EXPERT OPINION

Successes and future outlook for microfluidics-based cardiovascular drug discovery

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Introduction: The greatest advantage of using microfluidics as a platform for the assessment of cardiovascular drug action is its ability to finely regulate fluid flow conditions, including flow rate, shear stress and pulsatile flow. At the same time, microfluidics provide means for modifying the vessel geometry (bifurcations, stenoses, complex networks), the type of surface of the vessel walls, and for patterning cells in 3D tissue-like architecture, including generation of lumen walls lined with cells and heart-on-a-chip structures for mimicking ventricular cardiomyocyte physiology. In addition, owing to the small volume of required specimens, microfluidics is ideally suited to clinical situations whereby monitoring of drug dosing or efficacy needs to be coupled with minimal phlebotomy-related drug loss.

Areas covered: In this review, the authors highlight potential applications for the currently existing and emerging technologies and offer several suggestions on how to close the development cycle of microfluidic devices for cardiovascular drug discovery.

Expert opinion: The ultimate goal in microfluidics research for drug discovery is to develop 'human-on-a-chip' systems, whereby several organ cultures, including the vasculature and the heart, can mimic complex interactions between the organs and body systems. This would provide *in vivo*-like pharmacokinetics and pharmacodynamics for drug ADMET assessment. At present, however, the great variety of available designs does not go hand in hand with their use by the pharmaceutical community.

Keywords: cardiovascular, drug discovery, lab-on-a-chip, microfluidics

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1. Introduction

Cardiovascular system integrates three basic functional parts: a pump (heart) that generates the driving pressure to circulate the fluid portion (blood) through a set of vessels around the body (the vasculature). Functionally, the most important elements of the heart include cardiomyocytes, as well as fibrous skeleton and specialized cellular electrical conductance paths. Blood is a complex tissue composed of multiple cell types, including erythrocytes, immune cells and platelets, and acellular plasma in which several clinically important protein pathways take place, including coagulation, fibrin polymerization, complement activation and fibrinolysis. The strategic interface between blood and other tissues occurs at the level of vascular endothelium and is involved in multiple cell–cell interactions with blood cells, including platelets and immune cells, as well as with the above-mentioned protein pathways, whereas subendothelial cell types, such as vascular smooth muscles, respond to many environmental stimuli and regulate the vascular tone.

All the elements of the cardiovascular system are receptive to hemodynamic forces such as pressure and flow. Cardiomyocytes, blood cells and endothelial cells are all continuously exposed to shear stress, which regulates voltage-dependent

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Article highlights.

- The suitability of microfluidics for cardiovascular drug discovery lies in its ability to perform analysis under small volumes and controlled flow rate while also allowing generation of complex channel geometries and network of chambers for multi-cell and organotypic culture.
- Over the last several years, an unprecedented number of innovative microfluidic designs, such as endothelialized microfluidic platforms or heart-on-the-chip systems, with increasing bio-mimicry and high-throughput capabilities, have been developed.
- There is still an appreciable discordance between the needs of pharmaceutical industry to employ high-throughput quasi-*in-vivo* models derived from human tissues and the modest level of application of the available microfluidic platform in drug discovery pipelines.
- Specific strategies to close the development cycle of microfluidic platforms for cardiovascular drug discovery involve facilitating commercialization, encouraging open source publishing and in-house device application for drug testing by merging engineering and bio-pharmaceutical expertise within the same research groups.

This box summarizes key points contained in the article.

channels [1,2], and broad range of vascular functions ranging from inflammation and thrombosis to atherosclerotic plaque formation [3-6]. Therefore, physics of blood flow and flow conditions form the basis for all the interactions spanning the scales of molecules, cells, tissues and whole organ systems (**Figure 1**). Not surprisingly, determining cardiovascular drug action *in vitro*, using target-binding assays or effects on isolated cells grown under static 2D conditions, is a poor representation of the intact physiological milieu of *in vivo* vasculature. At the same time, high-throughput *in vivo* studies in mammalian organisms are not feasible due to ethical and economic considerations.

Microfluidics offers solutions to the above obstacle (**Figure 1**). The special role of microfluidic devices in cardiovascular drug discovery can be understood by analyzing blood rheological curves (shear stress vs shear rate), which show that blood is a non-Newtonian fluid at low shear rates, where Rouleaux formation and sedimentation increase its apparent viscosity. Blood's apparent viscosity depends also on the geometry of the vessel in which it is measured, according to the Fåhræus–Lindqvist effect, which must be taken into account when considering hemodynamics in capillaries. In order to design and test drugs, a microenvironment mimicking those intricate biophysical interactions is required. Second, in many experimental and clinical situations, for example, when multiple blood tests are required to monitor the efficacy or plasma levels of pharmacotherapeutics, in pediatric/neonatal patients, or in hypovolemic patients, it is imperative to limit phlebotomy-related blood loss. Microfluidic platforms, owing to their capability to analyze small volumes of whole blood or sera, open possibilities for small sample size in assessment of drug pharmacokinetics, or analysis of efficacy of anticoagulation and antiplatelet regimens.

In this review, we introduce the concept of microfluidics as an enabling platform for cardiovascular drug discovery, showcasing some of the recent developments in the field. Noteworthy, despite the long history of the concept on endothelialized microfluidics, its use in the field of cardiovascular pharmacology is relatively underrepresented. A Boolean search of PubMed with terms 'cardiovascular' AND 'drug' yields nearly 360,000 hits, but adding to this the term 'microfluidic' results in the list of 99 publications, many of which promise potential pharmacological applications that have so far not been realized. We hope that this review, by highlighting the advantages that microfluidics can offer in the field of cardiovascular drug discovery, will not only encourage biomedical researches to apply microfluidic platforms more broadly, but also initiate a new era of microfluidic-based designs that are user-friendly and application-driven.

2. Vasculature-on-the-chip: technology with great potential for cardiovascular drug discovery

The network of blood vessels within the circulatory system mediates a wide range of functions well beyond being merely the passive conduits for cellular and biochemical transport. They maintain a high degree of plasticity, including ongoing vessel formation and regression, constriction and dilatation, different levels of endothelial activation and responsiveness to endocrine and paracrine factors. Malfunction of vasculature underlies numerous pathologies, including atherosclerosis, arteriosclerosis, tumor growth and metastasis, retinopathy, sepsis and many others. Therefore, engineering vasculature *in vitro*, using approaches that allow mimicking the physio-anatomical complexity of human vasculature, has far reaching consequences for the discovery of drugs to treat such pathologies. There is a large and rapidly expanding concept of 3D quasi-*in-vivo* culture systems models, aimed at bridging the gap between conventional 2D cell cultures and animal models for high-throughput drug testing to limit high attrition rate of drug candidates late in the development process.

