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Multifactorial approaches to enhance maturation of human iPSC-derived cardiomyocytes.

**Kornél Kistamás¹, Anna Müller¹, Suchitra Muenthaisong¹, Federica Lamberto^{1,2},
Melinda Zana¹, Martin Dulac³, Filipa Leal⁴, Ali Maziz⁵, Pedro Costa⁴, Eiva Bernotiene⁶,
Christian Bergaud⁵, András Dinnyés^{1,2,7}**

¹ BioTalentum Ltd., Aulich Lajos Str 26, H-2100, Gödöllő, Hungary

² Department of Physiology and Animal Health, Institute of Physiology and Animal Nutrition, Hungarian University of Agriculture and Life Sciences, Páter Károly Str 1, H-2100, Gödöllő, Hungary

³ OSPIN GmbH, Helmholtzstraße 9, 10587 Berlin, Germany

⁴ Biofabrics Lda, Rua Alfredo Allen 455, 4200-135 Porto, Portugal

⁵ Laboratory for Analysis and Architecture of Systems - French National Centre for Scientific Research (LAAS-CNRS), 7 avenue du Colonel Roche, F-31400, Toulouse, France

⁶ Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Santariškių g. 5, LT-08406 Vilnius, Lithuania

⁷ Department of Cell Biology and Molecular Medicine, University of Szeged, Dugonics Sq 13, H-6720 Szeged, Hungary.

Correspondence

Dr András Dinnyés

BioTalentum Ltd., Aulich Lajos Str 26, H-2100, Gödöllő, Hungary

Email: andras.dinnyes@biotalentum.hu

ORCID ID: 0000-0003-4705-5327; kornel.kistamas@biotalentum.hu

ORCID ID: 0000-0001-8640-7802; anna.muller@biotalentum.hu

ORCID ID: 0000-0001-9354-0513; suchitra.polgari@biotalentum.hu

ORCID ID: 0000-0001-9974-1594; federica.lamberto@biotalentum.hu

ORCID ID: 0000-0001-5725-1300; melinda.zana@biotalentum.hu

ORCID ID: 0000-0003-3246-3707; martin.dulac@ospin.de

ORCID ID: 0000-0002-0510-1287; filipa.leal@biofabrics.com

ORCID ID: 0000-0002-2564-1109; amaziz@laas.fr

ORCID ID: 0000-0003-3114-9417; pedro.costa@biofabrics.com

ORCID ID: 0000-0002-1529-5513; eiva.bernotiene@imcentras.lt

ORCID ID: 0000-0003-0093-9600; bergaud@laas.fr

ORCID ID: 0000-0003-3791-2583; andras.dinnyes@biotalentum.hu

ABSTRACT

Cardiovascular diseases are the leading cause of death worldwide. Several strategies – small molecules, gene therapy, surgeries, cardiac rehabilitation – have been developed to treat patients with cardiac disorders or to prevent the disease. A novel promising tool is the application of induced pluripotent stem cell-derived human cardiomyocytes; however, their clinical application is hampered by their fundamentally immature characteristics. Although several approaches were recently proven to support certain aspects of cardiac maturation, it has been discovered that any kind of stimulus itself is insufficient to induce proper maturation of these cardiomyocytes. Therefore, researchers have developed multifactorial approaches linking biotechnology, life sciences and material sciences toolsets. In this review, we summarise the current state-of-the-art methodologies in cardiac differentiation and maturation with the application of biocompatible materials and automated cell culture systems combined with various electrical, mechanical, topological, and biochemical stimuli.

Keywords

hiPSC-CM, human induced pluripotent stem cells, cardiomyocyte, heart-on-a-chip, electrospun scaffolds, biocompatible materials.

Running title

Multifactorial maturation of human iPSC-derived cardiomyocytes.

Abbreviations

AHA, American Heart Association; Bry, brachyury; CAD, computer-aided design; CAST, Cardiac Arrhythmia Suppression Trial; CF₃SO₃, trifluoromethanesulfonate; CM, cardiomyocyte; cTnT, cardiac troponin T; CVD, cardiovascular disease; DBS, dodecylbenzenesulfonate; DAPI, 4',6-diamidino-2-phenylindole; DKK1, dickkopf Wnt signalling pathway inhibitor 1; EB, embryoid body; ECM, extracellular matrix; EMAPS, electromechanoactive polymer-based scaffolds; EOMES, eomesodermin; ESC, embryonic stem cell; FDA, US Food and Drug Administration; GelMA, gelatin methacryloyl; hCF, human cardiac fibroblast; HCl, hydrochloric acid; hESC, human embryonic stem cell; hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte; HUVEC, human umbilical vein endothelial cells; iPS, induced pluripotent stem cell; LiTFSI, lithium bis(trifluoromethanesulfonyl)imide; MEA, multielectrode array; MEF, mouse embryonic fibroblast; Mesp1, mesoderm posterior bHLH transcription factor 1; MPS, microphysiological systems; NCD, non-communicable disease; NME, New Molecular Entity; NRVM, neonatal rat ventricular myocyte; PANI, polyaniline; PCL, poly(ϵ -caprolactone); PEDOT, poly(3,4-ethylene-dioxythiophene); PEG, polyethylene glycol; PGA, poly(glycolic acid); PGS, poly(glycerol sebacate); PLA, poly(lactid acid); PLCL, poly(L-lactide-co- ϵ -caprolactone); PLGA, poly(lactide-co-glycolide); PPy, polypyrrole; rBMSC, rat bone marrow derived mesenchymal stem cells; REALM-DCM, Study of ARRY-371797 in Patients With Symptomatic Dilated Cardiomyopathy; SWORD, Survival with oral d-sotalol Trial; VEGF, vascular endothelial growth factor; WHO, World Health Organization.

Highlights

- Improving the maturation of hiPSC-CMs is of great importance as pluripotent stem cell-differentiated cardiomyocytes initially demonstrate a foetal phenotype.
- A biomimetic environment is needed with multiple stimuli to achieve higher maturation of hiPSC-CMs.
- Electrospun scaffolds could recapitulate the natural 3D environment for tissue engineering.
- Electrically conductive polymers are essential for cardiac tissue engineering.
- 3D printing and bioreactors support the long-term cell culture of hiPSC-CMs.

1. INTRODUCTION

Cardiovascular diseases (CVDs) remain the most prevalent concern in healthcare. CVDs affect the heart and vasculature, including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism, heart failure, atrial fibrillation, heart attack, stroke, or high blood pressure. According to the World Health Organization (WHO), more than two-thirds of all global deaths are caused by non-communicable diseases (41 million annually, NCDs). Almost half of the NCDs are caused by CVDs (17.9 million), far more than by cancer (9.3 million), respiratory diseases (4.1 million) or diabetes (1.5 million), making CVDs the chief killer diseases globally (one-third of all deaths) [1-2]. There have been calls for advocacy programs to raise awareness among policymakers, as cancer is still assumed as a superior health concern, despite the fact that 113 million people live with CVD within the member countries of the European Society of Cardiology [3]. Apart from CVD morbidity and mortality, CVDs represent a significant financial and economic burden worldwide. Estimates from the European Heart Network show more than 200 billion EUR cost for the EU economy annually [3], while the American Heart Association (AHA) reported costs of 555 billion USD (2016) within the US [4]. AHA estimates the projection of CVD costs by 2035 to an astonishing 1.1 trillion USD yearly, while nearly half of the population will live with some form of CVD [4]. Medical costs break down into direct (hospital and health care, medical services and devices, prescription drugs and nursing costs) and indirect costs (productivity loss, work loss).

Although there is an urgent and growing need for novel drug discoveries, therapies and treatments, infamous clinical trial failures obstruct the way of scientific and medical breakthroughs. This is best illustrated by the disappointing outcomes of the Cardiac Arrhythmia Suppression Trial (CAST) and Survival with oral d-sotalol (SWORD) trials, where the treatments increased the risk of mortality in patients assigned to the drug treatment [5-8]. Aside from safety concerns, trials might also fail due to lack of efficiency, as it was the case in a recent study [9-10]. Further concerns are raised as many of the failed, completed or uncompleted trials remain unpublished [11].

Consequently, only a handful of drugs targeting CVDs entered the market in recent years. More importantly, failure of a trial in the most expensive phase 3 carries an enormous economic burden [12]. In a 2018 research, 54% of the investigated phase 3 trials failed, 57% due to the lack of efficacy, 17% due to safety concerns while 22% because of commercial

reasons [13]. **Figure 1** shows the proportion of cardiac drugs progressing through the drug discovery pipeline compared to treatments for other diseases. There have only been 7 cardiovascular drugs approved by the FDA and introduced to the market over the last decade.

