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Title
Engineering and Evaluating Drug Delivery Particles in Microfluidic Devices

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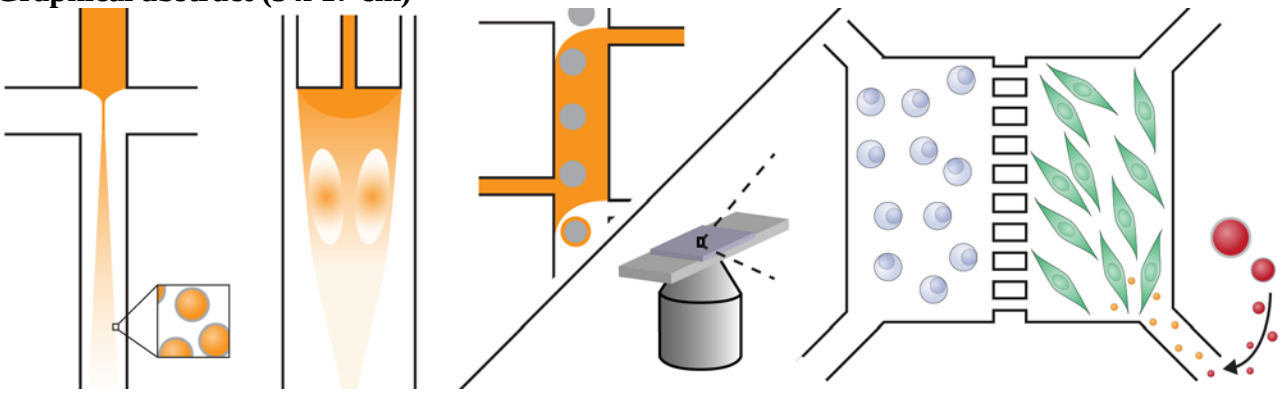
Graphical abstract (5 x 17 cm)



Note to editorial office
Figures made for double-column width: Fig 1, Fig 4, Fig 6
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Abstract

The development of new and improved particle-based drug delivery is underpinned by an enhanced ability to engineer particles with high fidelity and integrity, as well as increased knowledge of their biological performance. Microfluidics can facilitate these processes through the engineering of spatiotemporally highly controlled environments using designed microstructures in combination with physical phenomena present at the microscale. In this review, we discuss microfluidics in the context of addressing key challenges in particle-based drug delivery. We provide an overview of how microfluidic devices can: (i) be employed to engineer particles, by providing highly controlled interfaces, and (ii) be used to establish dynamic *in vitro* models that mimic *in vivo* environments for studying the biological behavior of engineered particles. Finally, we discuss how the flexible and modular nature of microfluidic devices provides opportunities to create increasingly realistic models of the *in vivo* milieu (including multi-cell, multi-tissue and even multi-organ devices), and how ongoing development toward commercialization of microfluidic tools are opening up new opportunities for the engineering and evaluation of drug delivery particles.

1. Introduction

Through nanotechnology, synthetic functional structures can be engineered at the nanometer-level, thus creating materials that can interact with, and influence, biological systems at their very core [1]. The application of nanotechnology to diagnose and treat diseases – nanomedicine – has moved from being solely an academic endeavor to making an impact in the clinic [2]. Examples include: (i) biomaterials for medical implants, such as nanocomposites used as dental fillers; (ii) *in vitro* diagnostics, such as gold nanoparticles that enhance sensitivity in genetic assays; (iii) *in vivo* imaging, such as superparamagnetic iron oxide nanoparticles for use as contrast agents in magnetic resonance imaging; and (iv) drug delivery, where nanostructured carriers can be used for the controlled delivery of therapeutics [1,2].

Encapsulating or attaching a therapeutic to an engineered drug delivery carrier can improve the safety and efficacy of a drug, thus enabling new and improved therapies [3-5]. However, the translation of engineered multifunctional drug delivery vehicles from *in vitro* to the preclinical and finally the clinical setting has proven to be a considerable challenge. A reason for this are the difficulties associated with predicting the behavior of an engineered carrier in a system as complex as the human body. Built up of a hierarchy of structures with functional dimensions that differ by many orders of magnitude, the human body is a multi-level, feedback-regulated compartmentalized system, both highly dynamic and interconnected. For example, receptor-ligand interactions at the nanometer scale can cause the release and distribution of hormones that can, ultimately, lead to organism-level changes. To work at, and understand, all of these length scales, especially at the smallest dimensions, requires a highly interdisciplinary approach [6].

In this review, we discuss current challenges facing particle-based drug delivery systems and review strategies where microfluidic technologies have been used to address some of these issues. We provide an overview of both the production and

evaluation of drug delivery particles, with a focus on microfluidics as an enabling technology. Emphasis is placed on how microfluidics can complement existing technologies by providing new ways to reliably and reproducibly engineer drug delivery particles and new *in vitro* models that can mimic important aspects of the *in vivo* situation. These features of microfluidic technologies that enable detailed analysis of mechanisms that govern interactions of particles with biological systems can facilitate the correlation of studies between *in vitro* and *in vivo*. Additionally, we provide an outlook of this growing interface between drug delivery and microfluidics, as well as discuss the impact of the evolution within microfluidics, from highly specialized "home-built" systems to easily accessible "off-the-shelf" instruments. This increase in accessibility is facilitating interdisciplinary work, thus accelerating the development of new and improved rationally designed drug deliver particles.

2. Drug delivery particles and challenges ahead

The objective of a drug delivery particle is to deliver a therapeutic to where it is needed, when it is needed. The archetypical example is to selectively deliver a cytotoxic compound to a tumor, at a high enough concentration and for long enough to kill the tumor, while at the same time leaving healthy tissue unharmed. A drug delivery particle can provide a different means toward realizing this, including: (i) facilitating formulation of the therapeutics; (ii) increasing specificity; and (iii) providing controlled release (Fig. 1). Multifunctional drug delivery particles therefore have the potential to enable the use of new drugs as well as to improve the performance of existing drugs.

