Microparticles in Contact with Cells: from carriers to multifunctional tissue modulators

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Abstract

For several decades microparticles have been exclusively and extensively explored as spherical drug delivery vehicles and large-scale cell expansion carriers. More recently, microparticulate structures gained interest in broader bioengineering fields, integrating a myriad of strategies that include (i) bottom-up tissue engineering, (ii) three-dimensional (3D) bioprinting, and (iii) development of tissue/disease models. The concept of bulk spherical micrometric particles as adequate supports for cell cultivation has been challenged, and systems with finely tuned geometric designs and (bio)chemical/physical features are current key players in impacting technologies. Herein, we critically review the state of the art and future trends of biomaterial microparticles in contact with cells and tissues, excluding internalization studies, and with emphasis on innovative particles' design and applications.

Glossary

3D printing: additive manufacturing enabled by computer-aided technology that allows the precise deposition of a binder material into a complex architectural structure in a layer-by-layer logic.

3D Spheroids: spherical-shaped multicellular aggregates with improved cell-cell and cell-extracellular matrix (ECM) interactions, closely mimicking the microenvironment found in *in vivo* tissues.

Bioink: a liquid/viscous biomaterial that may contain cells and/or biological molecules which is processed by bioprinting technology through material extrusion and deposition into a spatially controlled pattern, during which its viscosity and elastic character will increase.

Bioinstructive: with the ability to influence the behaviour of biological systems, including cells and tissues.

Capsules: a closed-like system separated from the outer environment by a membrane barrier – shell – surrounding a core that can be presented as liquid, hollow or matrix composed.

Cell Stacking: methodology for cell expansion based on the parallel growth of cells on piled up tissue culture flasks.

High-Throughput Screening: methods that allow a fast acquisition, processing and analyses of large amounts of data.

Injectable scaffold: supporting matrix that possesses suitable physical and mechanical properties to be injected through a syringe or a catheter and to perfectly fit and fill a certain defect without the need of invasive interventions.

Microcarrier: supporting matrix characterized by a high surface area-to- volume ratio, allowing large-scale expansion of anchorage-dependent cells and *in vitro* production of biologically-active molecules.

Microparticle: micrometric sized (ranging 1-1000 μm) particle that is extensively used in biotechnological and biomedical fields as drug/cell-delivery platforms.

Modular Tissue Engineering: engineering of hierarchical and biologically-functional structures with precise architectural features through assembly of modular building-blocks using a bottom-up approach.

Multi-compartmentalized particles: biomaterial within the micrometric size range comprising architectural features that enable different chemical compositions over its spatial extension. Well-established examples of multicompartmental particles include Janus particles and co-axial multilayer (onion-like) particles.

Off-the-shelf: amenable to be used directly without any substantial handling, and "as is", independently from any establishment of settings during a pre-order procedure.

On-demand: an action that is dependent on the application of external stimulus/stimuli by the user.

Organoid: *in vitro* miniaturized organ with self-organized organ-specific cell types in an accurate spatial manner that are able to replicate physiological functions.

Tethered: attached/immobilized onto a surface.

Xeno-free: free of xenogeneic (originated from a different species) or animal derived-components.

Microparticles as cell adhesive and modulating moieties

Over the past few decades, microparticles have gained increasing relevance in tissue engineering and biotechnological strategies. Apart from their mostly widespread application as drug delivery reservoirs with precise local targeting abilities and highly controlled release profiles, a very explored application of microparticles in direct contact with cells is their use as **microcarriers** (see Glossary) for large-scale expansion and differentiation of adherent cells in bioreactors. A plethora of chemical and structural

77 microparticles' formulations has been explored in the search for the most effective and
78 compliant cell expansion strategies, culminating in the exploitation of different types of
79 materials processed with completely different features [1]. The biotechnological value
80 of microcarriers was proven with high yield *in vitro* production of growth factors (GFs)
81 and other soluble molecules, as well as for the rapid expansion of clinically-relevant
82 cells, including stem cells [2,3]. Despite the promising reported outputs, advances in
83 microcarriers design and their optimization to adapt to **xeno-free** scalable and clinically
84 translatable setups, as well as their ability to modulate cell response, are still a growing
85 trend in several segments of biotechnology and biomedical fields [4].

86 More recent trends have been exploring the potential of micrometric particles beyond
87 the 'carrier' application. They have been successfully used as injectable/fit-to-defect
88 moldable systems proven to form adequate robust 3D structures for *in situ* tissue
89 regeneration [5]. Specialized activities including the ability to selectively recruit different
90 cell types and inducing highly localized responses through the presentation of cell
91 membrane-interacting domains (e.g. GFs) have been important in the advance of
92 vascularization strategies in tissue regeneration, on the fine spatial control over cell
93 differentiation, or on the induction of therapeutic potential [6–9]. The incorporation of
94 biomaterial microparticles into multicellular structures (e.g. **3D spheroids**) has allowed
95 the development of *in vitro* platforms for the generation of complex 3D tissue/disease
96 models, including multicellular tumor models [10–12]. Moreover, the advent of 3D
97 bioprinting brought new insights on possible applications of microparticles as
98 reinforcement units within **bioinks** produced to regenerate injured tissues [13].

99 With this review, we aim at providing a critical discussion about well-reported particle
100 design factors capable of modulating their (bio)chemical/physical and architectural
101 features (Figure 1), and how those characteristics correlate with cellular response
102 outputs [7,14]. Novel trends on microparticles design and engineering - including
103 controlled size, geometry, and anisotropy - will also be addressed, as well as their
104 potential on healthcare-related applications. We will discuss microparticles fabrication
105 and highly enabling technologies to produce finely modulated structures with tailored
106 chemical patterns, well-established surface area-to-volume ratios, complex geometries
107 and anisotropy, as well as multicompartmental features.

(Bio)physical and biochemical tailoring of microparticles: applications, needs and technical constrains

Microparticles with controlled (bio)physical aspects

It has long been known that biomaterial properties can affect and modulate several biological outputs [15]. By merely tuning physical properties of the particles such as size, geometry, anisotropy, topography, stiffness, porosity and compartmentalization, it is plausible to achieve specific biological responses (Table 1) [16–18]. However, the fabrication of microparticles with such desired and controllable physical attributes through conventional methods, namely through emulsion polymerization, still remains a challenge. To overcome these difficulties, various methods were developed in the search of a processes capable of rendering versatile particle with tuneable surface features and morphologies. Microsphere reshaping comprises a simple and scalable method to produce anisotropic complex-shaped particles based on the distortion of microspheres through film-stretching [19] or moulding techniques [20]. This technique uses spheres as the starting material and comprises two main steps: (i) liquefaction, where they are exposed to solvents/vapours or temperatures above polymers' glass-transition temperature and deformed until a desired shape is achieved, and (ii) solidification by extracting the solvent/vapours or by cooling the temperature of the system. Besides being an easy and versatile method, this method may induce damage to the properties and microstructure of the starting material through the exposure to aggressive solvents, which might affect biological activity. Enhancing the gentleness of the procedure can be achieved by using only the vapours of organic solvents, instead of the liquid form [20]. Electrohydrodynamic (EHD) co-jetting is another technique that exhibits great control over particles' anisotropy, size and shape [21]. Similarly to electrospinning, the application of an electric potential results in the stretching of a pendant droplet – the Taylor cone – allowing the formation of well-defined particles with great control over anisotropy, size and geometry, through rapid solvent evaporation. This technique often renders fibers and spheres, but control over several process parameters, such as flow rate and polymer concentration, enables the fabrication of disk- and rod-shaped particles. One particular feature of EDH co-jetting is the ability to produce chemically distinct and **multi-compartmentalized particles** which