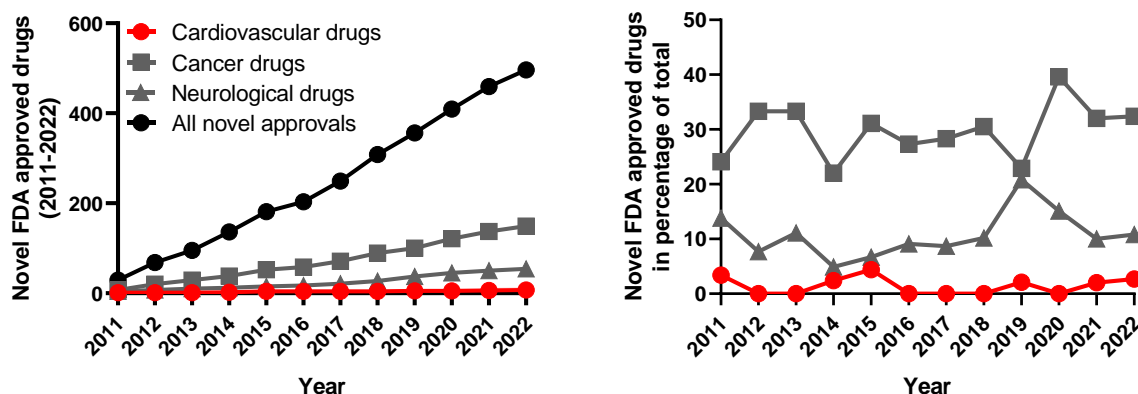


Figure 1.: Disheartening trends in the cardiac drug discovery pipeline.

(A) A cumulative comparison of the newly approved drugs by the US Food and Drug Administration (FDA), New Molecular Entity (NME) Drug and New Biologic Approvals in the field of cardiovascular, neurologic and cancer treatments between 2011-2022 [14-25]. Over the last decade, a strikingly low number of FDA-approved cardiac drugs entered the market, which is more prominent in the light of cancer drugs. (B) Drugs for treating neurological disorders and cancers take up ~10% and ~30% of the new approvals, respectively, while cardiovascular drugs are responsible for only ~1%.

Due to the above-mentioned factors, there is currently an urgency in the scientific community for a more relevant human-based model that can more accurately predict drug safety and efficacy before these candidates enter clinical trials. Novel approaches to manage CVDs are greatly needed, including regenerative medicine. In order to progress in modelling or regeneration, a deeper understanding of the developing healthy cardiac tissue niche is required. The native environment of cardiomyocytes (CMs) is comprised of the extracellular matrix (ECM) which is a complex network of proteins and other biomolecules. The composition of the cardiac ECM must dynamically adapt to provide the required signals at each step of maturation from immature foetal CMs to mature adult CMs. From a structural and functional point of view, mimicking this dynamic environment is generally accomplished with scaffolds combining fibrillar synthetic materials – which recapitulate the architecture and elasticity required for the matrix – and biomaterials (collagen, laminin, fibronectin, etc.) to promote the interaction between the CMs and the matrix. Intrinsically, this approach allows for more favourable conditions for maturation, but they are far from being sufficient. In fact, coupling with chemical, electrical, and mechanical stimuli is inevitable to reach a high degree

of maturation and implies the development of more sophisticated devices, in which the scaffolds are integrated, allowing a fine-tuning of these coupled stimuli over several weeks.

Here we review the current state of the cardiac differentiation protocols, a fruitful connection of biological and material sciences focusing on the state-of-the-art maturation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) lead by multiple stimuli.

2. ESSENCE OF HIPSC-CMS AND HIPSC-CM DIFFERENTIATION

Currently, preclinical *in vitro* or *in vivo* models barely recapitulate the human body. These experimental setups include 2D or 3D cell cultures or animal models. Many of the animal models apply small rodents, as their size and maintenance are less challenging compared to large mammals, and there is a huge variety of genetically modified laboratory rodents and small mammals for disease modelling. On the other hand, a significant limitation comes from the animals' anatomy, physiology, and pathophysiology. The behaviour of these cells and organs leads to interspecies differences and might markedly differ from that of the human. During excitation-contraction coupling of the CMs, the intracellular calcium concentration must elevate to initiate the contraction, while removing calcium from the cytosol is equally essential to let relaxation occur [26]. In this regard, rat or mouse models behave fundamentally differently from the ferret, dog, cat, guinea pig or human ventricles [27]. The positive staircase phenomenon (Bowditch effect) – a built-in autoregulatory mechanism of the human heart [28-29] – where elevated heart rate increases the force of contraction to increase cardiac output, is missing from rat hearts [30]. Large mammal models, such as canine or sheep hearts are frequently used in experimental cardiology [31-32], however, the lack of human models (due to obvious reasons) is yet to be solved.

The application of hiPSCs has become a powerful tool since the discovery of the technology for reprogramming somatic cells to a pluripotent state, thus providing an essentially unlimited cell source of human origin [33]. The potential to foster pluripotent stem cells led to the *in vitro* development of various cell types of the human body, such as muscle cells, neuronal cells, pancreatic cells, skin cells or even organs [34]. This customisable *in vitro* modelling creates an opportunity to emulate tissue-, disease-, or even patient-specific systems, while the application of iPSCs bypasses the ethical issues with the use of embryonic stem cells (ESCs).

The hiPSC-CMs are, therefore, potential cardiac models in healthy and diseased states and can serve as a human-relevant platform for developing cardioprotective drugs and *in vitro* cardiotoxicology methods. There is a growing interest towards hiPSC-CMs, which is represented by the escalated number of publications referring to hiPSC-CMs (**Figure 2**).

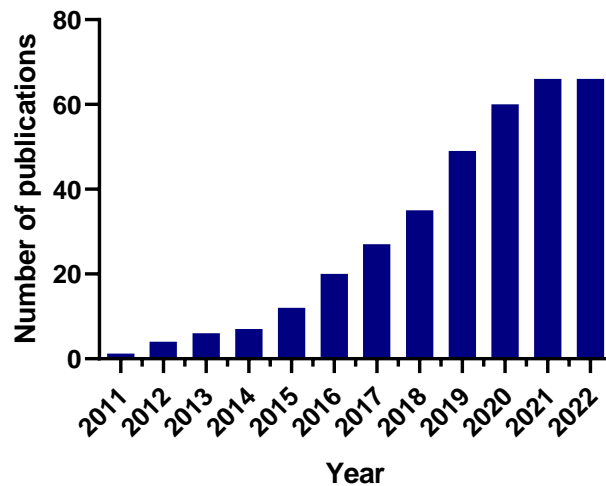


Figure 2.: Increasing number of hiPSC-CM publications.

There has been a tremendous increase in scientific publications focusing on hiPSC-CMs since the description of somatic cell reprogramming and cardiac differentiation. Data were obtained from PubMed, limited to 2011-2022; keywords were “hiPSC-CM”, the widely accepted and the most frequently used abbreviation for human iPS-derived cardiomyocytes.

There have been several approaches to generate CMs from stem cells over the last decade. During this process, the cells are committed to the mesodermal and cardiac cell fate. In the first studies, low yield and purity of beating cells were generated from ESCs in suspension cultures or with hanging-drop techniques, relying on spontaneous differentiation [35-36]. Subsequently, research groups applied increasingly sophisticated, chemically defined protocols on hiPSCs, shifting from sera, proteins and growth factors (DKK1, activin A, BMP4, foetal bovine serum, etc.) to the application of small molecules (CHIR99021, IWR-1, IWP-2, etc.), resulting in better controlled and more economic cell culture protocols [37-40].

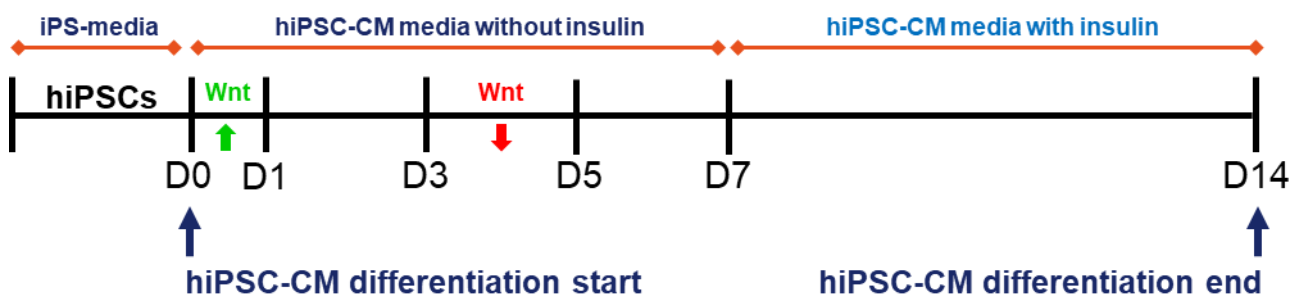


Figure 3.: A typical ventricular hiPSC-CM differentiation protocol.