The greatest advantage of using microfluidics as a platform for assessment of cardiovascular drug action is its ability to finely regulate fluid flow conditions, including flow rate, shear stress and pulsatile flow [7], while also providing means for modifying the geometry of vessels (bifurcations, stenoses, complex networks), the type of surface of the vessel walls, and for patterning cells in 3D tissue-like architecture, including generation of lumen walls lined with cells. With current design and manufacturing capabilities, vessel walls can be coated with components of extracellular matrix such as collagen, or with wild-type or genetically modified cultured endothelial cells, while micro-scale surface patterning can be used

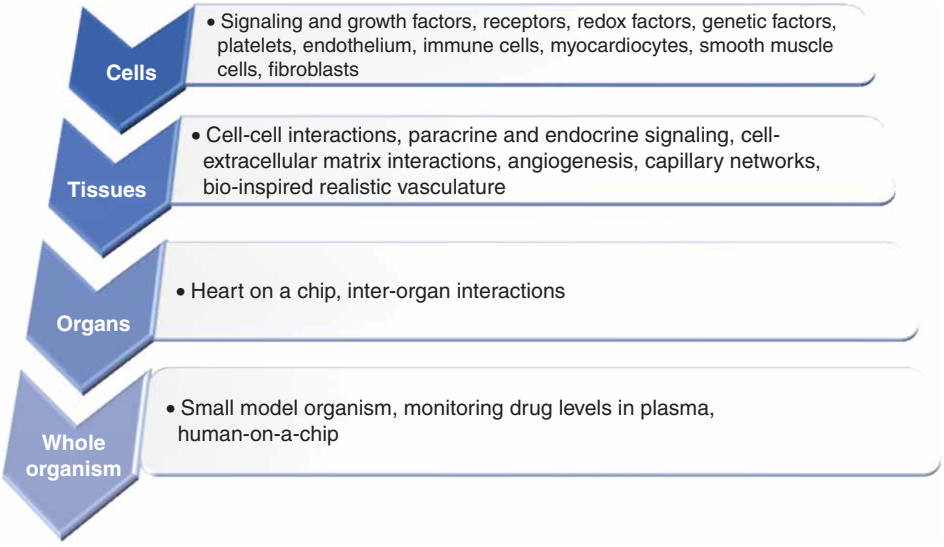

Figure 1. Cardiovascular drug discovery, viewed through the prism of interactions spanning the scales of single cells to an organism level, can be facilitated by microfluidics. Flow conditions affect basic physico-chemical interactions, signaling topology, cellular phenotype, cell–cell interactions, as well as whole-organ and system functioning. Microfluidic platforms can assist in elucidating drug action at all these levels.

to mimic such changes in human vasculature as focal vascular injury (**Figure 2A – D**) [8]. Such parallel plate microfluidic designs can combine the layer of relevant vascular adhesion molecules with shear stress and low volume of reagents/leukocytes to study leukocyte recruitment [9], with creative integration of porous barriers to separate different cell lineages seeded within the fluidic chambers allowing analysis of cell–cell interactions, occurring via paracrine factors, with potential to test drugs that interfere with such signaling pathways (**Figure 2E – G**). While introducing numerous possibilities for inter-cellular interactions, parallel plate flow devices do not accurately depict the geometry of microvascular networks *in vivo*. Advances in microvasculature mapping systems, Computer Assisted Design, and manufacturing, allowed the microfluidic design to evolve from the parallel plate, through arterial and venous-type bifurcations (**Figure 2H**) [10], to complex realistic models based directly on *in vivo* topology that recapitulate bifurcations, tortuosities as well as cross-sectional changes (**Figure 2G**) [11]. The detailed design of some of these platforms has been described in detail elsewhere [12-14] or can be obtained from the original publications, whereas here we focus on the conceptual evolution of the devices with particular emphasis on their increasing biological mimicry (including cellular and 3D complexity) and therefore applicability for predictive drug testing (**Figure 2**) [10,11,15-19].

The ultimate goal of the research efforts is to replace unethical, costly, time-consuming and lacking predictive power animal testing of drugs, and to develop 'human-on-a-chip' systems, whereby several organ cultures, including the vasculature, can mimic complex interactions between the organs and body systems and thus provide *in vivo*-like pharmacokinetics and pharmacodynamics for a new generation ADMET assessment. The concept of vasculature on the chip was applied to emulate various organs and structures for which microvasculature is at the hub of their physiological function, including lungs, liver and nephrons [20-22], and progressed through the stage of the multi-chamber micro cell culture systems [23-25], to the most recent multi-compartmental device of Schimek *et al.,* [26] that incorporates the pumping and transport functions of human cardiovascular system by embedding an on-chip micropump (to mimic heart) and a system of microchannels covered by human endothelial cells (to mimic vasculature), or a device of Moya *et al.* that allows self-assembly of microvasculature into an anastomozing and perfusing capillary network [27,28]. The advantage of this platform is the pulsatile nature and reversible pattern of shear stress and an organ-like vascularization, emulating the parameters encountered by endothelial cells *in vivo*.

Considering the rich selection of microfluidic platforms capable of mimicking many and varied aspects of human vasculature (**Figure 2**), their potential applications are far-reaching, and include assessment of drug effects on: i) endothelial permeability; ii) varied aspects of thrombus formation and blood coagulation (described separately in the next chapter); iii) interactions between blood components, endothelium and flow conditions; iv) role of rheological conditions (e.g., high velocity jets, flow across a narrow orifice at high velocity, flow from high pressure chamber to low pressure chamber) on formation of endothelial lesions; v) leucocyte diapedesis; vi) atherosclerotic plaque formation; vii) endothelial sprouting and angiogenesis; viii) interactions between endothelial and perivascular cells; ix) parasite adhesion to endothelium [29]; and x) endothelial wound healing [30]. Such devices can be also applied to assess the