can be advantageous for controlled drug delivery or cell targeting [22]. Moreover, this technique is compatible with both aqueous and organic solvents, enabling the processing of tailored particles using a wide range of polymers. Alternatively, microfluidics is a versatile method to obtain intricate particle designs with high precision. Besides being suitable for cell encapsulation with tuneable sizes and shapes [23], compartmentalized particles are also easy to attain. Structures with core-shell and multi-core organization, Janus and ternary set-ups [24], internal anisotropic features [25] have been obtained via microfluidics. Tailored porous structures were also achieved through insertion of porogens, such as fine oil droplets, or even through phase inversion [26]. Microparticles are often synthesized using difficult to remove oils, and seldom applied UV-polymerization strategies may be hazardous for biological applications and even to sensitive materials. Highly multifaceted structures may also be processed through microfabrication by the application of different techniques such as photolithography and soft lithography, using photomasks or elastomeric stamps/moulds, respectively [27]. These complex microarchitectures with high resolution have been assembled to fabricate fillable core-shell particles, providing a viable platform for a pulsatile and continuous release of soluble molecules [28]. More recently, the use of bioinspired and biomimetic platforms, namely superhydrophobic (SH) and superamphiphobic (SA) surfaces based on the high repellence of water and/or low-surface-tension liquids ('oils'), has driven the formation of liquid droplets with perfect spherical shape [29]. Inspired by such unique properties a new and cost-effective tool to produce engineered polymeric microspheres and **capsules** using mild conditions was created, allowing the fabrication of hierarchical systems [30]. To suppress the need of multiple pipetting or complex machinery and to allow the fabrication of non-spherical hydrogel particles, a droplet microarray platform combining SH or SA properties was developed [31]. This technique enabled the patterning and retrieval of microparticles with several different geometrical structures, including hexagons, triangles and even heart-shaped particles. Despite the multiple platforms and methods to produce particles with highly intricate and sophisticated structures, there is still a great need of a versatile system to enable the control and tuning of physical properties with high precision and resolution.

Microparticles with controlled biochemical cues

The control over cellular behaviour is dictated not only by the aforementioned physical aspects of the material, but also by (bio)chemical interactions (Table 1). Strategies based on the presentation of chemical domains to cells through microparticles often comprise the precise and a spatiotemporal delivery of soluble factors to achieve specific paracrine effects through soluble signalling [6,32]. The bulk of the microparticles can be used to encapsulate factors that may be diffused to the surface and be available to control cellular mechanisms [33]. However, the presentation of **tethered** biomolecules is found to improve the ability to direct and modulate cellular response by usually mimicking key components present in native tissues [34]. The most common practice is the immobilization of full-length extracellular matrix (ECM)-derived proteins, such as laminin and fibronectin, onto microparticles' surface aiming at cell matrix signalling replication and therefore, promoting cell adhesion [35]. Apart from mediating cell attachment and proliferation, these bioactive molecules can also provide signals that trigger cell aggregation and modulate cellular migratory behaviours depending on the selected coating. [36,37]. Another ECM-mimicking strategy is the use of decellularized tissue which can better recapitulate the innate microenvironment while providing a native-like and tissue-specific milieu [38]. Besides recreating cell-ECM contacts, it also has been developed systems that mimic cell-cell signalling with adsorption of cellular adhesion molecules (CAMs), namely E-cadherin fusion protein [39]. Being a key regulator of intrinsic cell-cell interactions, it is capable of mediating growth-promoting cell signalling pathways, promoting cell self-renewal, and improving induction or maintenance of stem cell multipotency. To simplify the workload bared by using full-length proteins, the use of biological motifs became widely popular for their relative ease of availability and lower cost of preparation [40]. Among other short peptides of interest, the cell adhesion properties of the RGD peptide (arginylglycylaspartic acid) have been intensely exploited in material functionalization. Present in fibronectin is also found in other ECM proteins such as laminin and vitronectin, has the ability to retain its cell-binding properties and to be recognized by several cell surface integrins, enhancing cellular adhesion [41,42]. Antibodies are another protein family of vast interest in tailoring the surface of microparticles due to their ligand binding specificity and their

ability to improve cell adhesion. It provides microparticles with additional and specialized activities, allowing a selectively recruit of different cell types and/or bioactive molecules [8]. This not only allows a better control over cellular function but also provides a feasible platform for specific cell isolation and capture from complex mixtures [9]. Likewise, immobilization of GFs has been a promising approach for providing cues in a well-controlled mode, overcoming limited efficacy shown by diffusional problems of soluble factors while inducing localized effects. For instance, immobilization of vascular endothelial growth factor (VEGF) proved to be a pro-survival agent for cell-based therapies [43] and tethered basic fibroblast growth factor (bFGF) and transforming growth factor β1 (TGF-β1) enhanced cell attachment and proliferation, and also stimulated locally chondrogenesis [44]. Moreover, coating of particles with a specific cell membrane is gaining attention, creating a cell-mimicking microparticle which emulates cell function. Acting as **'**synthetic cells', they have the ability to recapitulate biointerfacing activities of the natural cells [45,46]. Surface functionalization with other cues such as **bioinstructive** polymers have also been employed to modulate cellular responses and recreate a more native environment. Hyaluronic acid (HA) and poly-L-lysine (PLL) microparticles assembled through Layer-by-Layer (LbL) deposition was recently shown to increase cell-anchoring hotspots while simulating an ECM-like environment for the cells [11]. In addition to the different surface coating possibilities, the functionalization method is another key cell behaviour modulator. Stable covalent modifications have provided a stronger support for cells, leading to better cell attachment and spreading, while week and less stable coatings, as of those of surface adsorbed molecules, promoted a more efficient cell release and are more sensible towards cell migration [37]. Although surface modification through immobilization of various cues allows a fine-tuning over material bioactivity, many biological processes and mechanisms in which such decorative moieties play an important role are yet to unravel. Such know-how may help understanding how cell behaviour can be affected and what are the molecular mediators of such process.

Multidisciplinary Microparticles: translating processing know-how into useful applications

Microcarriers for the ex vivo *expansion of primary cells and stem cells*

As tissue regeneration approaches keep growing at a fast pace, cell-based therapies demand large quantity and high-quality cell numbers. In fact, it is estimated the need of millions to billions of cells per patient for the treatment of a disease. This is a result of the low cell retention in the defect area and also the significant shortage of cells in cell banks. Therefore, the development of an optimized cellular biomanufacture procedure to generate clinically-relevant cell numbers is in demand. A variety of methods for the large expansion of cells have been described, and the multi-tray system in culture flasks, also known as "**cell stacks**", is the most prevailing method. Adopting a scale-out, rather than a scale-up approach, requires a substantially amount of space and manual labour. To bypass such hurdles, microcarriers have been progressively replacing the conventional two-dimensional (2D) flat approach, and were found to outstand the performance of other expansion technologies [47]. In fact, not only achieved cell densities are significantly higher, boosting cellular yields in the overall process, as morphological aspects and mechanosensing properties of the cells can also be modulated by the surrounding environment, inducing changes in both cytoskeleton and nuclear dispositions, and altering cytokine production rate and expression levels of specific cell markers. Over the years, microcarrier culture within bioreactors have proved to be an easily scalable support for expansion of both primary and stem cells (Figure 2A) [2,48]. Their highly enhanced surface area enables an increase in cellular yields in a clinically-relevant time-frame, leading to an **off-the-shelf** approach to be used "**on-demand**". Moreover, the combination of process automation, control and monitoring leads to a more robust and cost-effective technology, replacing laborious and poorly controlled processes [49,50]. Consequently, a plethora of microcarriers with different physicochemical properties have been developed and commercialized in the search for the most effective and compliant cell expansion strategy (Table 2). Regardless of being a very promising approach, microcarriers are often employed in dynamic conditions which promotes hydrodynamic shear stress to the cells. Engineering of either hollow and highly-porous particles provide shelter and allow in-growth of shear-sensitive cells, while only the latter offers a larger culturing surface due to the skeletal structure with highly-interconnected pores, supporting cell attachment and promoting