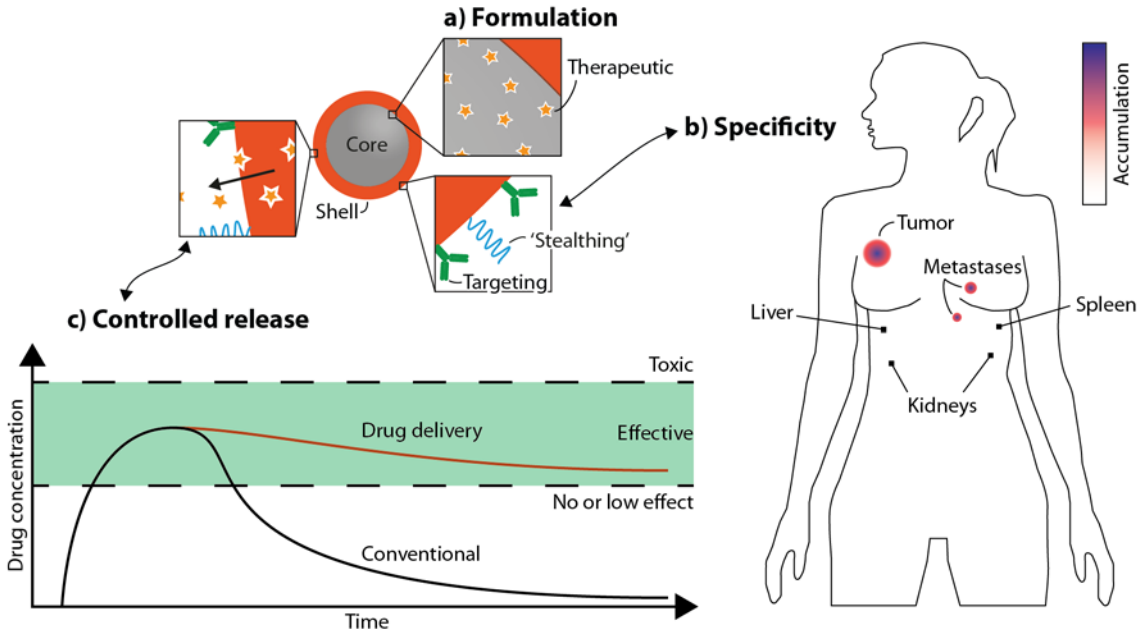

Fig. 1. Objectives for drug delivery particles, for example a core-shell nanoparticle. **a)** Facilitate the formulation of the therapeutic through engineered materials, for example by encapsulating a hydrophobic drug inside a hydrophilic shell. **b)** Increase the specificity of a drug, for example through the use of targeting and "stealthing" ligands. Ideally free drug should only be released at the intended site of action (e.g., tumor site for cancer drugs) with minimal accumulation at non-target sites, typically

including the spleen, the liver and the kidneys. **c)** Provide control over drug release kinetics, for example to keep the concentration of a drug within the therapeutic window for prolonged periods of time.

Despite the great promise of drug delivery, significant challenges still remain. Today, several particle-based drug delivery systems exist in the clinic and others are currently undergoing clinical trials [7-9]. However, these successes should be considered against a backdrop of many different research groups around the world that have developed a plethora of diverse drug delivery systems, and have proven effective in *in vitro* studies with only a very few having made it successfully past preclinical studies. A reason for this is the common discrepancy seen when comparing preclinical and clinical data, where remarkable advantages in efficacy for drug carriers seen preclinically almost completely disappear when moving to humans [10], although there are examples of successful preclinical-clinical correlations [11]. This discrepancy indicates the difficulty of extracting predictive information of drug carrier behavior and performance in the clinical setting using conventional models, information that is critical for the rational design and development of drug carriers.

It may be instructive to compare this situation to the pharmaceutical industry as a whole, which is facing unprecedented challenges due to a combination of scientific, economic and legal reasons, in what has been called the "pharmaceutical industry's grand challenge" [12,13]. A main reason for this is the high rate of expensive late-phase drug attritions, and therefore a key objective is to identify and eliminate unsuccessful drugs as early as possible. Part of the solution, as proposed by four major pharmaceutical companies, could be the development and increased usage of new and improved *in vitro* pharmacological profiling assays that can provide more accurate predictions of clinical performance [14]. Reliable *in vitro* assays with high predictive power of drug-performance in humans would not only prove valuable in preclinical evaluation, but could also inform and guide the initial research and development of new therapeutics.

To apply this to the field of drug delivery, several other factors need to be considered when evaluating engineered particles, in addition to the characteristics of the therapeutic to be delivered. Important factors affecting the behavior of drug delivery particles in a biological system include both physicochemical parameters of the particles as well as characteristics of the biological target environment [15-20]. Further, with each new functionality added to a particle – for example imaging or targeting moieties – the biological behavior becomes even more convoluted [21]. Delineating these complex relationships between design parameters and biological performance is one of the grand challenges within particle-based drug delivery and is critical for the rational design and development of carriers.

Developing *in vitro* models of the complex biological setting, to evaluate intricate particle-designs, in order to delineate convoluted particle-biological interactions may seem like a daunting challenge. However, being aware of such complexity can allow common pitfalls to be avoided, and focus placed on where most impact can be made [22-25]. Ideally, a reductionist approach could be applied, where the level of complexity can be tuned, as this would facilitate the study and formation of causative relationships. The conventional strategy, however, including standard cell culture and

animal models followed by clinical studies, allow only a limited reductionist approach (Fig. 2). For example, the step from a petri dish to an animal is arguably quite large. Reasons underlying a failure *in vivo* following a success *in vitro* can therefore be difficult to investigate, explain and rectify. Therefore, a key objective is the development of *in vitro* models that are sufficiently complex to realistically mimic aspects of the *in vivo* situation, yet simple enough to enable an understanding of underlying relationships and mechanisms.

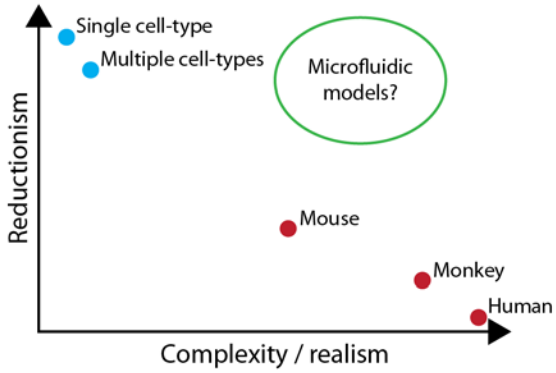

Fig. 2. Illustration of the trade-off between reductionism and complexity/realism for a few preclinical models in relation to the clinical setting (not to scale)(blue dots: *in vitro*, red dots: *in vivo*). Conventional methods typically require one to choose from models either enabling a reductionist approach or models with realistic/relevant results because of technological, economical and ethical constraints. Microfluidic models might provide opportunities to come closer to the clinical setting in relevance while retaining the power of *in vitro* reductionism.

3. Microfluidics as an enabling technology

Microfluidics is a multidisciplinary field where small amounts of fluids are handled in channels with dimensions typically from tens to hundreds of micrometers [26]. At these length scales novel, and sometimes nonintuitive, properties appear. Examples include laminar flow and the relative importance of diffusion. This is because the competition between various phenomena that dictate the behavior of fluids do not scale linearly with changes in dimensions [27]. Using soft lithography, microfluidic devices that exploit these features can be designed with both high precision and relative ease [28].