Recent studies about cardiac induction apply protocols with evolved cell culture media and small molecules decently timed throughout the first two weeks of cardiac development. Most protocols use CHIR99021 and IWP-2 to activate or inhibit the Wnt/ β -catenin pathway, respectively. This figure shows a representative example of a typically applied cardiac differentiation protocol.

The most frequently used hiPSC-CM differentiation (Gi/Wi protocol [37]) with the application of small molecules to generate ventricular subtype of cells via the biphasic modulation of the Wnt/ β -catenin pathway is shown on **Figure 3**. Briefly, Wnt activation is achieved by a transient application of the glycogen synthase kinase 3 β (GSK3 β) inhibitor (e.g. CHIR99021), which is followed by the administration of a Wnt inhibitor (e.g. IWR-1). The role of this biphasic modulation of the Wnt/ β -catenin signalling is to recapitulate those processes during embryonic heart development. Wnt regulates the mesoderm posterior bHLH transcription factor 1 (Mesp1) through the induction of mesendodermal markers (brachyury, Bry and eomesodermin, EOMES). Inhibition of Wnt *in vivo* is mediated by the Mesp1 activated dickkopf Wnt signalling pathway inhibitor 1 (DKK1) to promote the cardiac-lineage fate [41]. For a recent review, detailing the evolution of CM differentiation, see Lyra-Leite *et al* [42].

Characteristic immunocytochemistry images of hiPSC-CMs by staining early mesodermal and cardiac biomarkers are shown on **Figure 4**. Early biomarkers, Nkx2.5 and GATA4 show commitment to cardiac cell lineage from the mesoderm layer on cardiac precursor cells, while cardiac troponin T (cTnT) is a key marker for beating cardiac cells and for the development of the sarcomere structure. Co-staining with phalloidin marks the distinct f-actin expression in the hiPSC-CMs.

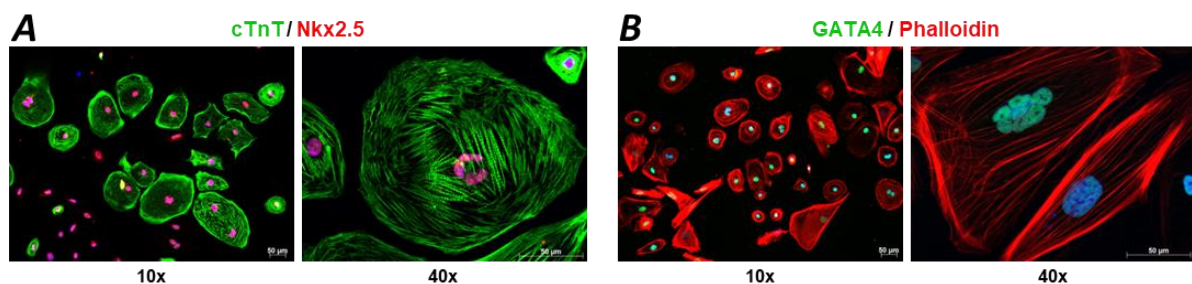


Figure 4.: Immunocytochemistry staining of D15 hiPSC-CMs.

Immunocytochemistry images show specific biomarkers for early-type hiPSC-CMs at day 15. The most frequently used marker for CMs is the cardiac troponin T (cTnT), which shows apparent striations (**A**, green) in case of the development of CM sarcomere structures. Mesodermal marker homeobox protein Nkx2.5 (**A**, red) is required for heart development. Transcription factor GATA4 (**B**, green) plays a role in the intrauterine cardiac development and drive foetal proliferation of CMs. Phalloidin staining (**B**, red) visualises the actin

filaments of CMs. 4',6-diamidino-2-phenylindole (DAPI) shows nuclear staining (**A, B**, blue). Images were taken on hiPSC-CMs generated by BioTalentum Ltd, at 10x and 40x magnification; scale bar, 50 μ m.

Besides identifying the differences within the cardiac induction techniques, it is also interesting to analyse the different protocols applied in the literature to achieve viable hiPSC-CMs, thus we present here the progress within the past 3 years. Between 2020 and 2022, 183 papers featured hiPSC-CMs, 17 out of them were review papers and 7 were computational simulations and modelling. Focusing on the original data articles, we found that the majority of the hiPSC-CM differentiation protocols (41%) applied small molecules to induce cardiac development, using CHIR99021 as the GSK3 β inhibitor to activate Wnt signalling and additionally, various forms of the Wnt inhibitors (IWP2, IWP4, IWR-1, C59, XAV939). Growth factors alone (Activin A, BMP4, bFGF) or in a combination with small molecules (CHIR99021 + Activin A) were presented in 2% and 7% of the research papers, respectively. The 2D chemically coated surfaces dominated the work, while 10% of studies were conducted in 3D arrangements (3D spheroid differentiation, growth in bioreactor tanks). Other methods (e.g. cultivation on the surface of mouse embryonic fibroblasts, MEF) were negligible in this context. Remarkably, a notable, 25% of the published papers used commercially available hiPSC-CMs. On the other hand, although commercially available cardiac differentiation kits offer a ready-to-use method, their application is not widely distributed (4% of the articles), possibly due to their relatively higher price and chemically undefined (non-disclosed) nature. **Table 1.** summarises the publications of hiPSC-CMs from 2020 to 2022.

	2020			2021			2022		
Total	59		Ref	64		Ref	60		Ref
2D - Small molecules (CHIR99021+IWP2-/IWP-4/IWR-1/C59/XAV939)	22	37%	[43-64]	26	41%	[65-90]	27	45%	[91-117]
2D - Growth factors (Activin A, BMP4, bFGF)	1	2%	[118]	2	3%	[75,119]	1	2%	[120]
2D - Combination of small molecules and growth factors	4	7%	[121-124]	6	9%	[125-130]	3	5%	[131-133]
2D - Other (MEF)	1	2%	[134]	0	0%		0	0%	
3D	7	12%	[118,135-140]	8	13%	[141-148]	3	5%	[149-151]
Review papers	5	8%	[152-156]	4	6%	[154,157-159]	8	13%	[160-167]
Computational modelling, simulations	1	2%	[168]	3	5%	[169-171]	3	5%	[172-174]
Commercially available hiPSC-CM differentiation kits	4	7%	[60,175-177]	4	6%	[79,178-180]	0	0%	
Commercially available hiPSC-CMs	17	29%	[118,181-196]	13	20%	[197-209]	15	25%	[210-224]

Table 1.: Distribution of the applied hiPSC-CM differentiation protocols.

The summary of the studies published in the last 3 years, focusing on the proportions of the different cardiac induction models. Some of the papers are represented more than once, as they contained two or more of the above listed applications. Data were obtained from PubMed, limited to 2020-2022, keywords were “hiPSC-CM”.

3. MULTIPLE APPROACHES TO LINK BIOMEDICAL AND MATERIAL SCIENCES FOR CM MATURATION, THE EMAPS-CARDIO CONCEPT

It is becoming increasingly evident that for hiPSC-CM maturation it is crucial to simulate physiological settings by recapitulating external stimuli that mimic the native microenvironment. Different mechanical characteristics of scaffold materials should be considered for engineering a functional myocardium *in vitro*, including choice of porosity, flexibility, fiber alignment, surface modification, hydrophilicity, and other techniques for the improvement of cell adherence and stimulation. The seeding cell density and the coating of the scaffolds with substances that improve cell adherence (e.g. laminin, fibronectin, Geltrex, etc.) are also of major importance to achieve a more advanced maturation stage. CMs have a range of cell–cell connections, including adherens junctions, desmosomes and gap junctions that are critical for electromechanical coupling and these interactions might be enriched at higher cell densities [225]. Furthermore, density-dependent cell alignment was also observed on a fibronectin-patterned surface [226]. To further foster hiPSC-CM maturation, engineered 3D tissues can be additionally subjected to various external stimuli, including electrostimulation, mechanical loading and incorporation of growth factors, adhesion molecules, platelet-rich plasma or other stimulatory ingredients [227]. The multiple approaches to be combined to mimic the natural environment and to foster the maturation of CMs *in vitro* are summarised in **Figure 5**.