multidirectional cell-cell interactions [1,5,51,52]. However, the traditional concept of the microcarrier as, solely, an expansion technology is becoming obsolete. This system can not only integrate a cell differentiation approach or even act as a transfection agent together with expansion, but also serve as cell delivery vehicles and as modular building-blocks for tissue regeneration (Box 1) [53–55]. Towards these approaches, different materials showcasing features as biodegradability and suitability for implantation were exploited. Those have been processed with different features in order to adapt to the selected application, while avoiding the need to harvest cells via enzymatic treatment which comprises one of the biggest liabilities of microcarrier culture [1,51,56]. Recently, the pursuit of an optimized xeno-free approach has gained momentum, promoting a facilitated translation to clinic setups for *in vivo* applications. Therefore, many strategies have been explored to develop xeno-free microcarriers using ECM-inspired synthetic coatings, such as vitronectin, albumin and laminin, avoiding the need of animal-derived components [50,57–59]. However, xeno-free carriers go beyond the synthetic ECM-based approach. The use of synthetic hydrogels (e.g. polyethylene glycol (PEG)) can offer a viable platform to engineer custom-made particles with the desired mechanical and degradability properties. [4] To this extent, various efforts have been taken to improve and upgrade this culture system beyond its well-established and traditional application as an expansion technology leading to a more wide-ranging and translatable setup suitable for *in vivo* regeneration.

Microparticles as building-blocks for tissue engineering and regeneration

Promising applications of microparticles have been reported for their use in the construction of *in vitro* tissue engineering models targeting drug screening and organ-on-a-chip platforms [60], as well as for *in situ* tissue regeneration as an 'one-fits-all' platform to minimize vastly invasive surgical interventions [5]. In fact, the need of an injectable/fit-to-defect moldable scaffold designed to accurately fill any defect site regardless of its shape is of the utmost importance, due to the complexity required to repair any irregularly shaped deformity (Figure 2B). Despite virtually being the simplest to administrate, 'bulk' hydrogel-based injectable systems can often fail to provide sufficient mechanical stability and durability to support anchorage-dependent cell proliferation and differentiation before the neotissue formation. The application of

biodegradable and biocompatible cell-laden microparticles as modular building-blocks could be a suitable and viable way to overcome such limitations [61,62]. For instance, a highly open porous particle with proper surface pores and interconnected passages which protected cells against stress during injection proved to be a viable method to host cell growth and to carry/deliver them to target sites [63]. Moreover, hydrogel-based systems often require prolonged periods of irradiation or the presence of toxic chemical cross-linkers for the *in situ* gel formation which can be damaging for cell survival. One of the employed strategies to surpass such obstacles comprises the use of particles with inducing gel formation properties where porous and biodegradable microparticles are used as cross-linker carriers to allow *in situ* hydrogel formation under physiological conditions [64]. Another approach was demonstrated by Yu and co-workers who fabricated chitosan microparticles as modular components for tissue engineering, with an ECM-like nanofibrous structure using a physical gelation process without resorting to any toxic or denaturizing agent [65]. Besides acting as cell-anchoring and delivery platforms, these particles can integrate specialized activities such as the ability to selectively recruit different cell types through presentation of bioinstructive moieties (e.g. antibodies and GFs) aiming a better control of cellular function [8,9]. Furthermore, they can also induce highly localized responses, modulating the surrounding microenvironment, acting as life-like 'synthetic cells' capable of communicating with their counterparts and induce biological functions, such as protein production [66] and even emulate stem cell function during tissue repair [45]. Self-assembly of multicellular aggregates with incorporated microparticles can establish interconnected networks and can lead to the formation of robust macroscopic tissue constructs with mechanical stability. For instance, gelatin microspheres were incorporated within self-assembled vascular tissue rings as GFs delivery vehicle and to improve its mechanical properties and morphology [6]. This potentiates the spatially control release of bioactive molecules to help overcome diffusion limitations and allows control of tissue structure and function in order to fabricate more intricate constructs, aiming at novel vascularization strategies. Nonetheless, the assembly process of these building units is not only achieved by cell-driven organization and ECM deposition. In fact, the material itself can be designed in order to improve interlocking ability between contiguous particles, enabling a rapid *in situ* tissue biofabrication [67]. Microparticles

have proven to be a suitable approach for the bottom-up engineering of complex 3D constructs and as a plausible injectable system for the *in vivo* regeneration of several tissues such as cartilage [5,14,64,68], bone [63,69–71] and heart [45,72,73]. However, a few drawbacks are yet to overcome regarding implantation within the body. As the structure of the engineered construct might not be perfectly uniform, those can be prone to clogging and cause a blockage in the needle, causing cells to be exposed to a stressful environment due to shear stress that happens during extrusion, culminating in a decrease of cell viability. Once in the body, the particles may exhibit low retention and fixation in the defect area, and may diffuse to other sites, prompting inflammation and embolization, or impeding the particles' from contacting the surrounding tissue and performing their pro-regenerative role [64]. The scaffolding material and the control over its degradation rate are two critical aspects that may help decrease such problems.

Incorporating microparticles in in vitro *3D Tissues and Disease Models*

The generation of tissue-like constructs or organotypic structures is a fast-growing field, remarkable for therapeutic effects on Regenerative Medicine, with the aim to regenerate or replace tissues and organs [6]. Moreover, these structures are also relevant for research purposes in areas that include cell biology - used to understand underlying cell mechanisms -, and in drug-screening as platforms for toxicity assessment [35,74]. Nowadays, 3D cell culture methods which typically comprises the generation of scaffold-free spheroids cellular aggregates are promising strategies to replace well-established 2D cell culture approaches. Although they can better replicate the physiological tissues' microenvironments in a spatially relevant manner, there are still some limitations that may be surpassed by introducing biomaterials, including micrometric particles, into the cellular constructs [11,75,76]. A common limitation of cell-exclusive aggregates is associated with the lack of vascularization, which limits the transportation and diffusion of nutrients, oxygen or even drugs compared to a vascularized native tissue. Additionally, engineered extracellular environments can fail to reproduce intrinsic signalling cues and the complex organization of the native tissue. Introducing microparticles within the cellular aggregates constitutes a viable way to modulate the biochemical and physical properties of the microenvironment (Figure 2C). In fact, they can act as reservoirs, providing local and controlled presentation of soluble

and tethered molecules. Apart from providing the typical structural support for cell growth [77], they can be loaded with small molecules or present tethered proteins in a precisely-controlled spatiotemporal and uniform manner [78]. This approach proves to be more efficient for morphogen delivery than the simple soluble delivery and it aids directly the differentiation of stem cells. The presentation of cell adhesion molecules also represents a plausible way to direct cell fate and enhance biological functions, due to activation of several signalling pathways [79,80]. The presentation of differentiating moieties is imperative to drive cell lineage commitment, and naïve biomaterials have proven to also influence cellular fate throughout aggregates [81]. Moreover, they can better control aggregate structure, improving its mechanical properties [82]. These mechanically-tailored particles can modulate cytoskeletal organization and subsequently alter intracellular mechanotransduction signalling cascades [83]. Furthermore, they can also act like sensors, reporting key characteristics of the local microenvironment, such as oxygen and pH levels or even protease activity [84,85]. To this extent, can offer a great way for scale-up approaches and **High-Throughput Screening** (HTS) platforms.