The possibility of engineering controlled fluidic microenvironments opens up interesting biological applications, as many important biological processes take place at the micrometer scale. Examples include the microvasculature [29], where gases, nutrients and waste products are exchanged, as well as important functional barriers and interfaces in major organs such as the kidney and liver [30,31]. Fluidic flows are an important part of both healthy and pathological conditions. This includes not only the more obvious flow of blood and lymph in the circulatory system, but also the interstitial flow taking place in virtually all soft tissues [32]. Key aspects of the biological setting therefore include both micrometer structures as well as well-controlled fluid flows.

In a typical microfluidic set-up, basic components include a syringe pump or a pressure source, and tubing connected to a microfluidic device that is typically fitted on top of a microscope slide. Cells and/or bacteria can be added to a device to create both simpler (such as a single cell-type cultured inside a straight channel) and more complex (such as different cell-types cultured in networks of interconnected channels) environments. By changing the conditions in the channel, for example by introducing compounds and particles in the flow, biological responses can be probed [33,34].

There are several advantages of using microfluidic devices to study the biological setting (Fig. 3). These include: (i) biologically relevant lengths scales; as discussed above, many important biological structures, as well as cells themselves, have length scales from tens to hundreds of micrometers; (ii) a high degree of control of geometry; well-developed and well-characterized microfabrication technologies, such as soft lithography [28], provides researchers with a large toolbox to manufacture specific designs in a reliable and reproducible manner; (iii) control of fluidics and the microenvironment; the intrinsic features of small volumes of liquid as well as fluid physics at these length scales [27] enables a high degree of control over the spatiotemporal environment of the cells, such as pulses or gradients of a stimulant; and (iv) real-time monitoring, microfluidic devices are typically made to fit on top of a standard microscope slide and as they are usually made of transparent glass and polymers they can be continually – and in real-time – monitored using standard microscopy techniques.

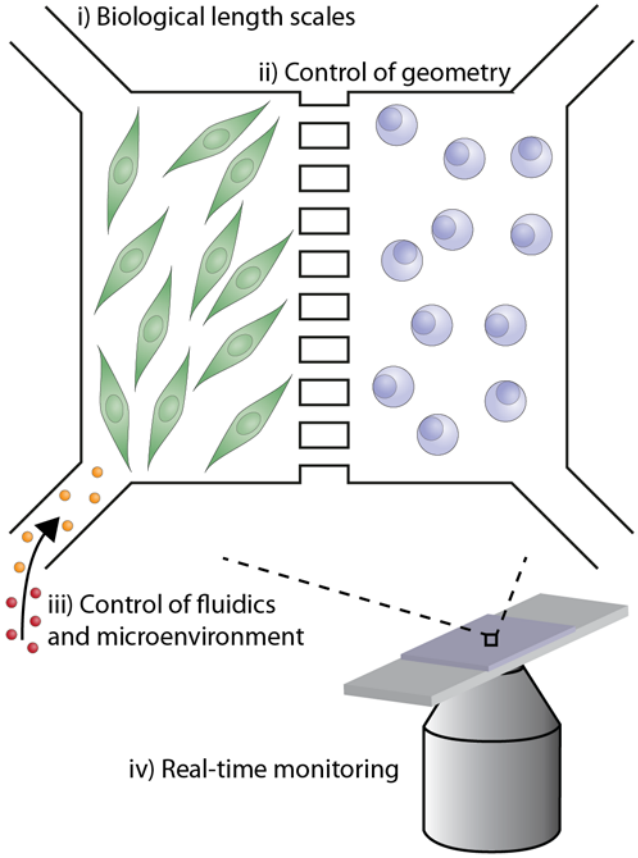

Fig. 3. Probing the response and interaction of two cell types (green and blue) in interconnected chambers during exposure to two pulses of compounds (yellow and

red). This is enabled by several features of microfluidic devices when applied to biomedicine, which include: (i) biologically relevant length scales; (ii) designed geometries; (iii) spatiotemporally, well-controlled microenvironments; and (iv) the option of real-time monitoring. These provide a means to recreate and probe important (patho-)physiological structures and conditions.

Capitalizing on these advantages, microdevices are emerging as a powerful tool for nanomedicine [35]. This includes microfluidic technologies used during both engineering and evaluation of drug delivery particles [36]. In engineering, including both synthesis and characterization, microfluidics provides new and improved methods of making particles. In the evaluation, as long as some considerations are made [37-39], microfluidics provides new and improved *in vitro* models, models that are starting to make an impact on our understanding of crucial interactions between drug delivery particles and biological systems.

4. Engineering drug delivery particles through microfluidics

Both the materials and the methods used to engineer drug delivery particles determine their properties. As all methods have both strengths and drawbacks, there is an ongoing process in improving existing – and developing new – techniques toward more reliable and reproducible production of particles with highly tuned properties. One example of an area where there is significant ongoing activity is the fabrication of polymer capsules [40].

Microfluidic devices offer new possibilities for the production of both micro- and nanoparticles [41-43]. Mass transport in fluids is governed by both viscous and inertial effects, with the latter being responsible for the nonlinearities that give rise to numerous instabilities, such as turbulence. However, when fluidic flows are miniaturized, as in microfluidics, some inertial effects become negligible [27]. This can be utilized to construct devices where fluidic flows interact in highly controlled and reproducible ways. This is difficult – or even impossible – to achieve at the macro scale. These types of devices have been used to create both organic and inorganic particles for biomedical applications [44,45].

In this section, we provide an overview of different microfluidic mechanisms that can be used to produce drug delivery particles. These include the microfluidic production of micro- and nanoparticles using: (i) droplets; (ii) flow focusing; (iii) microvortices; (iv) templated assembly; and (v) flow lithography (Fig. 4). We also discuss challenges in microfluidics where inherently small volumes can lead to low throughput and approaches that are being investigated to facilitate scale-up and future industrial-scale production.