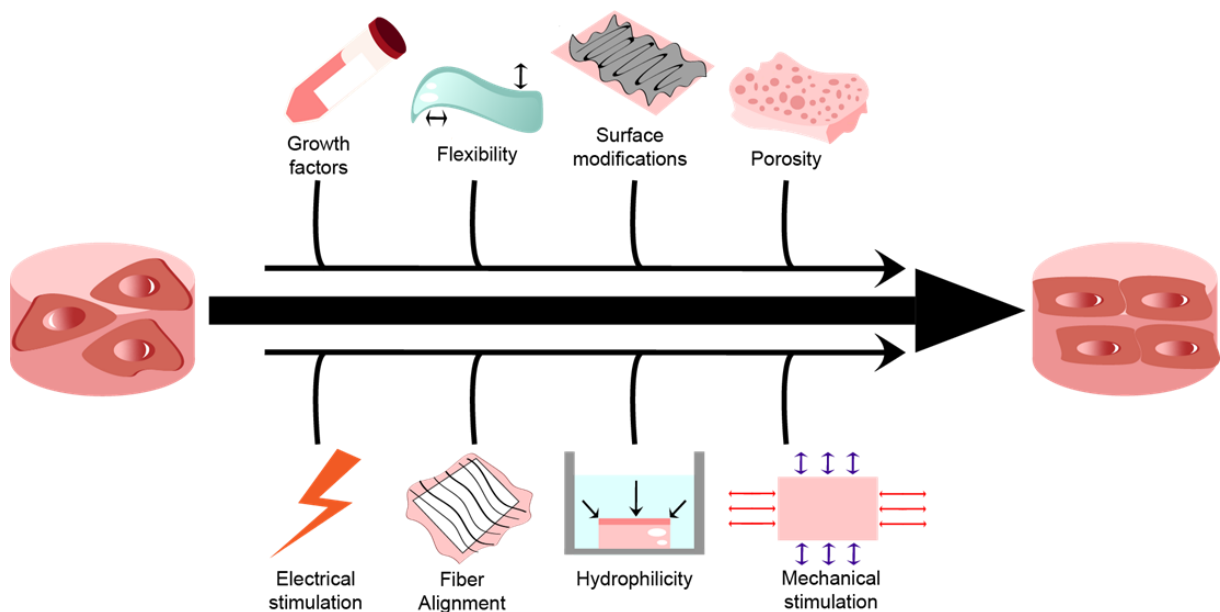


Figure 5.: Multiple approaches and their combinations to mimic the natural environment and to foster the maturation of hiPSC-CMs.

Maturation process must follow the *de novo* production of hiPSC-CMs to mimic the adult phenotype. Besides recapitulating the natural microenvironment and the ECM (i.e. topological stimulation), several stimuli are proposed to promote maturation, including mechanical, electrical, or biochemical stimulation, Optimisation of the physicochemical properties of the applied scaffolds may highly contribute to the development of the adult-like hiPSC-CMs (detailed in the text).

3.1 Mechanical properties of biomaterials to stimulate hiPSC-CM biological responses and maturation

Biomaterials used for CM maturation must simulate the natural microenvironment providing the ideal niche for the cells. The key properties of the scaffolds include biocompatibility, hydrophilicity, permeability, flexibility and the capacity to blend with other polymers [228]. Micro- (<50 μm in diameter) and macropores (>200 μm in diameter) are needed for cell penetration and diffusion of nutrients [228]. A pore size <60 μm facilitates endothelial cell penetration and colonisation, larger pores promote nutrient diffusion [229-231]. In addition, pore diameter >70 μm is needed for myocyte-penetration into the scaffold [232]. A possible solution to facilitate both might be creating scaffolds with pores of varying sizes. The end-diastolic Young's modulus (0.02-0.5 MPa) and the tensile strength (3-15 kPa) of the native myocardial tissue is relatively low, thus, the production of scaffolds with low tensile strength and mechanical properties might improve cell cultivation and contraction of the engineered tissue. On the other hand, surgical grafts are often failed due to breakdown because of the physiological loading in vivo. Based on these, intermediate tensile strength (1-2 MPa) was reported to be preferable over scaffolds with low or high tensile strength [233]. Furthermore, spring-like fibers showed increased elasticity and extensibility compared to straight fibers, suggesting a possible beneficial role in contributing to the contraction properties of the engineered cardiac tissues [232].

Scaffolds should also support aligned morphology of the fibers and the cells to mimic cardiac layout in myocardial tissue [232]. Aligned PCL fibers induced efficient hiPSC-CM alignment and improved sarcomere organisation [234]. High alignment of CMs can be achieved using micropatterns, where microcontact printing generated non-adherent polyethylene glycol (PEG) regions on Matrigel and fibronectin layered gold-coated glass slides, resulting in the highest hESC-derived CM and sarcomere alignment in the 30-80 μm gaps [235]. However, although multiple studies have demonstrated that aligned CMs may result in advanced cardiac phenotypes indicative of cell maturation, including the organisation

of sarcomeres and gene expression of sarcomere proteins, calcium handling proteins and ion channels, their calcium transient kinetics were slower than in cells grown on tissue culture polystyrenes [234].

Hydrophilic scaffolds are often used to increase cell attachment. To increase hydrophilicity of a polymer, additional surface modifications could be used, including polymer mixing (e.g. PEG, laminin fragments), coating (polydopamine coating) or plasma treatment [236-237]. Otherwise rat primary CMs prefer relatively hydrophobic surface (85 wt% P(lactide-co-glycolide) + 15 wt% poly(ethylene glycol)-poly(D,L-lactide) with a contact angle 60° [238].

3.2 Biomimetic external stimuli

Electrical stimulation can promote cardiac development and mediate the cardiac differentiation of hiPSCs by activating multiple pathways related to calcium signalling through the activation of Ca²⁺/PKC/ERK pathways [239-240]. Electrical pacing parameters also depend on the maturation stage – early maturation mimics foetal heart rate (110-160 bpm or 2-2.5 Hz) [241], while adult heart pacing could be mimicked by electric pulses of 2.5 V/cm at 5 ms pulse duration and at 1 Hz frequency [242]. Stimulation by electric pulses of 1-1.5 V/cm, 5 Hz induces spontaneous beating of hiPSC-derived embryoid bodies (EBs) [239]; one week of such stimulation resulted in upregulation of cardiac gene expression (Nkx2-5, GATA4, α MHC, CX43) and reduced expression of pluripotent markers (KLF4 and OCT4) in differentiated hiPSC-CMs [239]. Connexins are concentrated at the longitudinal ends of the cells during CM maturation (i.e. polarised). Engineered tissue rings were made of fibrinogen, Matrigel, thrombin and 1-day old Sprague Dawley rat ventricular or atrial CMs. Length, width, height, surface area, and volume of electrically stimulated CMs were not different compared to postnatal rat CMs on day 12. Stimulation increased the expression of CX43 (the membrane is stained positive for Cx43), but Cx43 polarisation was statistically lower than in adult CMs [243].

The application of electric stimuli has been shown to favour the formation of sarcomeres and improve the spontaneous and synchronous beating of cardiac cells. Composite conductive (2D and 3D) substrates for CMs were shown to decrease the duration of maturation and improve contractile and electrical properties, as demonstrated through the application of various approaches, including chemically synthesised conjugated polymers, single-walled carbon nanotubes or engineered nanowired 3D cardiac patches [244-246].

Besides electrical stimulation, mechanical stimulation can also be applied to the cells. Mechanical stimulation has been considered for cardiac maturation to mimic the mechanical forces that the cardiac tissue undergoes throughout development, as previously reviewed by Carlos-Oliveira et al. [247]. Hydrostatic stress is caused by blood pressure after birth, and it directly depends on the distance from the right atrium (the greater the distance, the higher the pressure due to the greater column). The hydrostatic pressure is compared to the right atrial pressure and it may vary from -10 mmHg in the head to +90 mmHg in the veins of the feet [248]. External mechanical loading mimics *in vivo* forces that affect CMs undergoing cardiac maturation during organ development. Physiologically, shear stress is generated by blood flow and promotes the formation and maturation of heart chambers, CM proliferation, and valvulogenesis. In embryos, shear stress is about $\sim 0.4\text{--}5$ dyne/cm² while in adult human organisms it is 10 to 26 dyne/cm² in arteries and 1.4–6.3 dyne/cm² in veins [249]. Shear stress is necessary for atherogenesis, while disturbed shear stress (low or unsteady blood circulation) is related to atherosclerotic plaque formation [250-251]. During CM maturation *in vitro* shear stress can be induced using a bioreactor system with laminar flow and physiological strain [252]. Cyclic strain mimics regular heart beating; physiological parameters are 5-10% elongation at 1 Hz frequency [253-254]. During hypertension, a 15% higher strain was estimated compared to conditions at normal blood pressure. Under hypertensive conditions, the amount of strain has been estimated to be 15% higher than under normotensive conditions. Cyclic strain can be applied on a monolayer (rat bone marrow-derived mesenchymal stem cells, rBMSC), seeded on elastic silicone membrane or on PLCL scaffolds with ES-derived CMs. In both experiments, 10% elongation resulted in increased GATA-4, Nkx2.5, Cardiac α -MHC, β -MHC, MEF2c gene expression [255-256].