Besides the modelling of healthy tissues [77], there is also the generation of several *in vitro* disease models [10–12,86]. Soker and colleagues demonstrated the creation of a liver-tumor hybrid organoid for tumor growth and as a metastasis model [10]. The use of a microgravity simulating Rotating Wall Vessel (RWV) bioreactor allied to cell culture onto HA and gelatin-coated microcarriers allowed the generation of 3D aggregates based on natural affinities resembling the physiological environment. The hydrogel-coated particles provided a scaffolding surface for cell growth while mimicking the naturally occurring ECM components, facilitating the suspended culture of adherent cells within the bioreactor and promoting an enzyme-free cell release through hydrogel degradability under mildly reductive conditions. In fact, expression of cell surface markers showed significant differences between 2D and 3D culture setups, where in the latter they were consistent with a metastatic phenotype, suggesting its higher relevance as accurate systems to create organotypic structures. Scaffold-free models often lack ECM-like cues and, therefore, there is a deficiency of pre-existing ECM components within the cell aggregate which prevents early ECM deposition, only to be cell-

assembled during culture periods, weakening the physical resistance. The incorporation of ECM-mimetics and spatial interconnectivity providers, namely instructive microparticles as cue providers to achieve on-demand biological responses, may improve the ability of the aggregates to better resemble the native physiology while affecting the synthesis of endogenous ECM, already at an early stage of the assembled constructs [12]. Such approach was also applied to engineer hybrid 3D *in vitro* lung tumour model with a robust architecture and an emulating tumour microenvironment which was possible through the incorporation of HA-coated microparticles [11].

Engineering microscaffold-based inks for 3D Bioprinting

3D Bioprinting is a promising biomanufacturing strategy that enables the fabrication of tissue-like constructs with custom-made architectures by the controlled deposition of a 'raw material' – bioink. However, since it is a relatively new technique there are still some challenges that need to be addressed. Besides the integration of a vascularized network within the constructs, another major challenge is to create functional and clinically-relevant grafts which requires the encapsulation of high amounts of cells [87,88]. Although hydrogels constitute the most desirable material type used for bioink manufacture, they are known for mostly providing highly hydrophilic and bio-inert microenvironments in which suspended cells are constrained to a round shape, regardless of cell type or native morphology that often result in cell depletion and low viability. Moreover, cell-encapsulation strategies in hydrogels are associated with cell constraints and fewer cell interactions due to inadequacy of cell spread and migration. Providing an anchor to support cell growth and proliferation has been suggested as a viable way to conquer this problem. A composite material comprising collagen microcarriers embedded in an alginate hydrogel containing collagenase provided not only a cell-affinitive interface but also sufficient cell spreading spaces upon collagen degradation [89]. Apart from these, most hydrogels are often portrayed as "soft" materials, lacking good mechanical properties for a proper bioprintability. While a hydrogel-based bioink can easily lose its structural integrity, a hybrid microscaffold-based ink, composed by cell-laden microspheres encapsulated in a thin agarose-collagen hydrogel layer, was developed to improve material stability during and post-print. The hydrogel acting as a glue to tightly pack the particles allowed a great improvement of

the compression strength compared to the scaffold-free hydrogel [90]. Inspired by the structural stability triggered by the capillary bridges found amid the wetted sand granules in sandcastle formation, Velev and colleagues developed an elastomeric ink composed of polydimethylsiloxane (PDMS) in the form of both precured beads coated with the uncured precursor liquid, which acts as a binding agent. [91] This capillary-based suspension ink renders extremely resilient, but delicate fibres with an excellent elasticity, and flexibility, and controlled porosity, holding a great potential in many biomedical applications. Burdick and colleagues also developed a granular bioink composed exclusively of densely-packed microgels [92]. In this work, cross-linked particles of various types of materials were extruded as stable filaments, either over a surface or within a hydrogel matrix, forming smooth aggregates without interparticle linkages, or even the need of any material as a binding agent. Systems such as injectable cell-laden microcarriers embedded in hydrogels (Figure 2D) have proven to not only provide platforms for cellular focal adhesion but also facilitate the cells to overcome gel enlacement and fully spread out into their natural morphology, maximizing cell-cell interactions while providing a structural support throughout the hydrogels' matrices [93]. In fact, a work from Mateos-Timoneda and colleagues shows the fabrication of living osteochondral constructs through bioprinting of mesenchymal stem cell (MSC)-laden polylactic acid (PLA) microcarriers encapsulated in gelatin methacrylamide-gellan gum bioinks [13]. It was demonstrated that PLA microcarriers not only allowed for highly cell-concentrated and viable structures but also improved bioink's compressive modulus, acting as reinforcement units that increase the mechanical strength of the gel without compromising the of the hydrogel network and its bioprintability. Furthermore, this system offered a high cell-anchoring surface that supported osteogenic differentiation and bone matrix deposition compared to cells suspended in the hydrogel system. In addition to these microcarrier/hydrogel hybrid bioinks, different types of materials can be exploited as the extruded material, replacing hydrogel-based inks. Considered as a "soft" material, the mechanical properties of hydrogels do not resemble those exhibited by hard tissues, such as bone. Müller an co-workers fabricated a biomechanically stable bioink with morphogenetic potential, suitable as a bone implant, composed by calcium polyphosphate (Ca-polyP) particles within a poly-ε-caprolactone (PCL) matrix [94]. PolyP promoted bone remodeling and regeneration, as PCL act as a

reinforcing material, hardening the scaffold to match that of the bone. Recently, bioprinting devices have been adapted and included in an automated bioassembly system allowing the generation of living constructs, suitable for clinical translation. A multistep bottom-up strategy that combined the fabrication of a layer-by-layer built scaffold and the co-assembly of cell-laden particles within the scaffold enabled the creation of complex hierarchical structures [95]. These evidences reveal the great potential held by microparticle incorporation within printable matrices through 3D bioprinting technology for the fabrication of biomedical models, although some improvements, encompassing nozzle clogging and possible toxic byproducts, are yet to be tackled [96].

Concluding Remarks and Future Perspectives

Microparticles are a multidisciplinary system that find application beyond the traditional delivery of drugs and other soluble molecules. Microcarriers with enhanced surface area proved their biotechnological value as an “off-the-shelf” approach for a rapid and efficient expansion and differentiation of countless clinically-relevant cells while their translation to the clinic remains a stumbling-block. Further research is expected to enable the design of advanced microparticles that showcase features, such as biodegradability, xeno-free set-ups and suitability for implantation to adapt to different applications (see Outstanding Questions Box). Several enabling technologies were explored to modulate microparticles’ physical and biochemical aspects and dictate several biological outputs. Still, standardized procedures that enable a precise correlation between material cues and their biological response are in great need to enlighten underlying mechanisms and predictable outputs. Microparticles with such desired features can easily find wider applications in many different fields, namely in bottom-up tissue engineering strategies as modular building-blocks to produce highly intricate 3D tissue constructs with great biological value, but also in 3D tissue and disease models as cue-providers to emulate the native environment, and in 3D bioprinting as reinforcement units of bioinks. Exciting novel trends comprise the use of completely synthetic polymeric hollow particles as life-like artificial cells capable of communicating with their counterparts and induce biological functions as protein production. The role of microparticles in synthetic biology is still to explore and may

bring outstanding breakthroughs in the development of completely autonomous or hybrid artificial biological systems.

Table 1. Interplay of microparticle (bio)physical and biochemical cues in cellular response driven by cell attachment to biomaterials.