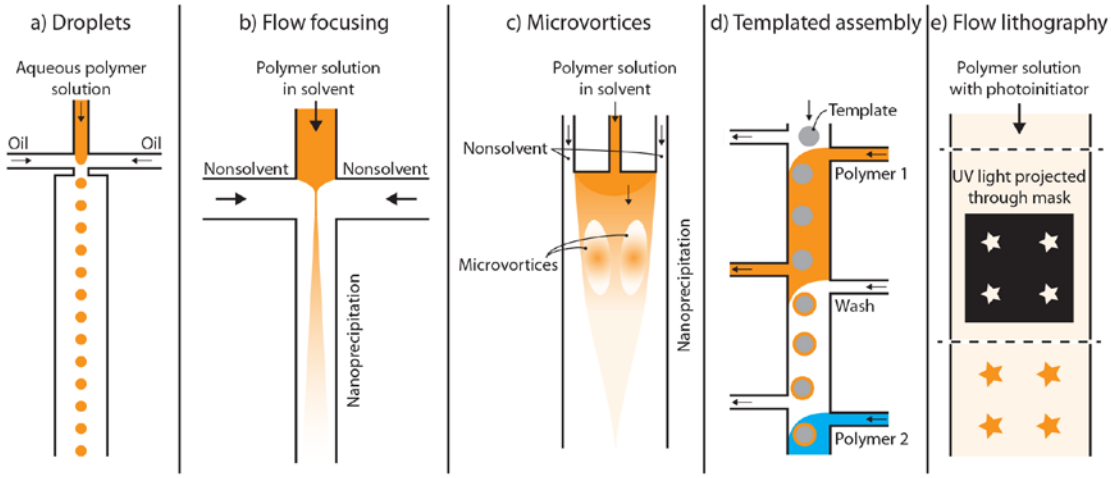

Fig. 4. Microfluidic methods to produce micro- and nanoparticles. **a)** Particles can be formed through droplet-formation in immiscible fluids. **b,c)** Flow focusing and microvortices can be used to create well-controlled liquid-liquid interfaces for nanoprecipitation of particles. **d)** In templated assembly, a template is moved between complementary polymer solutions to create multilayered particles. **e)** In flow lithography, UV light is passed through a shape-defining mask and projected down into a microfluidic channel where particles are formed through photopolymerization.

Droplet-based microfluidics are based on the use of immiscible fluids, including water/oil and liquid/gas, to create discrete droplets of precisely controlled size and composition (Fig 4a), and is a large field in itself [46,47]. The droplet generation is aided by the well-controlled interfaces and shear rates that can be produced in a microfluidic setting. One application of these emulsions is as templates to create microparticles for the engineering of drug delivery particles [48,49]. In a recent example, a microfluidic double emulsion technique was used to create core-shell microparticles through a one-step, solvent free process where a hydrophilic drug (doxorubicin hydrochloride) was encapsulated in an aqueous core surrounded by a lipid-shell containing a hydrophobic drug (paclitaxel) [50]. In another example, two different polymer solutions, with hydrophobicity tuned by using different ratios of poly(lactic acid)/poly(glycolic acid), were flowed side-by-side into a dispersing stream [51]. A droplet with two distinct halves ("Janus nanoparticle"), one from each solution, was formed and then released by shear forces from a continuously flowing dispersing stream. The particles subsequently solidified under nanoprecipitation in the dispersing stream and, by spiking the inlet polymer streams, this one-step method could encapsulate different therapeutics in each half of the resulting nanoparticles.

Flow focusing is the result of combining hydrodynamic forces with specific geometries and can be used to create micro- and nanoparticles (Fig. 4b) [52]. By hydrodynamically focusing a polymer in a solvent by a nonsolvent, tunable and rapid mixing can be achieved, leading to the tunable nanoprecipitation of particles. In an early example, poly(lactic-co-glycolic acid)-b-poly(ethylene glycol) was dispersed in acetonitrile and hydrodynamically focused using water in a microfluidic device with a "T"-geometry [53]. Flow rates, polymer composition and polymer concentration were found to be key parameters to optimize size, polydispersity and drug loading of the resulting nanoparticles. The same idea can also be used to generate nanoscale liposomes by having a lipid dissolved in isopropyl alcohol (IPA) in the central

channel with saline in side-channels [54], or to produce liposome-hydrogel hybrid nanoparticles using lipid-IPA in the central channel with poly(N-isopropylacrylamide) in saline in the side channels [55]. Multiple drugs can also be premixed prior to microfluidic flow focusing to prepare nanoparticles for combination drug therapy [56]. Materials with different solubility at different pH can also be used for nanoprecipitation. In a recent example, paclitaxel and chitosan, the latter hydrophobically modified using a lipid, was mixed under acidic conditions and then flow focused in a microfluidic device using side streams of water at basic pH, which induced nanoprecipitation and the formation of drug delivery particles [57]. Materials that require more complicated solutions, such as chlorinated organic polymer solutions and organic solvents, can also be used, for example by using glass microchannels [58]. To further improve the production of drug delivery particles, a few special geometries have been investigated in addition to the standard T-shape. This includes 3D hydrodynamic flow focusing where, in addition to the two lateral side streams, the center stream is also focused by two vertical streams [59]. Although this introduces some complexity, improved monodispersity and smaller sizes can be achieved as well as reduced problems with aggregation that can be seen using conventional 2D focusing. Further, special geometries have been used to increase the mixing rate and the throughput. Examples include staggered herringbone micromixers [60,61] and Tesla-type mixers [62]. In a recent example, 3D hydrodynamic focusing and micromixing were combined for the on-chip combinatorial synthesis of targeted drug delivery particles for cancer therapy [63]. Drug delivery particles with different size, zeta potential, ligand density and drug loading were all synthesized on-chip in a rapid and reproducible approach to form a library of 45 variants that were subsequently screened *in vitro* and *in vivo*.

Microfluidic devices operating at higher Reynolds numbers can be designed for the formation of drug delivery particles through controlled microvortices (Fig. 4c) [64]. For example, a central stream with poly(lactic-co-glycolic) acid in acetonitrile and side streams with lipid and lipid-poly(ethylene glycol) conjugates in 4% ethanol aqueous solution can be used to produce lipid-polymer hybrid nanoparticles at high Reynolds numbers (~ 150) [64]. By operating at these higher Reynolds numbers, where inertial effects contribute with convective mixing in addition to the diffusive mixing, a significant increase in throughput, reportedly a 1000-fold increase compared to conventional microfluidic diffusion-based syntheses, as well as improved reproducibility and homogeneity can be achieved [64]. This method has also been used for coencapsulation of multiple drugs and imaging agents as well as for the production of lipoprotein-derived nanoparticles, the latter by having apolipoprotein A in saline in the side channels and lipids plus imaging or therapeutic agents in a solvent (ethanol, methanol and/or chloroform) in the central channel [65],[66].