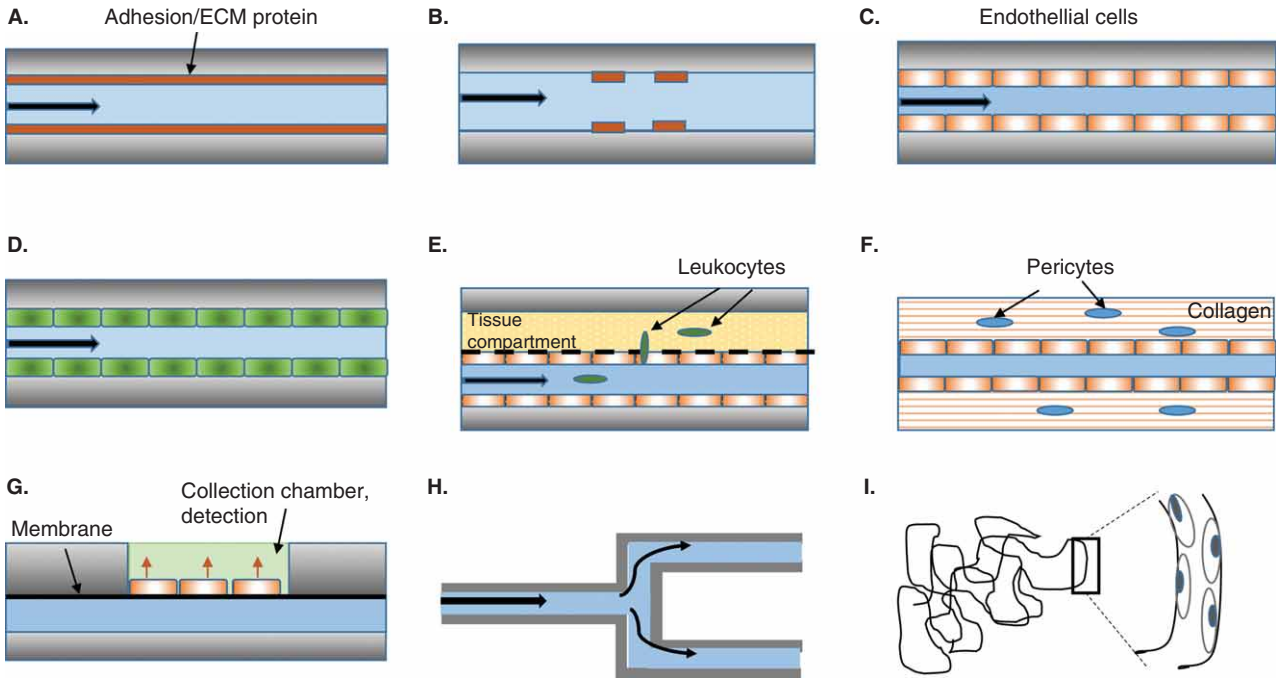

Figure 2. Design evolution in the field of vasculature on microfluidic chips (based on [10,11,15-19]). **A.** Microchannels coated uniformly with adhesion molecules/extracellular matrix substrates. **B.** Micropatterning of ECM patches (e.g., collagen) to mimic focal vascular damage. **C.** Whole surface of microfluidic channels covered by wild-type endothelial cells. **D.** Surface of channels covered by genetically engineered endothelial cells, for example, FRET sensor to detect caspase 3 activity. **E.** The cell–cell interaction mimicked on microfluidic devices can incorporate endothelialized channels, separated with a porous membrane from chemotactic factor-containing tissue compartments, and used for example, to study leukocyte diapedesis [9]. **F.** Endothelialized microfluidics fabricated in the matrix of collagen type I. **G.** Platform for studying paracrine interaction between cells flowing in the chamber (e.g., Red Blood Counts) and endothelial cells cultured in upper chamber, separated with a layer of polycarbonate membrane, coupled with detection of nitric oxide (NO) released by endothelial cells. **H.** Early on designs have also manipulated channel geometry to mimic arterial and venous bifurcations. **I.** Currently, channel geometry can be digitized based on *in vivo* microvascular networks.
ECM: Extracellular matrix.

specificity of endothelial drug targeting [31]. While it is discouraging to see that only a few of those designs deliver their promise of facilitating pharmacological discovery, there are several successes that deserve to be highlighted, including assessment of anti-thrombotic agents and angiogenesis-modulating drugs (see next two sections), and therapy targeting paracrine signaling (see below).

The microfluidic platform of Spence *et al.* (**Figure 2G**) has been employed to evaluate the effect of Red Blood Count storage conditions on endothelial release of nitric oxide (NO) [32], as well as to examine the mechanism behind the improved blood flow following hydroxyurea treatment in sickle cell disease [33]. Considering the importance of endothelium-derived vasodilating and vasoconstricting factors, such as NO, prostaglandins, endothelium-derived hyperpolarizing factor, endothelin or thromboxane, for regulation of vascular resistance, thrombosis, blood flow and blood pressure, with pathological implications in clinical conditions such as sepsis, hypertension, ischemic vascular disease and atherosclerosis, the device developed by the Spence's group holds great potential for pharmacological research. By adjusting the extracellular detection system to monitor a wider variety of compounds released from endothelial cells (the original platform employs extracellular NO probe), and by applying a wider range of stimuli in the flow channels, this microfluidic device could prove beneficial in studies of drugs targeting a whole range of paracrine signaling that affects endothelial function.

It will be of great interest to observe whether any of the recently described *quasi-in-vivo* systems, such as that developed by Rosano *et al.* (**Figure 2I**) [11], Schimek *et al.* [26] or Moya *et al.* [27], will be used for drug discovery. The effort towards designing such devices aligns with the NIH initiative of launching the Tissue Chip for Drug Screening, and with one of the sixteen engineering *Grand Challenges* for the 21st century (i.e., generation of '*improved systems to find effective and safe drugs*'), but we are yet to collect the pharmacological fruits of these technological developments. Importantly, the

issues of high-throughput and predictive power of tests performed with such platforms will be of concern to pharmaceutical industry, and therefore, it is critical to evaluate any new devices with respect to these parameters.