Different mechanical forces can be combined to mimic the natural mechanical forces, for instance, pulsatile flow-induced shear stress and physiological strain. In a mouse cell experiment, optimised conditions of 1.48 mL/min pulsatile flow with 5% cyclic strain at 0.33 Hz, from day 2 to day 12, not only induced cardiac gene expression (Actc1, Myh6, Myh7) in mESC-derived cells but also had the highest number of sarcomeric myosin-positive (MF-20) cells [252].

3.3 Integration of multiple approaches

Heart-on-a-chip technology has recently become an increasingly popular tool, combining controllable culture conditions, and the application of various stimulations and continuous parameter readouts [159]. This review discusses the multiple approaches that have

been shown to link biomedical and material sciences for CM maturation. However, currently only a few studies investigate their simultaneous application [159,257]. The most promising examples include a fully 3D printed and instrumented microphysiological device that provides continuous electronic readout of the contractile stress of multiple laminar cardiac micro-tissues [257].

In our recent, Horizon 2020 EU Framework Programme, the ElectroMechanoActive Polymer-based Scaffolds for Heart-on-Chip (EMAPS-Cardio) project, we aim to establish a realistic organotypic model both for healthy states and diseased models. A biomimetic microenvironment will be produced to grow and mature hiPSC-CMs, by combining biotechnology and biomaterial sciences. The system is equipped with beating hiPSC-CMs and sensors to closely monitor cell culture conditions and physiological parameters during the growth and maturation of the cells in a scaffold-cell arrangement placed either in a bioreactor or in an electromechanoactive polymer-based plate environment. Electrical, mechanical, topological, and biochemical stimulations allow us to examine physiology and develop realistic disease models in a human-based *in vitro* microenvironment (**Figure 6**). The following sections provide details on the components of this complex system.

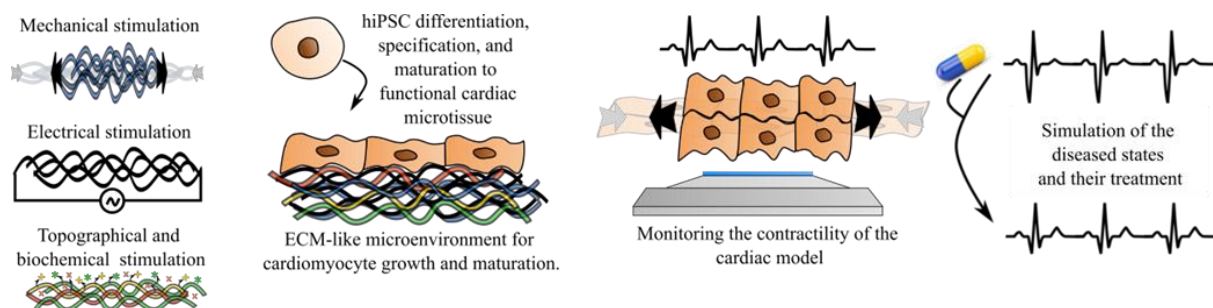


Figure 6.: The EMAPS-Cardio concept for matured human cardiomyocytes.

The concept aims to produce a platform for the growth and maturation of cardiac microtissues for realistic organotypic models in healthy and diseased states. Electromechanoactive polymer-based scaffolds (EMAPS) are combined with bioactive membranes to optimise the differentiation of the hiPSC to adult-like CMs. The cell-scaffold kit is then transferred to a 3D printed device and/or bioreactor (microfluidic system) to enable continuous monitoring of cardiac function. This platform aims to serve as the most realistic human heart model to help better understand heart diseases and cardiotoxicology screenings.

4. ELECTROSPINNING AND DRUG RELEASE FROM SCAFFOLDS

Several requirements – some of which are particular to the maturation of cardiac cells and tissues – must be met by artificial scaffolds in order to replicate the natural 3D environment for tissue engineering. While electrospinning is a straightforward and economical method that can meet most of these requirements, it does presuppose several specific material advancements, particularly in terms of physicochemical and biological qualities. By delivering a high voltage to a polymer solution that is drawn into a jet or stream and driven toward a collector, the electrospinning method is able to create polymer micro-nanofibers. The electrostatic forces stretch the electrically charged polymers to create fibers.

The first crucial step is to replicate the fibrillary structure of ECM, which directs cell arrangement and promotes the elongated, anisotropic shape of CMs by creating long, straight sarcomeres that control cell contractility [258]. With the best design, high-speed spinning drums or mandrel collectors can produce aligned electrospun nanofibrous scaffolds [259]. These scaffolds have continuous micro- and nanofibers, a high surface-to-volume ratio, a high porosity level, and a heterogeneous distribution of pore sizes, which are characteristics of natural ECM [260].

Another critical factor is the mechanical characteristics of the applied scaffolds. To support the myocardium's spontaneous contractions, ideal scaffolds should have a Young's modulus of tens to hundreds of kPa. Since many electrospun synthetic polymers, including PCL, PLA, PLGA, etc., have Young's moduli in the MPa range and yield points below 1%, they are not suitable for the maturation of cardiac cells. The necessary mechanical properties can be achieved using electrospun elastic polymers like PLCL, PGS, or natural polymers (chitosan, elastin, silk fibroin, gelatin), or mixtures of elastic and natural polymers (PGS/gelatin, PLCL/silk fibroin, etc.) [259]. A thorough analysis of the various polymers utilised for cardiac tissue engineering can be found in the review paper of Kitsara et al. [260].

Electrically conductive scaffolds have been found to naturally improve the synchronisation of cardiac cell beating in the myocardium due to electrical signal transmission through the scaffold [261]. In fact, the excitation-contraction coupling is facilitated, and intercellular gap junctions are better established in CMs cultivated on conductive 2D and 3D substrates. To create elastic conductive scaffolds, conductive fillers like carbon nanotubes, graphene, reduced graphene oxide, metallic nanoparticles, etc., or conducting polymers (CPs, see next section) are frequently utilised [262-263]. Another method involves utilising chemical or electrochemical deposition to coat the surface of the scaffolds with a layer of CPs.

Additionally, the application of electric stimuli that accelerate the maturation of heart tissues can be facilitated using conductive scaffolds. The structural and mechanical properties of myocardial function were shown to be enhanced by electrical stimulation [264]. Furthermore, the stimulation regime and duration significantly increased adult-like gene expression profiles in the stimulated tissue [265].

The modification of the scaffolds from both the outside and the inside of the fibers is another crucial topic. This can be carried out utilising physical, chemical, and biological techniques either during the construction of the scaffolds or afterwards. It can significantly enhance biocompatibility and how cardiac cells interact with scaffolds by improving cell adhesion, proliferation, etc. The simplest method is probably a post-construction treatment, which involves chemical alteration of the surface fibers by adding functional groups or changing those that already exist. For instance, to improve the interaction with negatively charged sticky proteins like fibronectin or laminin, positive charges can be produced using hydrochloric acid (HCl) [261]. Dopamine polymerisation on scaffold surfaces has also been investigated to enhance cardiac cell adhesion [266]. It is also an interesting alternative to modify surfaces using plasma [267]. The scaffolds can also be incubated in a solution containing biomolecules, such as fibronectin, chitosan, collagen, etc., to enhance their biological characteristics [268]. Other popular techniques include blend, coaxial, and emulsion electrospinning with biological additions [269]. They can be optimised to encapsulate substances like growth factors for drug release and have the advantage of causing a change within the electrospun fibers. Short sticky peptides from growth factors (e.g. Neuregulin-1) or laminin [270] can be encapsulated in polymer blends [271]. An effective technique for CM maturation involves the construction of core-shell fibers for the regulated release of active molecules with the timed secretion of suitable growth factors [272] and small molecule inhibitors [273]. In another study, core-shell electrospun nanofibers were created to enclose a bioactive form of VEGF [274].

Two additional points must be considered. The first one concerns the degradation of the scaffolds over time which can be a major issue. When employed for cardiac cell maturation, the properties of the scaffolds must be stable over several weeks or months, and using natural polymers can be problematic from that point of view. Finally, cell seeding can be extremely challenging and should be regarded as a crucial stage in the formulation of experimental protocols depending on the type of scaffold and the design of the device with which the scaffold is to be employed. The cell seeding must be preceded with potent

sterilisation procedures without harming the scaffold material (e.g. gamma irradiation, UV exposure, chlorine dioxide, ethylene oxide). The successful attachment of hiPSC-CMs to the scaffold surface also highly depends on the physicochemical properties (e.g. dry or pre-moistened, surface tension, electrospinning material, coating with ECM proteins) of the sheet and on the alignment of the nano- or microfibers (see previous chapter), while a higher amount of the cell culture media during cell seeding might reduce the density of cells on the scaffold surface and result in an unwanted attachment to the bottom of the culture dish. The cell seeding might be followed by slow migration into the deeper layers of the scaffolds, which – without functional vascularisation – limits the thickness of the samples due to the slowed or hampered diffusion of oxygen and nutrients [275]. In contrast to hydrogels, the cell distribution on porous scaffolds might also be inhomogeneous [276].