Templated assembly through layer-by-layer adsorption is a prominent technique through which multilayered films with tailored properties can be prepared, and this method has also been implemented in microfluidic devices [16]. Layer-by-layer buildup can be achieved by moving particle templates between complementary polymer solutions. In a microfluidic device this can be realized by having laminar streams of polymer solutions flowing side-by-side and using microfabricated structures to displace the particle templates back and forth [67]. Another strategy is to have the particle templates move through a central channel from which complementary polymer solutions are sequentially exchanged (Fig. 4d) [68]. This can

be achieved by cycles of adding and withdrawing solutions, but not the templates, from the main channel using side-channels where microfabricated structures keep the particles from exiting while allowing the polymer solutions to be withdrawn [68].

Flow lithography is a photolithographic technique where a pattern is projected into a photocurable polymer to form particles (Fig. 4e). In continuous-flow lithography, shape-defined photopolymerized microparticles can be made by passing UV-light through a transparency mask that is projected, using a standard microscope objective, down into a microfluidic device filled with monomer and photoinitiator [69]. The process can also be performed in a stop-start fashion [70]. Using flow lithography both the size and the shape of the particles can be tailored, as well as their degradation behavior [71]. This method has been used to encapsulate cells as well as to generate encoded particles for biomolecule analysis [72-74].

A key challenge in the translation of drug delivery particles from the lab to the clinic involves reliable and reproducible scale-up for industrial production. The inherently small dimensions and volumes of microfluidics, giving rise to the physics on which many of the advantages rest, such as laminar flow and well-controlled fluid-fluid interfaces, also pose challenges during scale-up toward industrial production [75]. Some processes, however, can be used in larger dimensions, allowing the use of millifluidic – instead of microfluidic – set-ups. Using a millifluidic system it has been demonstrated that grams of gold nanoparticles can be produced in a matter of hours, which is a thousand-fold improvement compared to common microfluidic techniques [76]. There are some microfluidic methods that can reach this high rate of production as well. One example is, as discussed earlier, by working at high flow rates and Reynolds numbers where controlled microvortices can be used to produce lipid-polymer hybrid nanoparticles allowing a production rate of ~ 3 grams per hour to be reached [64]. In addition to these advances toward higher rates of particle-production, a continuous fluidic platform also allows on-line implementation of other important stages, including post-synthesis functionalization and sterilization. In one example, hollow gold nanoparticles were synthesized, functionalized with poly(ethylene glycol) and sterilized with UV-light, all in the same continuous microfluidic platform [77].

In summary, microfluidics opens up the use of physics and chemistry that is difficult, or even impossible, to use at the macroscale. An important example is the relative lack of turbulence at the microscale, which enables well-defined and well-controlled fluid-fluid interfaces to be made and manipulated, thus enabling the use of interfacial processes, such as droplet formation and precipitation, in a more reliable and reproducible way. By complementing existing techniques, such as bulk emulsification and solvent displacement or centrifugation-based layer-by-layer assembly [40,78], microfluidic methods are already making an impact as seen by the examples highlighted above. These approaches are expected to play an important role in the production of new and improved drug delivery particles.

5. Evaluating bio-interactions of drug delivery particles through microfluidics

The objective of an engineered particle-based drug delivery system is to guide the interactions between a therapeutic and a biological system. Two important aspects of this are improving the localization and kinetics of drug exposure. Ideally, a drug

should only be released at the intended site of action, for example at a tumor for a cancer drug, and at a concentration and over a time frame that optimizes the therapeutic effect while minimizing toxicity. This dose-response relationship is different for different drugs but is critical for therapeutic effect. A well-investigated example is antimicrobial drugs, where usually one of several pharmacodynamics parameters, such as (i) peak concentration, (ii) area under curve or (iii) time over minimum inhibitory concentration, should be optimized [79]. To probe, understand, and ultimately direct these interactions is therefore of key importance in the development of drug delivery particles.

In this section, we first discuss how engineered *in vitro* microfluidic models can complement conventional methods. Secondly, we provide examples of how engineered particles behave under flow, including: (i) their mobility in constricted channels; (ii) their adhesion to cells; and (iii) their cellular uptake (Fig. 5). Finally, we highlight several studies where microfluidic devices have been developed to mimic human pathological conditions to investigate the behavior of engineered particles, with results subsequently validated *in vivo* using animal models.

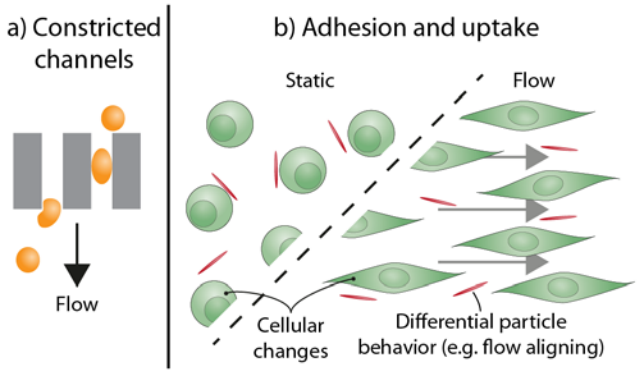

Fig. 5. Behavior of engineered particles under flow. **a)** Particles can be engineered with different properties (e.g., shape and softness) to behave differently in constricted channels. **b)** The adhesion and uptake of engineered particles is affected by flow conditions through changes in both cell behavior (e.g., cytoskeletal rearrangement) and particle behavior (e.g., flow aligning of non-spherical particles).

Conventional cell cultures and animal models have provided tremendous insights into interactions between drug delivery particles and biological systems, and remain the bedrock on which the field stands. However, there is a growing concern that these models have some significant limitations. For example, recently the genomic and transcriptomic landscape of the HeLa cell line, arguably the most commonly used cell line, was studied and strikingly aberrant characteristics were found [80]. Recent studies have also raised concerns on commonly used animal *in vivo* models and methods for the study of human conditions [81-84]. Therefore, there is a need to complement these techniques with methods that can capture other important aspects of human pathological states. Ideally these methods would allow the use of cells from more relevant cell sources, that can be difficult to maintain in standard culture dishes, in environments that more closely mimic human conditions.

Microfabrication technologies, in combination with microfluidics, offer tools through which aspects of an *in vivo* environment can be mimicked and responses to drug

exposure studied [85]. Exemplifying this are several microfluidic devices that have been developed to investigate dynamic spatiotemporal changes in drug exposure and corresponding biological responses. In one example, bacteria were grown in a microfluidic device mimicking naturally occurring bacterial niches and the heterogeneous microenvironment was studied as well as its influence on the emergence of antibiotic resistance [86]. In a similar example, drug gradients and motility of cancer cells within a microfluidic device were studied and it was shown that these aspects are important in the emergence of resistance to chemotherapy [87]. Microfluidic devices can also be used to study effects of drugs on multiple cell types in an integrated way, for example by culturing liver, colon and marrow cells in compartments connected by channels mimicking blood vessels [88]. Another multicompartment fluidic system, mimicking the *in vivo* fluctuations of antimalarial drugs, has recently been devised and demonstrated promise for the evaluation of compounds for the treatment of malaria [89]. Taken together, these studies demonstrate the potential of fluidic models to probe dynamic drug-bio interactions, thus increasing our understanding of processes that are difficult, or even impossible, to investigate using conventional methods.