Type of cue	Material/Moieties	Technique	Biological Response	Application	Ref
(Bio)Physical					
Size	Alginate	Aerodynamically-assisted jetting	Cell attachment and proliferation; Increase of microgel diameter led to a decrease of cellular growth; Cell differentiation exhibited no significant dependence on microgel diameter	Large-scale cell expansion	[97]
Geometry	-	-	-	-	Not Found[#]
Anisotropy	Poly-ε-caprolactone (PCL) with a distinct rough and a smooth surface on the opposite side	Micromoulding	Strong affinity to fibroblast over hepatocytes; The rough side absorbed large amount of proteins which enhanced cell-attractiveness, regardless of cell type;	Cell isolation and protein retrieving from a heterogeneous population	[17]

			Regulation of cell-adhesion and cell cycle-related genes		
Surface Topography	Polyethylene glycol diacrylate (PEG-DA) with wrinkled surface	Stop-flow lithography	Improved cell attachment and proliferation	Cell microcarriers; Cell physiological studies; Tissue engineering	[18]
Porosity	Polyhydroxyalkanoate (PHA)	G/O/W emulsion assisted with releases of carbon dioxide and ammonium bicarbonate degradation	High *in vitro* cell adhesion, continuous proliferation and improved differentiation of hMSCs; Supported osteoblast regeneration	Enhanced surface area cell carrier; Cell ingrowth and protection from shear stress; Tissue engineering as an injectable cell delivery system	[63]
	Chitosan	W/O emulsion-based thermally induce phase separation	Improved cell attachment, growth and spreading throughout the porous structure; Enhanced cellular activity and functions	Microcarriers for high-performance 3D cell culture	[1]
Compartmentalization	Various biodegradable polymers and a pH-responsive polymer.	Phase separation in microfluidics	-	Cell microcarriers;	[24]

				Selectively release therapeutic agents at acidic environments	
Stiffness	Polydimethylsiloxane (PDMS) with three different elastic moduli (soft, intermediate, stiff)	Curing of O/W emulsion non-crosslinked microdroplets; Different stiffness is attained adjusting the PDMS-curing agent ratio	Cell attachment and proliferation; Soft and stiff particles guided towards osteogenesis; Intermediate stiffness induced chondrogenesis, similarly to particle-free spheroids	Engineering toolkit for multicellular organoids in disease modelling and tissue engineering applications	[83]
Biochemical					
Antibody immobilization	Chitosan presenting anti-CD31 or anti-CD90	Aerodynamically-assisted jetting for particle fabrication and surface functionalization via Biotin/Streptadivin	Cell attachment and proliferation; Capture of HUVECs by CD31 and ASCs by CD90	Specific cell selection/ isolation from heterotypic cell populations	[9]
Growth factors immobilization	Collagen type I presenting bFGF or TGF-β1	Homogenization in Dispomix Drive system (Axonlab) for particle formation and functionalization via carbodiimide chemistry	Improved cell attachment and proliferation; Local stimulation of cells (chondrogenesis)	Expansion and chondrogenic differentiation	[44]

	Chitosan presenting Platelet Derived Growth Factor-BB (PDGF-BB), TGF-β1 and VEGF	Aerodynamically-assisted jetting for microsphere formation and functionalization via carbodiimide chemistry	Improved cell attachment and proliferation of ASCs	Tissue regeneration as an injectable cell delivery system	[8]
	Polystyrene-coated iron oxide microparticles presenting VEGF	VEGF immobilization via Histidine/Biotin/Streptavidin chemistry	Cell attachment and proliferation; Enhanced survival of outgrowth both in vitro and in vivo	Treatment of ischemic diseases	[43]
Biological motifs	Multi-armed PEG–vinyl sulphone presenting RGD	Microfluidic w/o emulsion for spherical microgel formation	Cell attachment and proliferation; Cell migration and integration in a 3D complex network	Tissue engineering as an injectable cell delivery system	[98]
	PEG-diacrylate (PEG-DA) presenting RGD	Polymer photo-polymerization and functionalization via acryloyl-PEG-RGDS	Improved cell attachment and proliferation	Tissue engineering of 3D vascularized microtissues	[42]
	RGD-coated PLA microcarriers	PLA particles were formed by atomization of the solution into droplets and then precipitated in a coagulation bath; RGD coating was achieved by either covalent modification or physiosorbed	Covalently-linked RGD showed a slight increase in cell adhesion and better cell proliferation capacity compared to the adsorbed coating;	Manipulation over cell adhesion and migratory potential of cells	[37]

				Surface adsorbed RGD enhance cell release, promoting a better cell migration ability		
Cell membrane-coated particles (Cell-mimicking microparticles)		Cellulose decorated with red blood cell membrane	Electrospraying for red blood cell-shaped microparticle formation and coating by sonication	Prolonged circulation time of the microparticles in the blood	Drug delivery	[46]
		PLGA decorated with cardiac stem cell membrane and secretome	W/O/W emulsion and membrane coating by sonication	Cell attachment and proliferation; Emulation of the paracrine and biointerfacing activities of cardiac stem cells	Therapeutic cardiac regeneration	[45]
Proteins	ECM-derived	Laminin- and fibronectin-coated melamine resin microparticles	Proteins were adsorbed to microparticles surface	Increased β-cells adhesion to fibronectin over laminin	Study islet cell biology	[35]
		Pancreatic decellularized matrix-coated PEG-co-PLL	Microfluidic for microspheres synthesis and absorption of decellularized tissue	Improved cell survival, expression of β-cell specific genes and glucose stimulated insulin secretion	Maintenance of β-cell phenotype and function *in vitro* for diabetes therapy	[38]
		Laminin- and vitronectin-coated PS particles with a PLL layer	Both laminin and vitronectin were adsorbed onto the particles	Combining the polyelectrolyte layer with the ECM protein, cell affinity was enhanced;	Generation of a large-scale cell expansion system under continuous agitation	[36]

				Laminin coating provide a better support for cell attachment and aggregation even under continuous agitation; Vitronectin coating requires a static pause to allow cell aggregation		
		Collagen-coated PLA microcarriers	PLA particles were formed by atomization of the solution into droplets and then precipitated in a coagulation bath; Collagen was covalently-linked and adsorbed onto carriers' surface	Covalently-linked collagen promoted a better cell attachment and proliferation; While adsorbed collagen promoted a mild cell attachment to the carrier, having a better cell release profile	Manipulation over cell adhesion and migratory potential of cells	[37]
	Cell adhesion molecules (CAMs)	PLGA decorated with E-cadherin fusion protein	O/W emulsion and solvent evaporation and protein immobilization via surface adsorption	Cell attachment proliferation and cytokine secretion	Cell expansion and controlled delivery of GFs	[39]
Polymers		PLL- and HA-coated PCL microparticles	O/W emulsion and solvent evaporation for PCL particles fabrication;	Cell attachment and proliferation	Tumour-ECM mimetic support;	[11]

		LbL deposition of PLL and HA for surface functionalization	Emulation of tumour environment by providing cell-ECM interactions and increased matrix deposition	Cell-anchoring hotspots; Study cell response to chemotherapeutics	

[#]Applications only found for cell internalization and drug delivery purposes.

Table 2. Physicochemical properties of commercialized microcarriers.

