprinting and melt extrusion AM. These technologies present different advantages and disadvantages. Inkjet-based bioprinting allows to reach very high resolution (< 100 μm) and to combine different biomaterials. The main disadvantage is the low structural and mechanical stability of the constructs. Laser-assisted bioprinting is a nozzle-free technique enabling high cell viability. However, this system is very expensive and the size of the fabricated structures is small for clinical applications. Lastly, extrusion-assisted bioprinting is a versatile technology that could be used with a wide range of different biomaterials. It is also economic, but the resolution that can be achieved is quite low (> 100 μm) [7]. One of the

most important drawbacks of bioprinting relies in the limited mechanical properties of the printed structures, that should also exhibit proper features (e.g., gas/molecule exchange) allowing cell encapsulation within the filaments. Furthermore, the printing of large-size structures is very complicated [13].
The melt extrusion AM technique is based on the use of thermoplastic polymeric materials. These materials exhibit a wide variety of mechanical and chemical characteristics, which can be combined to control the characteristics of the printed structures. The printing process is extremely fast, economical and highly repeatable [14]. The main limitations are the impossibility of printing cells due to the high temperature and the thermoplastic nature of the printed materials, and low resolution. However, the thermoplastic polymeric structures could be used as support to realize large-size constructs and to overcome the stability deficiency of biomaterial inks and bioinks [15]. The cells can then be grown on the printed structures or inserted using bioinks placed inside the constructs [16] [15]. For instance, a elastomer composed of poly-[glycerol sebacate] and poly-[ε-caprolactone] was printed with melt extrusion AM technique to realize scaffolds for cardiac application. The scaffolds had Young's modulus very similar to native myocardium (≈1 MPa) and the size of the fibres was about 300 µm. *In vitro* tests with rat cardiomyocytes, H9c2, showed good cell viability after 72 h, while *in vivo* tests confirmed a positive impact of the scaffold on cardiac tissues [17]. In another study, a complex and large structure (62.4 mm × 62.4 mm × 21.3 mm) was realized using polyvinyl alcohol and a melt extrusion AM system. The structure was filled with a hydrogel containing rat cardiomyocytes, H9c2, and umbilical vein endothelial cells. The survival rate of the cells was about 90% after 14 d of cell culture.

Within this context and with the aim to further advance the current state of the art, in this work we designed 3D structures based on a custom-made PU as potential frameworks for 3D cardiac tissue model establishment. A polyurethane consisting of a thermoplastic polyester and an amino acid-derived chain extender was used to combine the processability characteristics of synthetic polymers with the bioactive features of natural materials. Furthermore, it presented suitable mechanical characteristics for the fabrication of porous constructs with Young's modulus matching the requirements for cardiac tissue engineering applications [10]. The melt extrusion AM technique was then used to fabricate PU-based porous structures, relying on its high reproducibility, simplicity and low costs. The fiber size was consistent with typical values of the melt extrusion AM technology. To improve the resolution of the fibers it is possible to modify the printing process parameters. Surface modification resulted in a functional protein coat for cellular tests. The study of the mechanical properties of the printed structures and tests with human induced pluripotent stem cell-derived cardiomyocytes are currently underway. The synthesized PU and the used AM process have manifested excellent characteristics for the realization of large sized cellularized constructs.

V. Conclusion

The customized polyurethane showed excellent mechanical characteristics, good processability through melt extrusion additive manufacturing technique and suitability for surface functionalization with proteins. The developed matrices could pave the way towards the design of *in vitro* bioengineered cardiac tissue replicas. Interestingly, the chemical and technological versatility of polyurethane biomaterials, additive manufacturing techniques and surface functionalization procedures could be exploited to finely tune the physico-chemical properties of the fabricated structs, enabling cardiac tissue modelling in different physio-pathological conditions and at different degrees of ageing. In the end, this would significantly advance our knowledge of the mechanisms underpinning ageing and pathology onset and progression, while providing reliable tools for cardiotoxicity testing.

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