3. Microfluidics in hemostasis: testing anticoagulation and antiplatelet pharmaceuticals

A plethora of microfluidic-based designs have been developed to facilitate blood clotting assessment (e.g., prothrombin time – PT, activated partial thromboplastin time – aPTT), platelet activation (whether by means of surrogate signaling assessment, adhesion to substratum or aggregation), with potential for monitoring of anticoagulant and antiplatelet therapies [34]. Many of the designs are based on the principles of endothelialized cells outlined above, with functional modifications to suit the specific methodological requirements of blood coagulation and platelet formation testing, such as reagent mixing and type of coagulation trigger. The list of published designs is relatively extensive and includes platforms that perform: i) whole blood separation and plasma mixing with reagent, allowing expedited completion of blood coagulation testing [35,36]; ii) parallel PT and aPTT testing [36,37]; iii) real-time measurement of thrombin formation using fluogenic substrate and droplet format [38]; iv) assessment of whole blood coagulation on contact with defined surfaces (kaolin/type 1 collagen, focal collagen patterns) and at controlled shear rates [39,40]; v) whole blood coagulation testing on paper-based microfluidics [41]; vi) analysis of platelet aggregation, performed following on-chip re-calcification of whole blood [42], in label-free whole blood under conditions of multiple initial shear rates and in stenotic channels to determine occlusive and non-occlusive aggregation [43], or under conditions of strain-rate microgradients that mimic pathological changes in blood vessel geometry [44]; and vii) analysis of platelet adhesion to endothelial cells [45]. In addition, a microfluidic device for isolation of functional platelets, their labeling with fluorescent probes, and analysis of drug-mediated intracellular calcium flux (as a surrogate of activation) was developed back in 2005 [46].

Again the list of applications for the microfluidic devices in anticoagulant drug discovery is less impressive. In 2008, Neeves *et al*. developed a microfluidic method for focal patterning of flow channels with acid-soluble collagen and used this platform to study the effect of shear rates on platelet adhesion and aggregation (**Figure 2B**) [40]. This platform was subsequently used to assess the antiplatelet effects of several new agents, including an *ent*-diterpenoid glaucocalyxin A [47] and a new PI3K inhibitor in [48]. The main disadvantage of Neeves's design is the straight geometry of these channels, as it is at the sites of bifurcation and/or stenoses that thrombus formation is most likely to occur. This caveat was addressed to some extent by the Forest group [43], who developed a microfluidic-based platform featuring stenoses, and used it to study dose- and shear-rate-dependent drug effects on platelet activity as well as time to occlusion and time to thrombus detachment [49]. Using this approach, the authors showed, for example, that acetyl-salicylic acid (aspirin) has similar dose efficacy to eptifibatide (glycoprotein IIb/IIIa inhibitor) in terms of preventing occlusion at low shear rates, but fails at high shear rates [49]. This platform represents an approach to determine not only anti-thrombotic drug activity, but also preventive activity against formation of thrombotic emboli. Considering the clinical importance of both microvascular thrombosis, and the need to develop more specific treatments that target problem areas of the vasculature while limit potentially dangerous side effects, for example, associated with extensive gastrointestinal bleeding, it is surprising that microfluidic platforms have not been used more widely in antiplatelet drug discovery and screening. At the same time, considering the biophysical characteristics of endothelized microfluidic channels, caution needs to be exercised with regards to their use in studies of the pathophysiology and therapy of thrombosis in large arteries and veins.

4. Angiogenesis on a chip

The concept of vasculature-on-the chip can be also applied to study angiogenesis. The process of angiogenesis, or new blood vessel formation, is critical for many physiological processes, such as embryogenesis, wound healing, the female reproductive cycle. Angiogenesis can assure maintenance of adequate blood flow to tissues via newly formed collateral microvasculature to prevent slowly-developing ischemia. This can be beneficial, as occurs in coronary microvasculature in response to vessel stenosis, but also detrimental, as occurs during malignant angiogenesis that supports tumor growth, or in diabetic retinopathy. Angiogenesis is regulated by a multifaceted interplay between endothelial cells, extracellular matrix, and a plethora of signaling factors, such as VEGF. Despite this complexity, microfluidics could be successfully employed in the discovery of pro- and anti-angiogenic drugs, but design efforts need to focus particularly on mimicking well the extracellular microenvironment stimulating and encountered by the sprouting vasculature. This notion is mirrored in the evolution of designs that occurred over the years, with initial devices involving application of physical and biochemical stimuli within 3D hydrogel scaffolds integrated in microfluidic channels [50], and progressing to microfluidic devices such as that of Jeong *et al*., whereby the semi-interpenetrating network of collagen combined with hyaluronic acid – a system more closely mimicking the Extracellular Matrix – is used to monitor endothelial cell sprouting in a quantitative manner and under the influence of a well-controlled gradients growth factors [51]. While interesting, these designs are not particularly suitable for large-scale drug testing, as their preparation involves a time-consuming and laborious process, prone to variability due to the operator's technique, and they do not offer the necessary high-throughput. To improve user-friendliness and throughput of the