5. CONSTRUCTION OF ELECTROMECHANOACTIVE POLYMER-BASED SCAFFOLDS

The development of electrically conductive scaffolds based on CPs for cardiac tissue engineering has grown significantly in the last ten years [262]. Because of their intrinsic electronic and ionic conduction, they promote cell differentiation, proliferation, and maturation. They also favour synchronous contractions among cardiac cells increasing the expression of genes that encode key proteins involved in the regulation of the electrophysiological properties of CMs [261,277]. Moreover, electrical stimulation can be delivered through a CP-based scaffold with noticeable beneficial effects on differentiation and maturation (**Figure 7**) [278].

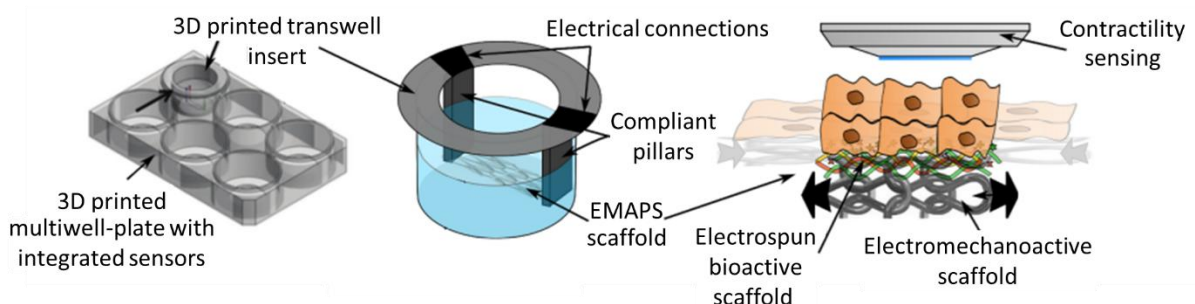


Figure 7.: Platform for integrated stimulation and continuous sensing.

Schematic illustration of the platform where electrical stimulation is combined with continuous sensing. During the fabrication process of the EMAPS (Electromechanoactive polymer-based scaffolds) actuators, their integration with bioactive scaffolds is achieved by

electrospinning of aligned microfibers followed by the vapour-phase and electrochemical deposition of conductive polymers and finished with the application of bioactive scaffolds, leading to a bioactive bilayer actuator. The prepared 3D printed transwell inserts are then ready to be integrated into a conventional cell culture plate for hiPSC-CM cultivation and evaluation of readouts.

The most commonly used CPs for tissue engineering are polyaniline (PANI), poly(3,4-ethylene-dioxythiophene) (PEDOT), and polypyrrole (PPy) with electrical conductivities up to 105 S/cm [262,279]. PPy is usually considered the most promising for cardiac tissue engineering because of its ease of synthesis, high conductivity in physiological conditions, and well-known biocompatibility. PPy has been coated onto electrospun scaffolds or incorporated into the fibers, and both ways have been used with various materials, including silk fibroin [280-281].

Their surface properties can be easily tuned through the binding of bioactive molecules using adsorption, entrapping, or covalent binding [278]. For example, PPy surfaces can be doped with peptides from laminin (p20 and p31) to enhance the differentiation of ESCs [282] and PPy-incorporated cardiogel was shown to result in a substantial improvement of cardiac functional properties [283]. The key issue with these approaches is to keep the biomolecules attached without impacting their bioactive roles.

Apart from being conductive, an exciting feature of CPs is that they can be used for electromechanical actuation in biomedical applications [284]. The actuating principle of CPs resembles the physiological muscles, in terms of being wet, soft, and electrically stimulated and controlled. CPs undergo a volume change upon electrochemical oxidation and reduction by applying a low potential usually under 1 V. The volume change is predominantly caused by the motion of ions and solvent in and out of the polymer matrix during electrochemical cycling. This process can produce significant stress from 3-5 MPa and strain of a few percent. Furthermore, the use of scaffolds with aligned fibers will translate the volume change into a uniaxial strain leading to a more efficient mechanical stimulation of cardiac cells.

However, for an efficient electromechanical actuation, CPs often have too high Young's modulus ($E \approx 100$ -300 MPa) and low elasticity due to their brittle backbones. Here, the conductive scaffold, directed towards cellular mechanical stimulation, requires specific features. These include having elastic properties, and a low Young's modulus so not to hinder the electrically driven volume change in the actuating polymer. Moreover, particular attention

must be paid to obtain a strong adhesion of the deposited CPs to avoid delamination and creep during actuation.

To overcome these issues, different approaches have been reported to enhance the properties of CPs using appropriate dopants, or by incorporating the CP into cross-linked elastic scaffold structures. Different doping ions can be employed to enhance the electrical and electromechanical properties of CPs. In the case of PPy, for example, dodecylbenzenesulfonate (DBS), trifluoromethanesulfonate (CF₃SO₃), or Lithium bis(trifluoromethanesulfonyl)imide (LiTFSI) have been used as dopants and will change the electromechanical properties of the PPy. It was shown that PPy doped with DBS exhibits the best performance for electromechanical actuation [285]. PPy electrochemical actuators can operate in physiological liquids which makes them extremely relevant for the electromechanical stimulation of cardiac cells [286-287].

Electrochemical synthesis of CPs is the preferred means for obtaining redox-actuating materials, due to its simplicity, good selectivity, reproducibility, and high actuating performances. The electrochemical deposition route requires the attachment of a separate electrically conductive electrode to the scaffold (e.g. metal coatings) followed by the deposition of the main actuating layer. This solution is, however, hampered by additional manufacturing costs and delamination issues. Temmer et al. developed a metal-free two-step combined chemical-electrochemical polymerisation strategy to prepare the actuating material on a non-conductive membrane [288-289]. A first chemically synthesised CP layer (synthesised through dry vapor phase deposition or wet chemical route) forms an electronic electrode surface, allowing the following electrochemical deposition of the main actuating layer of the CP. This synthetic pathway ensures good adhesion between the scaffold membrane and the CP. Harjo et al. used the combined polymerisation process to coat PPy on the surface of electrospun gelatin–glucose nanofibers to prepare a linear actuator [285]. The results of the electromechanical actuation showed that the obtained actuators have good actuation strain ($\approx 20\%$) and stress (≈ 0.15 MPa) with high stability [285].

Gelmi et al. followed the combined chemical-electrochemical polymerisation strategy of PPy to develop electrospun conductive scaffold mats that can deliver electrical as well as mechanical stimulation to hiPSC-CMs upon electrochemical oxidation and reduction processes of PPy in cell media [287]. The material is comprised of a poly(lactic-co-glycolic acid) (PLGA) fibrous scaffold coated with the CP PPy. The authors reported an increased expression of cardiac markers for stimulated compared to unstimulated protocols. Similarly,

E. Kerr-Phillips reported cross-linked electrospun rubber fibers with electrochemically controllable pore sizes using the CP PEDOT [290].

6. MICRODEVICES SUPPORTING hiPSC-CMS, COMMERCIALY AVAILABLE DEVICES AND CHALLENGES

The 3D cultivation of hiPSC-CMs towards their mature stage is considered a very promising model for drug safety testing as it mimics the complex *in vivo* architecture, notably by reproducing closely the ECM microenvironment [291]. As described above, the maturation process of hiPSC-CMs requires the help of several crucial biophysical stimuli, namely, mechanical, electrical, topological and biochemical cues [292]. Therefore, there is a rising demand for the development of proper cultivation platforms, able to provide such biophysical stimuli and environment simultaneously [293]. Moreover, an adequate sensing of hiPSC-CMs autonomous contractility and excitability is of major importance as they are crucial indicators to evaluate CM functions during various treatments [294]. An overlook at the existing market permits a glimpse of a few commercially available devices that can cultivate hiPSC-CMs.

Devices based on microelectrode array (MEA) measurement of CM monolayer are often used for electrophysiological measurement including drug testing for pharmacology evaluation to characterise the electrophysiological changes and to detect functional cardiotoxicity [295-296]. These are powerful tools for toxicity screenings with several plate formats available (up to 96-well plates). These field potential or impedance recording machines provide readouts such as electrical activity, contractility and cell viability [295]. Despite the functional readouts, the environment provided by MEAs is fundamentally different from the *in vivo* ECM, while important stimuli (e.g. mechanical, topological) are missing.

The currently available CM culturing devices in the market offer a number of essential stimuli and sensing for the physiological cultivation and maturation of hiPSC-CMs [297-301]. These engineered heart tissues and heart-on-a-chip devices can be supported by hydrogel substrates and equipped with flexible wires to record active force and passive tension, calcium transients and action potentials combined with contractility sensing, to generate 3D-engineered cardiac tissues from hiPSC-CMs, used subsequently for pharmacology studies [302].