Microfluidic devices can be used to investigate the behavior of engineered particles in constricted channels. Intravenous injection is a common route for systemic drug delivery and microfluidics can be used to create models of the vasculature [90]. Using these models, particle behavior in the microvasculature and in tumor vessels can be investigated [91,92]. This includes mechanobiological aspects of the *in vivo* setting and the impact of particle geometry and mechanics [93,94]. By changing the shape and stiffness of a microparticle, the ease with which they can squeeze through a small capillary can be tuned [95-100]. Red blood cells are another well-studied example, for example the impact of healthy and pathological states on their behavior in flow [101]. Generally, red blood cells can squeeze through blood microcapillaries smaller than their size, but become mechanically trapped (especially in endothelial slits 0.5 – 1 micrometer in diameter in the spleen) if they are stiffer [102]. This has important implications for the performance of engineered particles *in vivo*, as has been demonstrated using red-blood-cell shaped hydrogel particles where differences in stiffness caused differences in both biodistribution and circulation half-life in a mouse model [103].

Another important aspect of the behavior of engineered particles under flow is their interactions with cells. These interactions have important implications for the toxicity observed for some engineered particles [104]. An example is the discrepancy in observed toxicity between *in vitro* and *in vivo* models for quantum dots [105]. Interestingly, a difference in toxicity can also be seen when comparing static and fluidic *in vitro* methods [106]. A possible reason for this are sedimentation effects that are much more pronounced under static than fluidic conditions, effects that have been shown to strongly influence cellular association and uptake [107].

The adhesion of engineered particles to cells under flow conditions is influenced by both design parameters and flow conditions. The use of targeting ligands can provide an “anchoring effect” to enhance the specificity. However, an increase in flow rate also decreases the number of adhered particles, even in the presence of targeting molecules [108-110]. Shape effects have also been shown to be important. Examples include particles of different geometries (spheres, discs and rods) exhibiting different

adhesion profiles [111], and filaments tens of nanometer in diameter and micrometers in length demonstrating flow-aligning effects minimizing their cell interactions [112]. By combining the knowledge of how shape-effects and targeting ligands affect cell adhesion under flow, particles with higher specific and lower nonspecific accumulation under flow can be engineered [113].

The cell response to engineered particles under different flow conditions is influenced both by differences in shear stress and by a differential particle uptake by the cells. For example, the presence of flow increases the delivery rate of liposome–DNA plasmid complexes [114], while changes in shear stress modulate the cytotoxicity of engineered particles [115,116]. This difference in cellular responses is partly due to changes occurring in cells adapting to flow conditions. It has been shown that endothelial cells undergo significant cytoskeletal rearrangements when cultured in a flow environment, which is more similar to the cell physiology *in vivo* compared with the static cell culture environment [117], with the formation of actin stress fibers implicated as an important mechanism affecting particle internalization pathways and profiles [118-120]. This flow-mediated modulation of particle behavior has important implications for drug delivery, as it affects how cells interact with and process engineered particles.

In addition to studies discussed above, many of which are of a more fundamental nature, there have also been some studies where microfluidic devices that mimic pathological conditions of the *in vivo* situation have been developed and used to evaluate engineered particles with results validated *in vivo* using animal models. Here, we highlight a few examples of these studies and discuss the different steps involved.

In a study by Korin *et al.* [121], shear-activated drug delivery particles were designed and developed using a microfluidic model of an obstructed blood vessel and the efficacy was confirmed both in a microfluidic emboli model and in a mouse emboli model (Fig. 6). In the first step, pathological shear stresses associated with obstructed small blood vessels were identified and a microfluidic model mimicking these conditions was designed (Fig. 6a). Computational fluid dynamics simulations were used to refine and verify the design, which was then realized through soft lithography (Fig. 6bc). This microfluidic device was then used to evaluate the shear-responsiveness of microaggregates (~ 4 μm diameter) of poly(lactic-co-glycolic acid) nanoparticles (~ 180 nm) under physiologically relevant shear stresses (Fig. 6d). Nanoparticles were then coated with a thrombolytic drug before assembly into microaggregates, forming drug delivery particles. Following injection of emboli into another microfluidic device, these drug delivery particles were infused and were shown to accumulate at, and dissolve, the blood clots (Fig. 6e). In contrast, treatment with soluble thrombolytic drug at equivalent concentration had negligible effect. The efficacy of the drug delivery particles were subsequently validated in an *ex vivo* whole-mouse-lung ventilation-perfusion model. It was shown that drug delivery particles needed a hundred-fold lower concentration for a therapeutic effect compared to soluble drug. The therapeutic effect of the drug delivery particles was also validated in living mice by infusion of the particles, or the soluble drug, following injection of preformed fibrin clots. All seven mice in the control group (soluble drug) died within an hour while >85% (six out of seven) of mice treated with drug delivery particles survived without any symptoms (Fig. 6f). This clear increase in efficacy, combined with a rapid clearance and low diffusion, which could help to minimize

unwanted bleeding and neurotoxicity, illustrates how microfluidic models can facilitate the design and development of new types of drug delivery particles, for example by targeting pathological hemodynamic states.