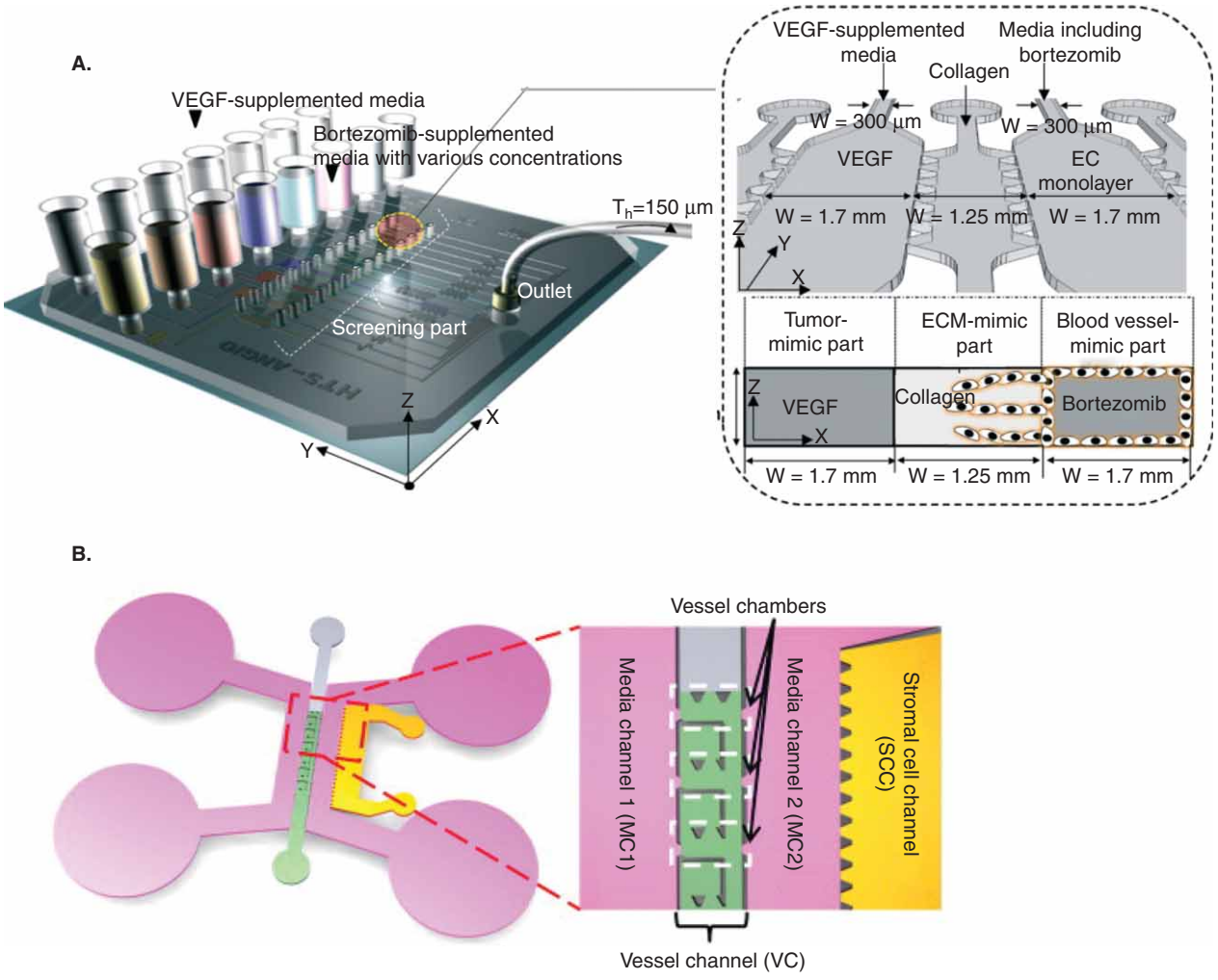

Figure 3. Towards higher throughout in angiogenesis-on-a-chip. A. Schematic overview of the QMAS system, with embedded gel cages housing hydrogel scaffolds, cell culture chambers with confluent endothelial cell monolayers and separate channels for VEGF-supplemented media and for media with added candidate or reference drugs. **B.** Schematic overview of a microfluidic system for engineering microvessel arrays, in which endothelial cells are placed on left sidewall of vessel channel and sprout towards the stromal cell channel, traversing the vessel channel which is filled with fibrin gel.

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ECM: Extracellular matrix.

angiogenesis-on-a-chip devices, Kim *et al*. have recently fabricated the quantitative microfluidic angiogenesis screen platform incorporating multiple parallel 3D hydrogen scaffold matrices with a manifold layer for consistent gel loading, and a perfusion-based system to achieve stable concentration profiles within each matrix (**Figure 3A**) [52]. A proof-of-concept application of this platform involved analysis of endothelial cell response to treatment with several concentrations of bortezomib, an approved anti-angiogenic agent [52]. This is a significant technological step forward in terms of validating angiogenesis-on-a-chip devices for pharmaceutical use, but still remains at the stage of a medium-throughput device. Considering the amount of compounds that needs to be tested during drug development pipelines, further increase in throughput is needed before such technology can convincingly provide a solution to drug discovery, validation and optimization. Another alternative to achieve high-throughput capabilities in angiogenesis is offered by the device of Lee *et al*. (**Figure 3B**), developed as a platform for assessing drug effects on vascular permeability [53]. As the platform utilizes the process of microvessel formation via a natural angiogenic process, stimulated by growth factors secreted by co-cultured stromal cells, this device offers several advantages for studying anti-angiogenic

drugs, such as simple and modular design that facilitates ease of increasing the number of test points [53].

5. Heart-on-chip technology

Analysis of cardiac muscle physiology, such as contractility and electrical properties, is important for two facets of drug development, that is, to test cardiac pharmaceuticals (e.g., inotrops, chronotrops) as well as to assess potential cardiotoxic effects of non-cardiac candidate drugs. Constraining single cardiomyocytes in micro-channels within microfluidic devices has proven useful for measuring extracellular potential [54], transmembrane currents [55], and for prolonged field stimulation of single cells [56], coupled with a range of metabolic measurements (Reactive Oxygen Species generation, calcium spikes, intracellular pH) based on cell staining with fluorescent probes (see **Figure 4A and B** for examples of designs). While suitable as a proof-of-concept, and for many aspects of basic studies of cardiomyocyte physiology, assays that employ single cells usually do not have enough predictive power to be embraced by the pharmacological industry (unless single cell survival is of clinical importance), and instead networks consisting of multiple cardiomyocytes need to be employed [57]. Therefore, an increasing emphasis is now being placed to produce quasi-*in-vivo* cardiac tissue models on microfluidic devices, using cardiomyocyte networks and substrates that mimic the elastic modulus of the heart. These 'beating heart-on-a-chip' platforms are based on engineered, anisotropic ventricular myocardial cells, that mimic the aligned, laminar structure of ventrical myocardium, cultured on a deformable elastomeric thin film cantilevers, forming muscular thin films originally developed by Feinberg *et al.* (**Figure 4C**) [58]. As it has been recognized that the lifetime and physiology of cardiomyocytes is greatly affected by the type of substratum on which they grow, the cantilevers have been produced from several different materials, including polydimethylsiloxane polymer [59,60], alginate hydrogel or gelatin hydrogels [61], with the latter providing benefit of long-term culture without decline of contractile function. These devices are capable of measuring contractility and propagation of action potential and could thus be useful for testing drugs targeted to exert inotropic or chronotropic effects. The proof-of-concept pharmacological assessment using such microfluidic heart-on-a-chip was conducted by Agarwal *et al.* [59], who tested changes in contractile output upon treatment with a range (1 nM – 100 μM) of doses of a non-selective β-adrenergic agonist isoproterenol. Although the development of heart-on-a-chip platforms is a relatively recent concept, its potential applications are far reaching, and may well extend beyond simple drug testing. With further developments in material sciences, allowing improved functionality of bioengineered cardiac tissue – as has been shown for example with the addition of carbon nanotubes [62] – these heart-on-a-chip platforms may be a step towards providing cures for arrhythmias, and perhaps even as alternatives to donor-derived tissue and organ transplants.