Microcarrier	Manufacturer	Material	Surface Feature	Shape	Size[#]/Pore size (µm)	Density (g/mL)	Surface Area (cm^2/g)	Storage Conditions	Harvesting method
Positively Charged (protein-free)									
Cytodex 1™	GE Healthcare	Dextran	DEAE	Spherical	147-248/n.a.	1.03	4400	RT	Trypsin
DE-52	Whatman™	Cellulose (biodegradable)	DEAE	Cylindrical	L 130 x D 35 /n.a.	0.9	6800	RT	Trypsin
DE-53	Whatman™	Cellulose (biodegradable)	DEAE	Cylindrical	L 130 x D 35 /n.a.	1.1	6800	RT	Trypsin
QA-52	Whatman™	Cellulose (biodegradable)	Quaternary Ammonium	Cylindrical	L 130 x D 35 /n.a.	1.2	6800	RT	Trypsin
Hillex®	SoloHill	Polystyrene	Cationic Trimethyl Ammonium	Spherical	160-200/n.a.	1.09-1.15	-	RT	Trypsin
Hillex II (HLX II-107)	SoloHill (Thermo Scientific)	Polystyrene	TEA	Spherical	160-180/n.a.	1.12	515	RT	Trypsin
Plastic Plus (P Plus-102-L)	SoloHill (Thermo Scientific)	Polystyrene	Uncoated	Spherical	125-212/n.a.	1.034-1.046	360	RT	Trypsin
FACT III (FACT 102-L)	SoloHill (Thermo Scientific)	Polystyrene	Uncoated	Spherical	125-212/n.a.	1.02	360	RT	Trypsin
Non/Negatively Charged (protein-free)									
Enhanced Attachment	Corning	Polystyrene	CellBIND Treatment	Spherical	125-212/n.a	1.022-1.030	360	4ªC	Trypsin
Plastic (P 102-L)	SoloHill (Thermo Scientific)	Polystyrene	Uncoated	Spherical	125-212/n.a.	1.02	360	RT	Trypsin
2D MicroHex™	Nunc	Polystyrene	Nunclon™ Treatment	Flat hexagons	L 125 x W 25 /n.a.	1.05	360	RT	Trypsin

SphereCol®	Advanced BioMatrix	Type I Collagen (bovine) (bioegradable)	Uncoated	Spherical	100-400/n.a.	1.022-1.030	-	2-10ºC	Trypsin
G2767	Merck (former Sigma Aldrich)	Glass	Uncoated	Spherical	150-210/n.a.	1.03	-	RT	Trypsin
G2517	Merck (former Sigma Aldrich)	Glass	Uncoated	Spherical	90-150/n.a.	1.03	-	RT	Trypsin
G2892	Merck (former Sigma Aldrich)	Glass	Uncoated	Spherical	90-150/n.a.	1.04	-	RT	Trypsin
Collagen Coated									
Collagen (CGEN 102-L)	SoloHill (Thermo Scientific)	Polystyrene	Type I Collagen (porcine)	Spherical	125-212/n.a.	1.02	480	RT	Trypsin
Cytodex 3™	GE Healthcare	Dextran	Denatured Type I Collagen (porcine)	Spherical	141-211/n.a.	1.04	2700	RT	Trypsin
ECM Coated									
ProNectin® F (Pro-F 102-L)	SoloHill (Thermo Scientific)	Polystyrene	Recombinant Fibronectin	Spherical	125-212/n.a.	1.02	-	RT	Trypsin
Synthemax® II	Corning	Polystyrene	Synthemax® II	Spherical	125-212/n.a.	1.022-1.030	360	4ºC	Trypsin
Macroporous									
Cultispher-G™	Thermo Scientific	Gelatin (biodegradable)	Uncoated	Spherical	130-380/10-20	1.03	40000	RT	Trypsin
Cultispher-S™	Thermo Scientific	Gelatin (biodegradable)	Uncoated	Spherical	130-380/10-20	1.03	75000	RT	Trypsin
Cultispher-GL™	Thermo Scientific	Gelatin (biodegradable)	Uncoated	Spherical	130-380/50-70	1.03	-	RT	Trypsin
Cytopore 1™	GE Healthcare	Dextran	DEAE	Spherical	200-280/30	1.03	11000	RT	Trypsin
Cytopore 2™	GE Healthcare	Dextran	DEAE	Spherical	200-280/30	1.03	11000	RT	Trypsin

High density									
Cytoline™	GE Healthcare	Polyethylene & Silica	Uncoated	Lens-shaped	L 2100 x W 750 /10-400	1.32	-	RT	Trypsin
Temporary									
Dissolvable Microcarriers	Corning	Polygalacturonic acid crosslinked with calcium ions	Denaturated Type I Collagen (Porcine) or Synthemax® II	Spherical	200-300, fully hydrated	1.02-1.03	5000	RT/4ºC	Bead dissolution by EDTA-chelation of calcium ions, exposing polymer chains to pectinase

Abbreviations: n.a. - not applicable; EDTA – Ethylenediaminetretacetic acid; DEAE - Diethylaminoethyl; TEA - Triethylamine; RT – Room temperature - Data not found
[#]Swelled (When applicable)

Box 1. From modular building-blocks to 3D macroscopic tissue architectures.

Native tissues are characterized by being very intricate systems composed of different cell types conducting a specific function and arranged in a highly ordered structure with a distinct and defined spatial distribution. From a bottom-up tissue engineering strategy perspective, micrometric sized particles, specially, cell-laden particles, reveal a great potential to be used as modular building-blocks to recreate complex tissue functionalities via development of hierarchical and biologically-functional structures [99]. To this extent, several biofabrication techniques such as bioprinting and bioassembly have been explored to engineer organomimetic cellular constructs [100–102]. The automated assembly of the micromodule units is generated through cell-driven organization, by material-material assembly, or hybrid cell-material interactions, usually applied as an injectable platform in a microfabricated mould. Cellular-driven assembly can be accomplished by cell-coated particles, as previously demonstrated by Matsunaga and colleagues, where cells were seeded over collagen particles and injected into a designed mould, promoting cell-cell adhesions [103]. Additionally, cells could migrate and grow within the scaffolding material. This approach expands the potential of these repeating units allowing the development of a more realistic and dynamic microenvironment through co-culture techniques, allowing encapsulation and seeding of different cell types aiming the formation of vascularized tissues. Microparticle annealing, on the other hand, allows the fabrication of a covalently-linked 3D scaffold with interconnected networks of pores suitable for cell migration and integration with the surrounding tissue [41,104]. Providing sufficient space for the cells to expand and proliferate through the construct, this novel biomaterial can circumvent the need of material degradation before neotissue growth. Another particle-driven assembly strategy is based on the direct assembly of, so called, lockyballs, specifically designed to have hoops and loops to enhance random interlocking between neighbouring particles, promoting different levels of flexibility and mobility of the resulting structure. [67]. Due to being a hollow structure and having a very porous wall, these microscaffolds allow an efficient cellularization which allied to the singular architectural features allows a rapid *in situ* tissue construct biofabrication. Although the assembly of macrotissues is often made in a randomly-packed manner, the precision and control over the organization of

the building-blocks to produce highly-ordered structures is of great importance in order to recreate accurate tissue-like constructs with physiological significance, such as the anisotropy existent in several tissues and organs [42,105]. These exceedingly precise architectures can be magnetically-driven, through microfluidics and molecular recognition [106,107]. Moreover, a guidance procedure with a clear-cut precision was developed by Yang and co-workers with the ability to manipulate the modules almost in a Lego-like manner into very sophisticated 3D designs [108]. This Tetris-style assembly reveals the undoubtable impact that geometrically designed microstructures may have in the assembly of functional biological structures with complex hierarchical and highly spatially-organized features, closely resembling architectural aspects of the native tissues found within the human body.