Bioreactors offer promising options to reach the expected degree of complexity and maturation of hiPSC-CMs [303-305]. The possibility to apply an extensive array of actuators and sensors, which can be manipulated by automated systems allows close monitoring of the long-term cell cultures. Such automated cell culture system can provide control over the bioprocesses in a sterile environment without the need for manual interventions, thus reducing the risk of contaminations. Besides sterile surfaces, long-term hiPSC-CM cultivation requires additional well-controlled parameters, such as optimal temperature, stable level of nutrients in the cell culture medium, removal of by-products and control of pH, i.e. environmental control. Fluidics systems of bioreactors allow continuous replacement of the cell culture medium to avoid sudden changes (i.e. nutrient shock) [303], while administration of certain drugs is also available for treatments and measurements. Furthermore, the modularity of the bioreactors and precision techniques such as 3D printing make it possible to tailor the tissue chamber for specific purposes, including integration of different stimuli [306-308]. **Figure 8** illustrates a possible design of a tissue chamber inside a bioreactor adapted to hiPSC-CMs cultivation and maturation. The tissue chamber can be equipped with a pneumatic membrane and elastic (or even bioactive) scaffolds, delivering the topological and biochemical cues to the hiPSC-CMs. The mechanical stimulation is generated by the expansion of the pneumatic membrane, controlled by a flux of air underneath the membrane. Electrodes integrated into the tissue chamber are responsible for the current injection to evoke the electrical stimulation, which can support T-tubule formation and positive force-frequency relationship to promote the maturation of hiPSC-CMs, while a precise camera system evaluates autonomous contractions of the hiPSC-CMs. Finally, automation of the bioreactor controls and monitors the events in the tissue chamber and fluid path for a distinct long-term culture.

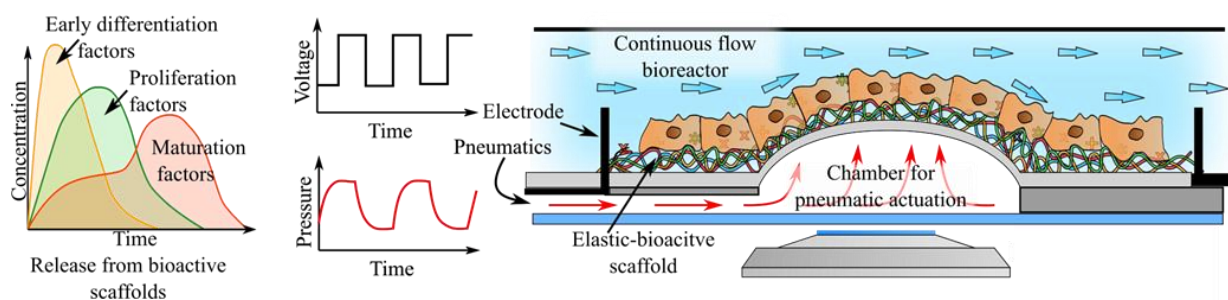


Figure 8.: Bioreactor platform for long-term cell culturing of hiPSC-CMs.

Schematic illustration of a bioreactor platform where elastic bioactive membranes are integrated into the tissue chamber of the bioreactor, allowing biochemical, mechanical, topological, and electrical stimulation of hiPSC-CMs to achieve higher maturation.

7. ADDITIVE MANUFACTURING FOR IN VITRO CARDIAC TISSUE MODELLING

Additive manufacturing, also known as 3D printing, is a cutting-edge technology that produces objects layer-by-layer from a computer-aided design (CAD) model [309]. Their use in tissue engineering has increased over the years, both in the production of biomaterials for the repair and regeneration of tissues and in *in vitro* tissue modelling. Despite this, no 3D printed devices for cardiac tissue modelling were found to be commercially available, so an overview of the literature was carried out to investigate the current state-of-the-art.

Engineering cardiac tissues still represent a significant challenge due to its heterogeneous, anisotropic, complex, and hierarchical structures, which is critical for transitioning from pre-clinical trials to clinical practice. Conventional cell culture methods (i.e. 2D and static culture) play a vital role in research but have several limitations since they inaccurately represent the tissue *in vitro*. Bioprinting has emerged to overcome these limitations by spatially controlling the 3D structures of artificial tissues and, thus, fabricating reproducible tissues. Indeed, this technique is able to create unidirectionally aligned 3D microfibers (anisotropic arrangements) for cardiac tissue engineering by varying the distance between the fibers deposited in the y direction while keeping the distance in x direction constant (**Figure 9A**) [310]. A simple approach for generating endothelialised myocardium tissue involves (1) printing, using the aforementioned arrangement, a bioink containing human umbilical vein endothelial cells (HUVECs) embedded in a mixture of alginate and Gelatin methacryloyl (GelMA), (2) organisation and migration of the HUVECs into a layer of confluent endothelium surrounding the microfibers (**Figure 9B**) and (3) seeding and culture of CMs into the bioprinted scaffold [310]. By using this procedure, cardiac tissue demonstrated improved cellular alignment and synchronous spontaneous beating after 48h of culture. Notably, the incorporation of the bioprinted tissue on a microfluidic device, i.e. endothelialised-myocardium-on-a-chip, prompted its use as a platform for cardiovascular drug screening. Despite all that, the published *in vitro* model has some limitations, namely the use of animal CMs and the lack of a hollow lumen in the endothelialised microfibers which affects the functionality of the endothelium.

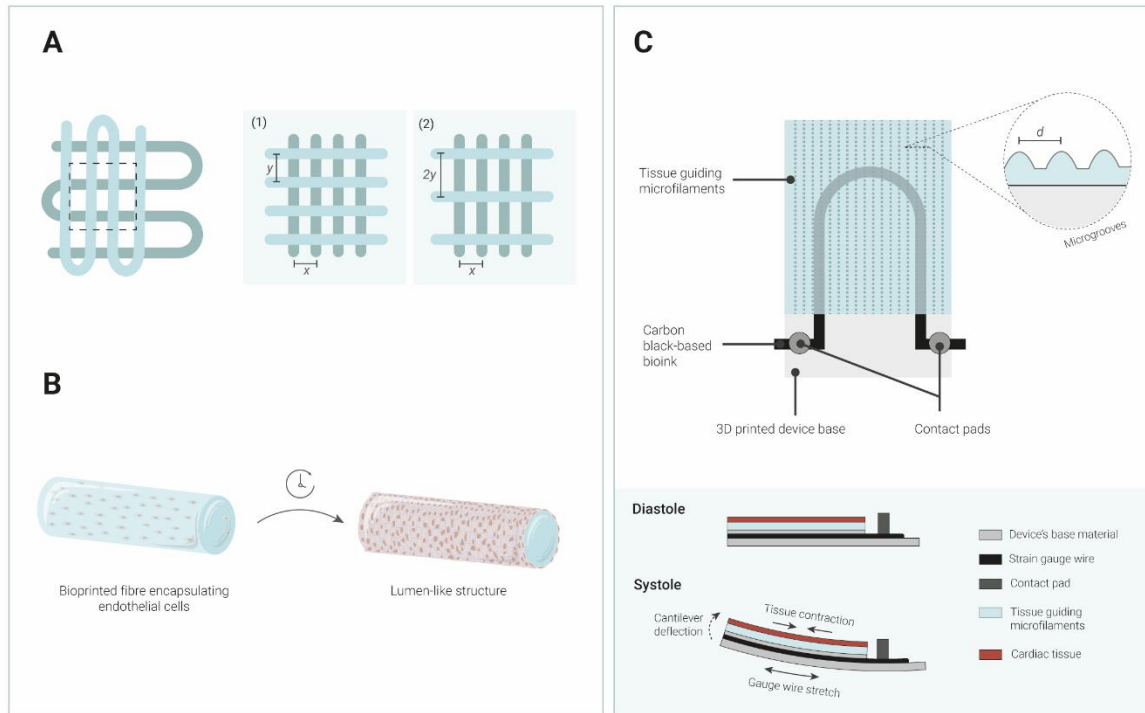


Figure 9.: Schematics of additive manufacturing methods to achieve organised and functional cardiac tissue.

(A) Scheme of the y -distance variation technique to obtain an anisotropic arrangement of the cardiac tissue: (1) arrangement unlikely to improve unidirectional cell alignment; (2) pattern capable of promoting cell alignment. (B) Organisation and migration of endothelial cells to the fiber surface forming a lumen-like structure. (C) Illustrative representation of the 3D printed sensor and its principle. The contraction of the cardiac tissue deflects the cantilever, stretching the strain gauge which, in turn, generates a resistance alteration that is proportional to the contractile tension of the tissue. d – spacing between filaments.