6. Linking the micro with the macro: microfluidics for small model organism-based drug discovery

Drug discovery studies are unquestionably most relevant when performed in an intact physiological milieu, with all its intercellular, multi-organ and multi-system interactions. To breach the gap between *in vitro* cellular assays and highly regulated, low-throughput rodent and higher animal models, small vertebrate organisms such as zebrafish (*Danio rerio*), particularly in their embryonic form, are increasingly deployed in drug discovery pipelines. Several transgenic zebrafish lines have been established that are particularly useful for assessment of drugs targeting vasculature or cardiac tissue. For example, Tg(Fli1a: enhanced green fluorescent protein [EGFP]) transgenic line expressing EGFP in endothelial cells represents an excellent and rapid biotest to visualize development of characteristic patterns of intersegment vessels. The interspecies concordance between zebrafish and human is relatively good, and the predictive power of zebrafish-based candidate drug testing for efficacy and safety liabilities is particularly high when combined with post-test verification of intra-organism drug levels.

At present, the major hurdle to widespread deployment of zebrafish embryo and larvae in large-scale drug development projects is the lack of enabling high-throughput analytical platforms. Most experiments are still performed in multiwell microtitre plates and require laborious and time-consuming manual manipulation of specimens and solutions. Furthermore, static culture of embryos can lead to a significant bias in toxicity studies due to for example, drug adsorption to the surfaces and also secondary effects from the secreted embryo metabolites. Lastly, microtiter plate culture is not conducive to precise specimen positioning and immobilization, both necessary for applications such as high-resolution imaging, electrophysiology measurements or precise anatomical mapping of physiological processes.

In order to spearhead vascular drug discovery with the use of transgenic zebrafish as a model, automated platforms need to integrate embryo in-test positioning by customizing the fish tanks to match exactly the size of the embryos and thus restrict their movement (necessary for high-content imaging), as well as embed fluidic modules for continuous drug and oxygen delivery to embryos. Several groups have pioneered the concept of microfluidics for small model organisms such as zebrafish [63-70]. Wlodkowic group, in particular, has developed a range of automated microfluidic systems suitable for real-time analysis of transgenic zebrafish embryos [66,67,69,70]. The most notable example is a fully integrated microfluidic drug discovery platform with an array of 3D micro-mechanical traps that actively capture and immobilize single embryos using a low-pressure suction. The microfluidic system was designed as a monolithic and fully integrated device with no moving parts. The chip-based device incorporates embedded

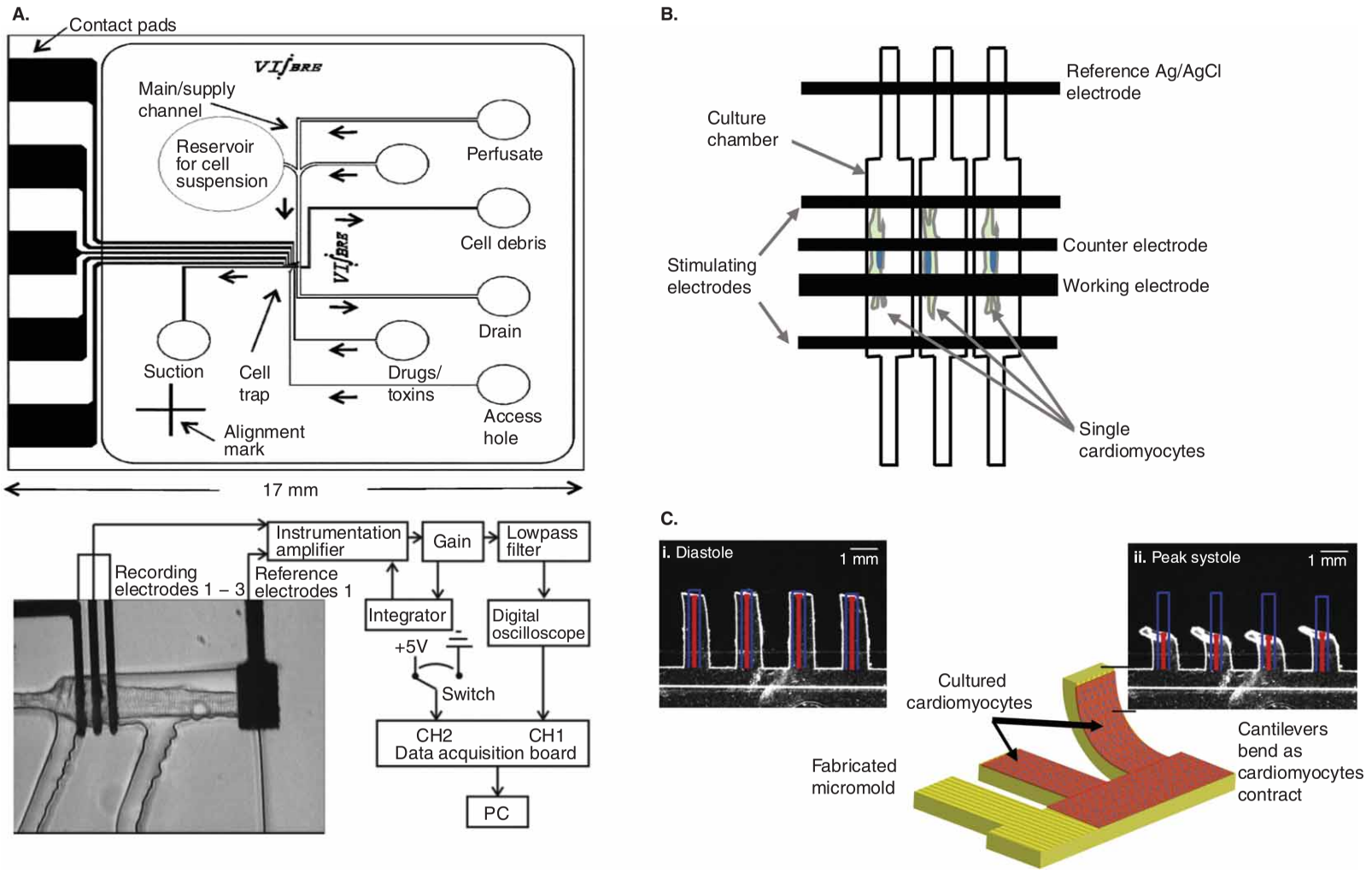

Figure 4. Heart-on-chip platforms utilize microfluidics and miniaturization to perform single cell physiological assessment and to reproduce coordinated functioning of cellular networks. A. Schematic overview of a microfluidic platform for extracellular potential recording from single cardiomyocytes. The cell suspension is applied to the reservoir in the upper left side of the system, and single cells are drawn by pressure gradients from there into the cell trap located at the centre. Lower panel shows a setup of measurement electrodes and electronics for recording the extracellular potentials. **B.** Microfluidic platform combining cell culture microchambers with an array of sensor electrodes and stimulating microelectrodes for obtaining electrochemical and optical measurements from electrically stimulated single cardiac cells. Based on [56]. The original device consists of 15 culture chambers, whereas here 3 chambers are shown to depict the principle. **C.** Schematic representation of the fabrication and function of muscular thin films consisting of an engineered layer of cardiac myocytes cultured on micromolded cantilevers. The photographs depict cantilevers with cultured cardiac tissues that are relaxed at diastole, or bent away from the glass coverslip during systole.