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References

1 Huang, L. *et al.* (2018) Porous chitosan microspheres as microcarriers for 3D cell culture. *Carbohydr. Polym.* 202, 611–620

2 Rafiq, Q.A. *et al.* (2017) Process development of human multipotent stromal cell microcarrier culture using an automated high-throughput microbioreactor. *Biotechnol. Bioeng.* 114, 2253–2266

3 Oliveira, M.B. and Mano, J.F. (2011) Polymer-based microparticles in tissue engineering and regenerative medicine. *Biotechnol. Prog.* 27, 897–912

4 Dias, A.D. *et al.* (2017) Microcarriers with Synthetic Hydrogel Surfaces for Stem Cell Expansion. *Adv. Healthc. Mater.* 6, 1700072

5 Wang, Y. *et al.* (2018) Fabrication of nanofibrous microcarriers mimicking

extracellular matrix for functional microtissue formation and cartilage regeneration. *Biomaterials* 171, 118–132

6 Strobel, H.A. *et al.* (2017) Cellular Self-Assembly with Microsphere Incorporation for Growth Factor Delivery Within Engineered Vascular Tissue Rings. *Tissue Eng. Part A* 23, 143–155

7 Le, L. V. *et al.* (2018) Injectable hyaluronic acid based microrods provide local micromechanical and biochemical cues to attenuate cardiac fibrosis after myocardial infarction. *Biomaterials* 169, 11–21

8 Custódio, C.A. *et al.* (2014) Functionalized Microparticles Producing Scaffolds in Combination with Cells. *Adv. Funct. Mater.* 24, 1391–1400

9 Custódio, C.A. *et al.* (2015) Cell selective chitosan microparticles as injectable cell carriers for tissue regeneration. *Biomaterials* 43, 23–31

10 Skardal, A. *et al.* (2015) Liver-Tumor Hybrid Organoids for Modeling Tumor Growth and Drug Response In Vitro. *Ann. Biomed. Eng.* 43, 2361–2373

11 Ferreira, L.P. *et al.* (2018) Bioinstructive microparticles for self-assembly of mesenchymal stem Cell-3D tumor spheroids. *Biomaterials* 185, 155–173

12 Brancato, V. *et al.* (2018) 3D breast cancer microtissue reveals the role of tumor microenvironment on the transport and efficacy of free-doxorubicin in vitro. *Acta Biomater.* 75, 200–212

13 Levato, R. *et al.* (2014) Biofabrication of tissue constructs by 3D bioprinting of cell-laden microcarriers. *Biofabrication* 6, 035020

14 Morille, M. *et al.* (2016) PLGA-based microcarriers induce mesenchymal stem cell chondrogenesis and stimulate cartilage repair in osteoarthritis. *Biomaterials* 88, 60–69

15 Mitragotri, S. and Lahann, J. (2009) Physical approaches to biomaterial design. *Nat. Mater.* 8, 15–23

16 He, Y. and Park, K. (2016) Effects of the Microparticle Shape on Cellular Uptake. *Mol. Pharm.* 13, 2164–71

17 Zheng, H. *et al.* (2018) Biodegradable Anisotropic Microparticles for Stepwise Cell Adhesion and Preparation of Janus Cell Microparticles. *ACS Appl. Mater. Interfaces* 10, 36776–36785

18 Li, M. *et al.* (2016) Wrinkling Non-Spherical Particles and Its Application in Cell Attachment Promotion. *Sci. Rep.* 6, 30463

19 Meyer, R.A. *et al.* (2015) An automated multidimensional thin film stretching device for the generation of anisotropic polymeric micro- and nanoparticles. *J. Biomed. Mater. Res. Part A* 103, 2747–2757

20 de Alteriis, R. *et al.* (2015) A method to tune the shape of protein-encapsulated polymeric microspheres. *Sci. Rep.* 5, 12634

21 Bhaskar, S. and Lahann, J. (2009) Microstructured Materials Based on Multicompartmental Fibers. *J. Am. Chem. Soc.* 131, 6650–6651

22 Bhaskar, S. *et al.* (2010) Towards Designer Microparticles: Simultaneous Control of Anisotropy, Shape, and Size. *Small* 6, 404–411

23 Choi, C.-H. *et al.* (2016) One-step generation of cell-laden microgels using double emulsion drops with a sacrificial ultra-thin oil shell. *Lab Chip* 16, 1549–55

24 Min, N.G. *et al.* (2016) Microfluidic Production of Uniform Microcarriers with Multicompartments through Phase Separation in Emulsion Drops. *Chem. Mater.* 28, 1430–1438

25 Qiu, Y. *et al.* (2015) Microfluidic-based fabrication, characterization and magnetic functionalization of microparticles with novel internal anisotropic structure. *Sci. Rep.* 5, 13060

26 Udoh, C.E. *et al.* (2016) Microporous Polymer Particles via Phase Inversion in Microfluidics: Impact of Nonsolvent Quality. *Langmuir* 32, 8131–8140

27 Choi, C.-H. *et al.* (2015) Controlled fabrication of multicompartmental polymeric microparticles by sequential micromolding via surface-tension-induced droplet formation. *Langmuir* 31, 1328–1335

28 McHugh, K.J. *et al.* (2017) Fabrication of fillable microparticles and other complex 3D microstructures. *Science* 357, 1138–1142

29 Neto, A.I. *et al.* (2018) Patterned superhydrophobic surfaces to process and characterize biomaterials and 3D cell culture. *Mater. Horizons* 5, 379–393

30 Costa, A.M.S. and Mano, J.F. (2017) Solvent-Free Strategy Yields Size and Shape-Uniform Capsules. *J. Am. Chem. Soc.* 139, 1057–1060

31 Neto, A.I. *et al.* (2016) Fabrication of Hydrogel Particles of Defined Shapes Using Superhydrophobic-Hydrophilic Micropatterns. *Adv. Mater.* 28, 7613–7619

32 Heidariyan, Z. *et al.* (2018) Efficient and cost-effective generation of hepatocyte-like cells through microparticle-mediated delivery of growth factors in a 3D culture of human pluripotent stem cells. *Biomaterials* 159, 174–188

33 Martins, C.R. *et al.* (2018) Multifunctional laminarin microparticles for cell adhesion and expansion. *Carbohydr. Polym.* 202, 91–98

34 Custódio, C.A. *et al.* (2014) Engineering Biomolecular Microenvironments for Cell Instructive Biomaterials. *Adv. Healthc. Mater.* 3, 797–810

35 Bernard, A.B. *et al.* (2014) Controlled local presentation of matrix proteins in microparticle-laden cell aggregates. *Biotechnol. Bioeng.* 111, 1028–1037

36 Lam, A.T.-L. *et al.* (2014) Cationic surface charge combined with either vitronectin or laminin dictates the evolution of human embryonic stem cells/microcarrier aggregates and cell growth in agitated cultures. *Stem Cells Dev.* 23, 1688–703

37 Levato, R. *et al.* (2015) Role of ECM/peptide coatings on SDF-1α triggered mesenchymal stromal cell migration from microcarriers for cell therapy. *Acta Biomater.* 18, 59–67

38 Li, W. *et al.* (2013) Microbead-based biomimetic synthetic neighbors enhance survival and function of rat pancreatic β-cells. *Sci. Rep.* 3, 2863

39 Zhang, Y. *et al.* (2016) Enhanced Biological Functions of Human Mesenchymal Stem-Cell Aggregates Incorporating E-Cadherin-Modified PLGA Microparticles. *Adv. Healthc. Mater.* 5, 1949–1959

40 Wronska, M.A. *et al.* (2016) Adding Functions to Biomaterial Surfaces through Protein Incorporation. *Adv. Mater.* 28, 5485–5508