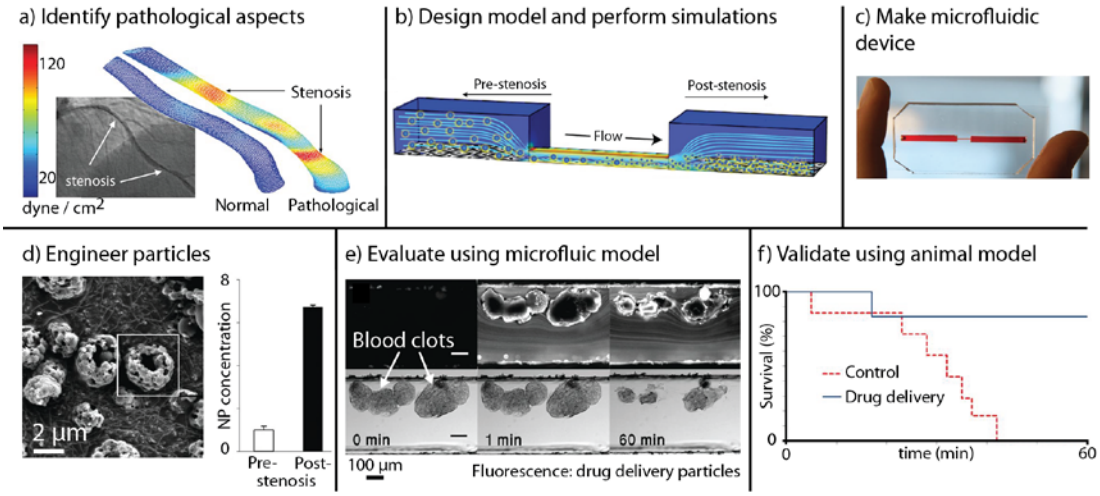

Fig. 6. Using microfluidic devices during engineering and evaluation of drug delivery particles. **a)** In vivo pathological aspects are identified and quantified. **b)** A microfluidic model mimicking these conditions is designed and simulations are performed to refine and validate the design. **c,d)** The microfluidic device is then made and used during the engineering of particles. **e)** A second microfluidic device is used to evaluate the thrombolytic performance of the drug delivery particles. **f)** The results are validated using an *in vivo* mouse model. Adapted with permission from [121]. Copyright 2012 The American Association for the Advancement of Science.

Microfluidic devices have also been used to evaluate nanoparticle accumulation at tumors under physiological flow conditions, providing important insights into the mobility and retention of nanoparticles in tissues [122]. In that study, multicellular tumor spheroids were first cultured in 96-well plates and then loaded into a microfluidic device (Fig. 7). Each spheroid produced, and was surrounded by, a non-uniform layer of extracellular matrix (ECM) that acted as a physical barrier between the cells and the medium. The penetration into spheroids of gold nanoparticles (NPs) functionalized with poly(ethylene glycol) (PEG) in various sizes (40, 70, 110 or 150 nm in diameter) was investigated. It was shown that the nanoparticles with smaller dimensions (40 and 70 nm) entered the spheroids and accumulated in the interstitial spaces to a greater extent than the larger ones, suggesting size is a key factor governing tissue penetration. By functionalizing the nanoparticles (40 nm) with transferrin (Tf), the accumulation was further improved by more than an order of magnitude (both inside the spheroid and in the ECM) compared with PEG-NPs. Interestingly, Tf-NPs showed an enhanced retention compared with PEG-NPs during washing, indicating a significant “anchoring effect” by the targeting molecule. It was also shown that the tissue accumulation was responsive to the flow rate. A nine-fold increase in flow rate resulted in a two-fold increase in both PEG-NP and Tf-NP accumulation. However, it was noted that both Tf-NPs and PEG-NPs accumulated predominantly in the ECM surrounding the spheroid, and that the change in accumulation under different flow rates occurred almost exclusively at the spheroid’s outer layer. This indicates that diffusion is the main mechanism through which

nanoparticles penetrate the spheroids. These findings on size dependence, tumor accumulation and targeting effects were further validated in an *in vivo* murine xenograft tumor model, suggesting microfluidic models can be used to mimic physiological microenvironments to deepen our understanding of complex biological interactions. Moving forward, this capability could play a part in addressing important clinical challenges. For example, it can be envisaged that microfluidic devices could be used to help elucidate underlying mechanisms of drug resistance, as tissue distribution and drug release kinetics influence resistance, and therefore help guide future developments in particle-based drug delivery [123,124].

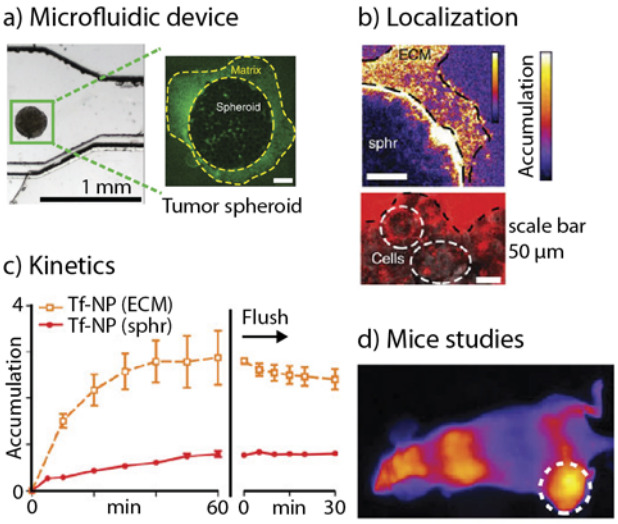

Fig. 7. a) Microfluidic tumor-model to probe tissue transport of engineered particles. **b,c)** Localization and kinetics of tissue accumulation of engineered particles was evaluated. It was observed that nanoparticles predominately accumulated in the extracellular matrix (ECM) surrounding a tumor spheroid (sphr) and that a targeting ligand (Tf) provided an "anchoring effect", reducing efflux during flushing/washing. **d)** Results obtained using the microfluidic *in vitro* model was largely in agreement with those obtained in an *in vivo* mouse model. Adapted with permission from [122]. Copyright 2013 Nature Publishing Group.

In a study by Kim *et al.* [125], a microfluidic device with two compartments separated by an endothelial layer with controllable permeability, was used as an atherosclerotic endothelium model to study the translocation of engineered particles, and the results were compared to an *in vivo* rabbit model (Fig. 8). Permeability was monitored through transendothelial electrical resistance using four integrated electrodes. An inflammatory cytokine (TNF-alpha), involved in the pathogenesis of atherosclerosis, was infused into the device at concentrations seen clinically, which caused disruption of intercellular junctions and the endothelial permeability to increase. Different flow rates, with different resulting shear stresses, could also be used to modulate the permeability. Lipid-polymer hybrid nanoparticles were infused into the device and their translocation across the endothelial layer was monitored. Interestingly, a significant correlation between nanoparticle translocation and endothelial permeability was observed. Rabbits with induced atherosclerotic lesions were used to study the *in vivo* behavior of the engineered particles and using near infrared fluorescent imaging, particle translocation was investigated both in microfluidic

devices and in rabbit aortas. A high degree of correlation ($r^2 > 0.8$ and $P < 0.0001$) was observed between permeability and particle translocation for both methods, indicating the validity of the microfluidic model. Taken together, the results demonstrate that particle translocation primarily occurs at sites of increased permeability, which has important implications for targeting of drug delivery particles to atherosclerotic plaques. Further, the similarities between the microfluidic model and the *in vivo* model demonstrates the potential of the former as a tool to probe the translocation of engineered particles in a highly controlled, and physiologically relevant, manner.