The orientation of the CMs is of great importance for heart contraction and, consequently, for the efficient pumping of blood. Additive manufacturing is ideal to produce guiding scaffolds for this application, as it allows control over the architecture of the object. The study of Iwanaga et al. demonstrated this by achieving oriented and synchronous CM-contracting tissue constructs after 3D printing fiber-like acellular scaffolds and culturing them with CMs [311]. Another study printed a microscopic, hollow, cylindrical, and acellular scaffold to guide the tissue structure composed of hiPSC-CMs [312]. This structure was incorporated into an organ-on-a-chip, and the scaffold was able to sustain substantial tissue contractions and allowed flow generation without collapse over the course of a week. The use of 3D printed acellular scaffolds with subsequent cell culture may be particularly beneficial compared with bioprinted cellular ones, as the mechanical and thermal stresses that occur in the bioprinting process can affect cell viability and proliferation [313].

The bioprinting process involves the optimisation of several aspects, especially the bioink's properties. Cell spreading, proliferation and morphogenesis are highly dependent of the stiffness and porosity of the bioink [314]. Studies on microfluidic bioprinting of 3D cardiac tissue constructs found that HUVECs had improved cell spreading on constructs with an elastic modulus of around 40 kPa and when embedded in a bioink with a lower elastic modulus, cells maintained a spherical shape after 10 days of culture [315]. Evidently, the optimal bioink's mechanical properties differ for each cell type and, therefore, must be tailored according to the specific tissue to be mimicked [314].

Cellular co-culture systems have been widely used to study and design complex multicellular models by mimicking *in vivo* cell-cell interactions and cell signalling [316]. Native tissues are composed of several cell types that work synergistically to achieve the full functionality of that tissue or organ, making it, therefore, crucial to replicate this multicellularity in *in vitro* models. In a study by Alonzo et al., organoids containing human CMs, microvascular endothelial cells and cardiac fibroblasts were bioprinted to resemble the *in vivo* myocardium more closely and investigate the role of microgravity in disease initiation and development [317]. They showed that the interaction between CMs, endothelial cells, and fibroblasts has effects on the cardiac remodelling process and cardiac function during development and disease. In addition, the 3D printed constructs resembled the striated structure of *in vivo* cardiac tissue at day 21, and exhibited high maturity and complexity, indicating that the scaffold supported the growth and development of myocardial tissue.

Although extensive efforts have been made towards the bioprinting of cardiac constructs, most studies still use animal primary cells, or human cell lines [310-311,317]. Human stem cells are a fundamental resource for a physiologically relevant *in vitro* system, but their combination with bioprinting technology still remains a challenge, mainly because human stem cells eventually lose cell elongation, differentiation and functionality [318-319]. Overcoming these challenges, i.e. the successful bioprinting of hiPSC-CMs with enhanced cell differentiation, distribution, and functionality, will increase the speed and reduce the cost of drug testing by limiting number of cells required and will improve the clinical relevance of these tests by replacing the animal models which are not faithfully representing the human physiology [319].

The majority of the current microphysiological systems (MPS) lack integrated sensors and their fabrication involves multi-step lithographic methods. 3D printing can solve this exhaustive manufacturing by producing the different parts in a single or just a few

programmable steps. This was demonstrated in a study by Lind et al., where a fully printed MPS with soft strain gauge sensors for the continuous electronic readout of the contractile stress of human cardiac tissue was obtained using six functional inks [257]. In seven automated sequential steps, the device was produced and contained three main structures: the strain gauge wire, the tissue guiding microfilaments and the electrical leads and contact pads (**Figure 9C**). The results showed that microfilaments guided the CM self-assembly into anisotropic tissues and that strain gauges provided reliable readings of tissue contractile stress, thereby indicating great potential for *in vitro* tissue engineering and drug screening tests. Yong et al. developed a similar approach, but instead of manually seeding the cells, they bioprinted a bioink composed by hiPSC-CM and human cardiac fibroblasts (hCFs) in parallel to the bi-pillar anchors and onto the sensor [320]. This platform enables high-throughput screening, can be connected wirelessly to a smartphone, and can monitor the contractile force and beat rate of the engineered cardiac tissue. Clearly, simultaneously printing sensors and cardiac tissue constructs bring advantages in terms of reproducibility, control over tissue structures, versatility, customisation, and real time measurements. In another study, Wang et al. designed a bioprinted cardiac tissue construct integrated with a 3D printed micro-spring to measure contractile forces directly [321]. Using this force sensor, the researchers were able to monitor the contractile force generated by the tissue construct and assess the effect of drug dosage on the contractile force of the bioprinted heart tissue. In addition, a PCL frame was 3D printed to act as an anchoring point to attach the cell-laden hydrogel and facilitate the development of muscle fibers as well as guide their alignment. Overall, a uniformly organised, centimetre-scaled and aligned cardiac tissue was designed, bearing great potential for pharmaceutical and regenerative medicine applications.

8. CONCLUSIONS

The aim of the recent studies is to develop cellular systems in microenvironments that serve as more accurate human-relevant *in vitro* models to increase the efficacy and translational value of drug development and cardiotoxicity safety screenings. Besides generating human-based platforms, an outstanding benefit lies in the compliance with the principles of the 3Rs (Replacement, Reduction and Refinement in animal research) as millions of laboratory research animals are used in preclinical studies. These developments are corroborated by an urgent need for appropriate *de novo* CMs due to the limited

regeneration capabilities of the heart. The production of *de novo* CMs is solved to date, however, there are cumbersome attempts to bring these cells to the desired maturity status.

There have been several attempts to develop the proper structural basis for the hiPSC-CMs to grow and age, from electrospun scaffolds, heart-on-a-chip devices and muscular thin films through cardiac cell sheets and engineered heart tissues to the de- and recellularised heart models. The construction of these systems provides physical support and contact to the cells, while additional stimulations help the long-term maturation goals by electrical, mechanical, or biochemical cues. On top of that, besides *in vitro* experiments and developments, validation of clinical-grade hiPSC-CMs is crucial to prepare these cells for clinical use and transplantation into patients with CVDs.

The use of additive manufacturing strategies in cardiac tissue engineering is promising; however, the functionality and complexity of these tissues are still insufficient to replicate native tissue accurately. Undoubtedly, this technology is in its infancy and challenges such as the low resolution of printing machines, reproducibility, and the lack of robust myocardium-targeted bioinks need to be overcome before the devices can enter the market. Nevertheless, as technology evolves, further development of additive manufacturing strategies and their integration with innovative biomaterials and stem cell therapy is expected, which could foster the development of more complex tissues with broader applicability in medical, pharma, and other fields.

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CRedit AUTHORSHIP CONTRIBUTION STATEMENT

Kornél Kistamás: Conceptualisation, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualisation, Supervision. **Anna Müller:** Formal analysis, Investigation, Data Curation. **Suchitra Muenthaisong:** Investigation. **Federica Lamberto:** Investigation. **Melinda Zana:** Writing - Review & Editing, Supervision. **Martin Dulac:** Writing - Original Draft. **Filipa Leal:** Writing - Original Draft. **Ali Maziz:** Writing - Original Draft. **Pedro Costa:** Writing - Original Draft, Supervision. **Eiva Bernotiene:** Writing - Original Draft, Supervision. **Christian Bergaud:** Writing - Original Draft, Funding acquisition, Supervision. **András Dinnyés:** Writing - Review & Editing, Supervision, Funding acquisition.

DISCLOSURE OF INTEREST

A. Dinnyés is founder and CEO of BioTalentum Ltd. K. Kistamás, A. Müller, S. Muenthaisong, F. Lamberto and M. Zana are employed by BioTalentum Ltd.

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1. Dr. Nazha Hamdani
Department of Cellular and Translational Physiology, Institute of Physiology, Ruhr University Bochum, 44801 Bochum, Germany
nazha.hamdani@ruhr-uni-bochum.de
2. Dr. Christian Hellmich
Institute for Mechanics of Materials and Structures, TU Wien (Vienna University of Technology), Karlsplatz 13/202, 1040 Vienna, Austria
Christian.Hellmich@tuwien.ac.at
3. Dr. Maria Kitsara
Technology Transfer Consultant at PRAXI Network/Foundation for Research & Technology-Hellas (FORTH), Greece
kitsara.m@gmail.com
4. Dr. Marco Rasponi
Biomechanics Group, Department of Electronics, Information and Bioengineering, Politecnico di Milano, Italia
marco.rasponi@polimi.it
5. Dr. Mark Mercola
Department of Medicine and Cardiovascular Institute, Stanford University School of Medicine, Stanford Cardiovascular Institute, 240 Pasteur Drive, Module 3300, Stanford, CA 94304, US
mmercola@stanford.edu

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