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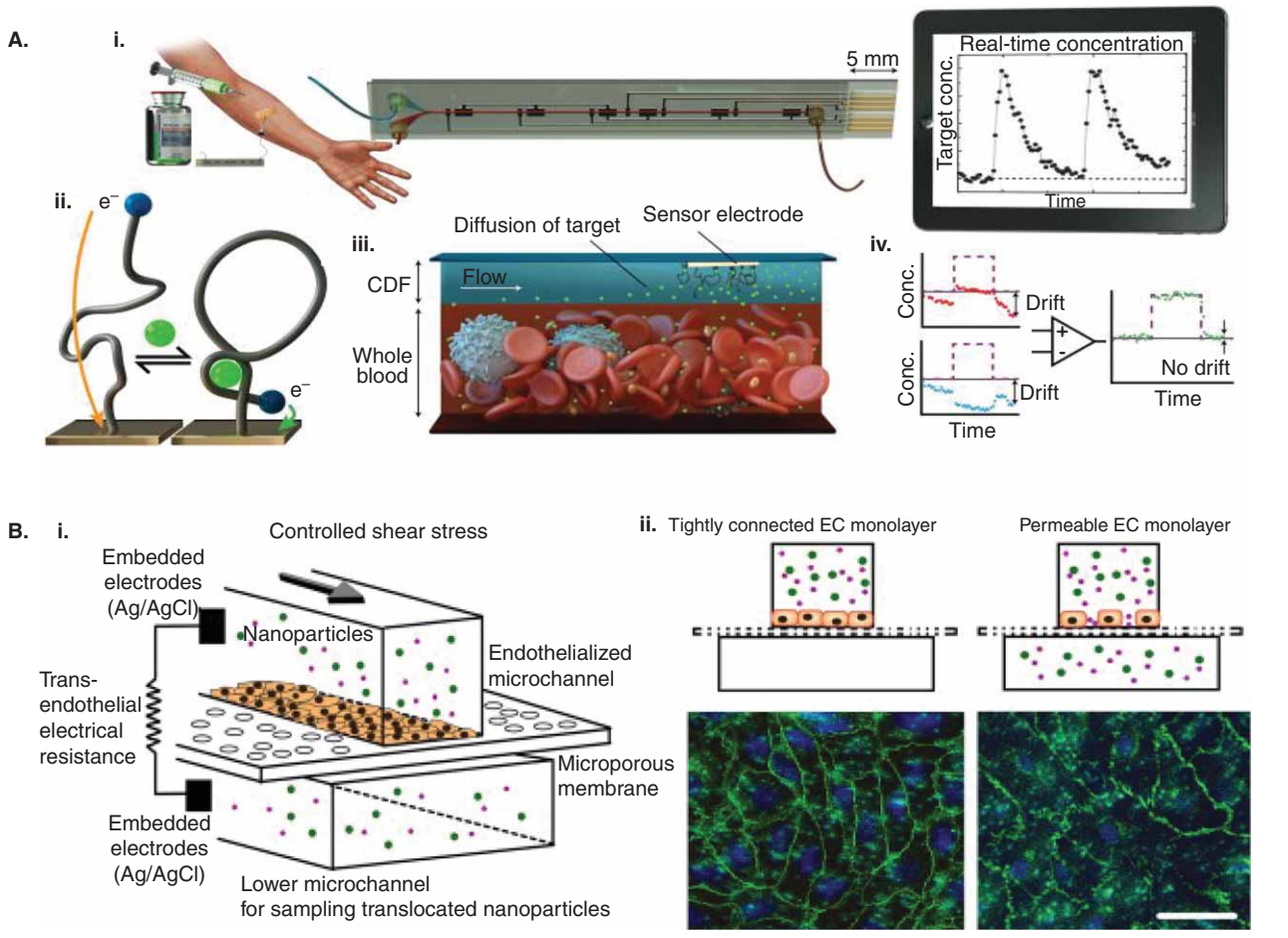

Figure 5. Microfluidic platforms for monitoring drug level in the bloodstream. A. Microfluidic electrochemical detector for *in vivo* continuous monitoring (MEDIC) of circulating drug level. i) The intended clinical setup; ii) the aptamer probe attached to the gold electrode undergoes conformational change upon drug (green) binding, which in turn increases the rate of electron transfer between an electrochemical redox reporter (blue) and the electrode, causing current changes over time; iii) verticaly stacked laminar flow of buffer (blue) and blood (red) forms a continuous diffusion filter (CDF) which selectively allows diffusion of drugs while excluding large blood-borne particles; iv) single-on (red) and single-off (blue) both exhibit significant change in response to a pulse of drug. **B.** An endothelialized microfluidic device for probing microvessel permeability. i) Schematic of the device, consisting of two-layer microfluidic channels that are separated by a porous membrane which provides substratum for growing endothelial cells (ECs). ii) Upon stimulation by inflammatory factors, shear stress or disruption of intercellular connections, the monolayer of ECs becomes highly permeable, and thus translocation of drugs (here nanoparticles) across the layer into the lower chamber can be monitored.

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B. Reproduced with permission from [75].

piezoelectric microdiaphragm pumps, embryo trapping array with suction manifold, drug delivery manifold and optically transparent indium tin oxide heating element to provide optimal temperature during embryo development. A miniaturized USB fluorescent microscope was integrated for low-resolution and low-cost imaging together with solid-sate LED illumination. In addition, a motorized rack and pinion drive was embedded within the stage that can drive the chip for imaging. PC and interconnected embedded ARM microcontroller controlled all sensor inputs and actuator outputs. Together with embedded sample and waste reservoirs, this system brought Lab-on-a-Chip for small model animal bioanalysis a step closer to realization of full analytical automation. The authors have also for the first time successfully demonstrated that integrated chip-based device can be applied to kinetic analysis of anti-angiogenic compounds using transgenic Tg (fli1a:EGFP) embryo. Before these platforms can be adopted for large-scale drug discovery projects further automation and miniaturization is necessary, involving zebrafish embryo sorting, extended physio-behavioral analysis (to detect any

off-target side effects), and post-test organism recovery to assess drug concentration and thus limit the false-positive and false-negative effects due to enhanced or poor drug uptake.