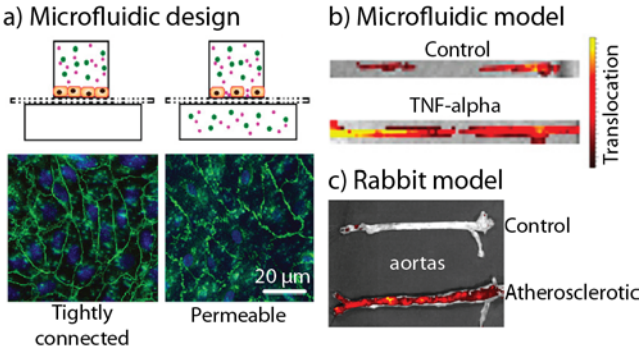

Fig. 8. Microfluidic and animal models of atherosclerosis to probe endothelial translocation of engineered particles. **a)** An endothelialized microfluidic device with controllable permeability was engineered. **b,c)** Integration of data from a microfluidic *in vitro* model and a rabbit *in vivo* model provided insights into the extravasation of engineered particles with implications for the treatment of atherosclerotic plaques. Adapted with permission from [125]. Copyright 2014 National Academy of Sciences USA.

In an early study by Huh *et al.* [126], a microfluidic device was used to reconstitute the air-blood alveolar-capillary interface of the human lung by culturing human alveolar epithelial cells and pulmonary microvascular endothelial cells on each side of a membrane, facing air or liquid microfluidic channels, respectively. Physiological breathing motions were recreated by applying cyclical mechanical lateral stretching over the cells. Nanotoxicology studies were then performed by exposing the epithelial side to 12 nm silica nanoparticles. Exposure to the nanoparticles resulted in a significant increase in expression of a leukocyte adhesion molecule (ICAM-1), reactive oxygen species production, and translocation of the nanoparticles across the alveolar-capillary interface. Importantly, both the inflammatory responses and the translocation of nanoparticles were significantly augmented by the application of simulated breathing motions, an effect that could not be studied in controls using conventional transwell cultures. To validate the microfluidic model, a whole-mouse-lung ventilation-perfusion model was used. The results were in agreement with what was observed using the microfluidic model. The system presented in this study has also been used to investigate the toxicity of drugs and in the discovery of new drug candidates [127].

In summary, microfluidic devices provide new methods through which engineered particles can be evaluated. Both for more fundamental studies, such as how they behave under flow in constricted dimensions and how flow affects their interaction

with cells, but also in more applied studies, such as how engineered particles behave in more complicated dynamic pathological settings, including the tumor microenvironment and at the site of a blood clot. By integrating microfluidic assays with conventional *in vitro* and *in vivo* assays, a more complete understanding of the spatiotemporal performance of drug delivery particles can be reached, an understanding that is essential for the rational design of particles with highly defined drug release kinetics localized to a target site.

6. Conclusion and outlook

Engineered drug delivery particles have over the last half a century moved from being primarily within the realm of science fiction into technological reality [128]. This development has been fueled by convergence of several disciplines, including biology, chemistry, engineering, materials science, and medicine. This highly interdisciplinary nature introduces some significant challenges but also provides ample opportunities, provided that researchers from disparate fields can coalesce around a common focus [129]. Illustrating this is the field of microfluidics as discussed in this review, where microfabrication techniques (commonly used by physicists) are used to recreate physiological environments (requiring biology expertise) to investigate drug delivery particles (typically designed and made by materials scientists and chemists).

A key feature of microfluidic devices when used to evaluate drug delivery particles is the ability to model physiological settings with variable complexity. The starting point can be a very simple model that only captures a part of the (patho-)physiological setting, which can then be expanded by adding components to mimic more complicated scenarios. Bioengineering techniques such as 3D cell culture and tissue engineering [130,131], as well as recent advances in applying microfluidic devices for purification and manipulation of stem cells [132-134], provide powerful tools that can help generate increasingly sophisticated and realistic models. Prominent examples are so-called “organ-on-a-chip” microdevices [135,136]. In these devices, multiple cell types are cultured in environments microengineered to reconstitute the tissue arrangement observed in specific organs. Examples of organs from which microfluidic devices have been based include the lung, liver, kidney and heart [137]. The use of these microfluidic devices holds promise for the development and translation of new therapies by allowing one to probe the behavior and response of multiple tissues, and even multiple organs, in an integrated and highly controlled and defined setting [138-141]. However, as the model becomes more and more realistic the results can become more relevant, but at the cost of more complicated operation and more convoluted behavior which can make conclusive relationships difficult to elucidate. This flexibility of microfluidic devices thus allows optimization of the trade-off between simplicity and realism to a degree not possible using conventional *in vitro* and *in vivo* methods, therefore opening up new and improved complementary assays to illuminate important relationships and mechanisms governing particle-bio interactions.

For microfluidics to continue to increase its impact on the field of drug delivery, the development toward increased and simplified access for non-experts is important. Even though there are laudable efforts to disseminate know-how (e.g. [28,142-144]),

significant hurdles still exist for researchers without prior microfluidic experience before they can apply microfluidic technologies. Part of the challenge lies in the differences in handling between a microfluidic and a standard cell culture. This means that currently existing cell culture skills and expertise might not be directly translatable to the microfluidic setting. For example, the sealed nature of typical microfluidic devices can complicate seeding and attachment of cells, and changes in media are commonly performed using fluidic-based injection instead of conventional pipetting. One can compare this to the history of the flow cytometer that started out as custom-built instruments requiring highly specialized knowledge from its users in optics, electronics and fluidics and then evolved into commercialized "black box"-type instruments that only required more general knowledge of the strengths and limitations by the day-to-day user [145]. In large part due to this, flow cytometry is today one of the most widely used and powerful techniques in biomedical research. In light of this, recent developments within microfluidics toward commercially available off-the-shelf, out-of-the-box tools are highly interesting. In the present review, most studies discussed use devices produced by the researchers themselves, typically through soft lithography [28], but there are also several examples of studies using devices and set-ups from commercial sources. This includes a parallel plate chamber from Cytodyne (La Jolla, CA, USA) [114], a microfluidic platform from Cellix (Dublin, Ireland) [119], and a microfluidic flow chamber from Ibidi (Munich, Germany) [116]. Another example of interest is the microfluidic platform developed by CellASIC (Hayward, CA, USA) [146,147] and commercialized by Millipore (Billerica, MA, USA).

In summary, as new and more comprehensive biological *in vitro* models are developed at the same time as the commercial availability of microfluidics is increasing, microfluidics is poised to make a significant impact by enabling new and improved ways of engineering and evaluating drug delivery particles.

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