7. Microfluidics for plasma drug level monitoring and drug delivery

Every drug discovery pipeline involves also pharmacokinetic analysis whereby drug bioavailability, metabolism and drug elimination are assessed. The small sample size requirements of microfluidic platforms are extremely attractive from the perspective of pharmacokinetic studies, for example, for creating AUC curves while limiting phlebotomy-related blood loss, and for point-of-care individualized plasma drug concentration assessment in order to limit side-effects for example, in patients with abnormal kidney function or in cases of novel poly-pharmacotherapy. Serum drug concentration has important implications for cardiovascular health, not only by ensuring the therapeutically-effective doses have been achieved, but also to protect the patients from cardiotoxicity, one of the most important side effects of pharmaceuticals. The quest for real-time monitoring of therapeutic agents in the bloodstream has led to the development of several designs that need highlighting, including a promising new microfluidic platform equipped with aptamer-based sensors [71] and capable of continuous monitoring of blood cocaine levels [72] and also a wide range of circulating drugs, including doxorubicin and kanamycin (**Figure 5A**). Microfluidic platforms have been also integrated with sensors of liposome-based nanoparticles to monitor liposomal drug loading and delivery [73]. In addition, digital microfluidics has been applied to multiplexed extraction and analysis of pharmaceutical compounds in dried blood spot samples [74]. Finally, early this year, Kim *et al.* [75] developed an endothelialized chip (**Figure 5B**); i) with controllable endothelial permeability (**Figure 5B**) ii) and validated its performance in assessing nanoparticle drug delivery across endothelium using an *in vivo* rodent model as a reference.

In addition, microfluidics is lending solutions for drug delivery approaches, by means of producing stable microbubbles directly in the vasculature, using a flow focusing microfluidic devices [76,77]. This approach of *in situ* microbubble production, in combination with clinical ultrasound exposure, holds great promise for focal therapy of atherosclerosis, either with pharmaceutical agents [76] or as a new means for gene delivery [78].

8. Conclusion

The advantages of microfluidics for studies of cardiovascular physiology and pathophysiology, and their amenability for pharmacological modifications, have been recognized for a long time, resulting in a vast array of designs for specific, clinically relevant applications. The platforms developed to date include single-cell arrays for assessment of cardiomyocyte function, endothelialized devices creating vasculature-mimicking structures with increasing complexity of cellular composition and geometries, organs-on chip platforms that allow monitoring of contractile functions, and platforms for monitoring cardiovasculature in whole small model organisms. Finally, microfluidic devices emerge as useful solution to pharmacokinetic analysis of small volume samples. Despite such a suite of devices available for use in drug discovery, their application has so far remained predominantly in the purview of biomedical engineers and has not resulted, as yet, in ground-breaking pharmacological discoveries.

9. Expert opinion

As current users and developers of innovative microfluidic platforms, we observe that the immense expansion of the published designs does not go hand-in-hand with practical pharmacological applications. Is the message lost in translation? This is one of the reasons behind such underperformance of microfluidics – technology is evolving, but there are only a few groups that combine engineering, biological, pharmacological and medical expertise under one roof. Another reason is that many of the microfluidic platforms appear complex in their fabrication process – even if this is not the case for bioengineering groups, biomedical researchers inexperienced in microfluidic fabrication find the process too daunting and turn instead to readily available off-the-shelf platforms. We can refer to this phenomenon as workshop-to-bench gap of bioengineering science, in analogy to the well-recognized bench-to-bed gap in biomedical sciences.

Considering the scarcity of commercialization efforts, and with so many promising microfluidic platforms joining the ranks of merely a good publication topic never to be taken outside of academic use, there is a widening discrepancy between an increasing number of available designs and the willingness to use them. Only a limited number of microfluidic platforms have been commercialized, and these notable exceptions include CYTONOME or CellASIC, but endothelialized microfluidics and heart-on-chip technologies are still outside the purview of entrepreneurial efforts. Apart from overt commercialization of microfluidic platforms, there are two further solutions to complete their cycle of development: open-source protocol-type publications [66], containing step-by-step explanation on microfluidic platform fabrication thus allowing potential future end-users to use the platform for drug discovery without the need to buy patent rights or collaborate internationally [15,79], and an in-house drug testing, whereby teams developing microfluidic platforms engage also in drug assessment to spearhead the use of their platform, showcase its applicability, and perform technological fine-tuning based on directly experiencing the needs of an end-user.

Finally, there are several fields in cardiovascular drug discovery that have so far escaped the focus of the microfluidics

community. This includes the assessment of drugs for regeneration of endothelial glycocalyx, a meshwork of surface-bound and loosely attached glycoproteins, sulphated proteoglycans and glycosaminoglycans that protects endothelium from shear stress and is involved in flow-induced mechano-transduction [80]. The unique feature of glycocalyx is that it responds purely to mechanical stimulation in a form of vascular wall shear stress. The explosion of interest in endothelial glycocalyx as a new drug target led it to be dubbed the most important early prevention target for Cardiovascular Disease. Despite this, current studies addressing the properties of endothelial glycocalyx are limited to rodent and porcine *in vivo* models and traditional *in vitro* cultures, and have not, as yet, employed the microfluidic synthetic vasculature models. These platforms would be valuable to study drug-induced reversal of glycocalyx dysfunction, and also to determine the effects of different resuscitation fluids as compared to fresh frozen plasma, under varying dynamic states.

Microfluidics offers unprecedented investigative advantages, allowing fine manipulation of fluid flow characteristics, including flow rate and shear stress, to mimic arterial, capillary and venous flow conditions. Therefore, microfluidics serves as an excellent tool to study rheological behavior of blood and its analogues. While still limited, there have been noteworthy reports on design and fabrication of microfluidic devices for analysis of rheological behavior of blood [81] as well as for evaluation of blood analogue solutions [82].

While offering the insight into those interesting aspects of cardiovascular biology that can be served well by developing novel, specialized microfluidic platforms, we maintain our emphasis on the need to close the existing gap between the rapid growth of Lab-on-the-Chip designs and the stagnancy in their application by biomedical and pharmaceutical research community. With so many technological advances made in the field of miniaturization, additive 3D manufacturing and electronics, our primary goal should now be to integrate the existing platforms into automated, user-friendly, simple, portable and cost-effective systems that will find their way into the biomedical laboratories.

Declaration of interest

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