41 Griffin, D.R. *et al.* (2015) Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks. *Nat. Mater.* 14, 737–744

42 Wang, H. *et al.* (2017) Assembly of RGD-Modified Hydrogel Micromodules into Permeable Three-Dimensional Hollow Microtissues Mimicking in Vivo Tissue Structures. *ACS Appl. Mater. Interfaces* 9, 41669–41679

43 Aday, S. *et al.* (2017) Synthetic microparticles conjugated with VEGF165 improve the survival of endothelial progenitor cells via microRNA-17 inhibition. *Nat. Commun.* 8, 747

158 44 Bertolo, A. *et al.* (2015) Growth Factors Cross-Linked to Collagen Microcarriers
159 Promote Expansion and Chondrogenic Differentiation of Human Mesenchymal
160 Stem Cells. *Tissue Eng. Part A* 21, 2618–2628
161 45 Tang, J. *et al.* (2017) Therapeutic microparticles functionalized with biomimetic
162 cardiac stem cell membranes and secretome. *Nat. Commun.* 8, 13724
163 46 Hayashi, K. *et al.* (2018) Red Blood Cell-Shaped Microparticles with a Red Blood
164 Cell Membrane Demonstrate Prolonged Circulation Time in Blood. *ACS*
165 *Biomater. Sci. Eng.* 4, 2729–2732
166 47 Shekaran, A. *et al.* (2015) Enhanced in vitro osteogenic differentiation of human
167 fetal MSCs attached to 3D microcarriers versus harvested from 2D monolayers.
168 *BMC Biotechnol.* 15, 102
169 48 Gupta, P. *et al.* (2016) Optimization of agitation speed in spinner flask for
170 microcarrier structural integrity and expansion of induced pluripotent stem
171 cells. *Cytotechnology* 68, 45–59
172 49 Borys, B.S. *et al.* (2018) Scale-up of embryonic stem cell aggregate stirred
173 suspension bioreactor culture enabled by computational fluid dynamics
174 modeling. *Biochem. Eng. J.* 133, 157–167
175 50 Eicke, D. *et al.* (2018) Large-scale production of megakaryocytes in microcarrier-
176 supported stirred suspension bioreactors. *Sci. Rep.* 8, 10146
177 51 Confalonieri, D. *et al.* (2017) * An Injectable Recombinant Collagen I Peptide-
178 Based Macroporous Microcarrier Allows Superior Expansion of C2C12 and
179 Human Bone Marrow-Derived Mesenchymal Stromal Cells and Supports
180 Deposition of Mineralized Matrix. *Tissue Eng. Part A* 23, 946–957
181 52 YekrangSafakar, A. *et al.* (2018) Hollow microcarriers for large-scale expansion
182 of anchorage-dependent cells in a stirred bioreactor. *Biotechnol. Bioeng.* 115,
183 1717–1728
184 53 Srinivasan, G. *et al.* (2018) An integrated biomanufacturing platform for the
185 large-scale expansion and neuronal differentiation of human pluripotent stem
186 cell-derived neural progenitor cells. *Acta Biomater.* 74, 168–179
187 54 Hsu, C.Y.M. *et al.* (2018) An Integrated Approach toward the Biomanufacturing
188 of Engineered Cell Therapy Products in a Stirred-Suspension Bioreactor. *Mol.*
189 *Ther. - Methods Clin. Dev.* 9, 376–389
190 55 Martin, Y.H. *et al.* (2017) A novel system for expansion and delivery of human
191 keratinocytes for the treatment of severe cutaneous injuries using microcarriers
192 and compressed collagen. *J. Tissue Eng. Regen. Med.* 11, 3124–3133
193 56 Li, C. *et al.* (2016) Alginate/PEG based microcarriers with cleavable crosslinkage
194 for expansion and non-invasive harvest of human umbilical cord blood
195 mesenchymal stem cells. *Mater. Sci. Eng. C* 64, 43–53
196 57 Fan, Y. *et al.* (2017) Engineering Xeno-Free Microcarriers with Recombinant
197 Vitronectin, Albumin and UV Irradiation for Human Pluripotent Stem Cell
198 Bioprocessing. *ACS Biomater. Sci. Eng.* 3, 1510–1518
199 58 Rodrigues, C.A. *et al.* (2018) Scalable culture of human induced pluripotent cells
200 on microcarriers under xeno-free conditions using single-use vertical-wheel™
201 bioreactors. *J. Chem. Technol. Biotechnol.* 93, 3597–3606
202 59 Badenes, S.M. *et al.* (2017) Long-term expansion of human induced pluripotent
203 stem cells in a microcarrier-based dynamic system. *J. Chem. Technol. Biotechnol.*
204 92, 492–503

60 Zhang, C. *et al.* (2009) Towards a human-on-chip: Culturing multiple cell types on a chip with compartmentalized microenvironments. *Lab Chip* 9, 3185
61 Leferink, A. *et al.* (2014) Engineered Micro-Objects as Scaffolding Elements in Cellular Building Blocks for Bottom-Up Tissue Engineering Approaches. *Adv. Mater.* 26, 2592–2599
62 Yajima, Y. *et al.* (2017) Collagen Microparticle-Mediated 3D Cell Organization: A Facile Route to Bottom-up Engineering of Thick and Porous Tissues. *ACS Biomater. Sci. Eng.* 3, 2144–2154
63 Wei, D.-X. *et al.* (2018) A Micro-Ark for Cells: Highly Open Porous Polyhydroxyalkanoate Microspheres as Injectable Scaffolds for Tissue Regeneration. *Adv. Mater.* 30, 1802273
64 Liao, J. *et al.* (2017) Injectable Alginate Hydrogel Cross-Linked by Calcium Gluconate-Loaded Porous Microspheres for Cartilage Tissue Engineering. *ACS Omega* 2, 443–454
65 Zhou, Y. *et al.* (2016) Chitosan microspheres with an extracellular matrix-mimicking nanofibrous structure as cell-carrier building blocks for bottom-up cartilage tissue engineering. *Nanoscale* 8, 309–317
66 Niederholtmeyer, H. *et al.* (2018) Communication and quorum sensing in non-living mimics of eukaryotic cells. *Nat. Commun.* 9, 5027
67 Silva, K.R. *et al.* (2016) Delivery of Human Adipose Stem Cells Spheroids into Lockyballs. *PLoS One* 11, e0166073
68 Correia, C.R. *et al.* (2016) A Closed Chondromimetic Environment within Magnetic-Responsive Liquified Capsules Encapsulating Stem Cells and Collagen II/TGF-β3 Microparticles. *Adv. Healthc. Mater.* 5, 1346–1355
69 Duan, B. *et al.* (2017) Hierarchical Microspheres Constructed from Chitin Nanofibers Penetrated Hydroxyapatite Crystals for Bone Regeneration. *Biomacromolecules* 18, 2080–2089
70 Correia, C.R. *et al.* (2017) In vivo osteogenic differentiation of stem cells inside compartmentalized capsules loaded with co-cultured endothelial cells. *Acta Biomater.* 53, 483–494
71 Correia, C.R. *et al.* (2016) Semipermeable Capsules Wrapping a Multifunctional and Self-regulated Co-culture Microenvironment for Osteogenic Differentiation. *Sci. Rep.* 6, 21883
72 Mealy, J.E. *et al.* (2018) Injectable Granular Hydrogels with Multifunctional Properties for Biomedical Applications. *Adv. Mater.* 30, 1705912
73 Chen, M.H. *et al.* (2018) Injectable Supramolecular Hydrogel/Microgel Composites for Therapeutic Delivery. *Macromol. Biosci.* 19, 1800248
74 Ferreira, L.P. *et al.* (2018) Design of spherically structured 3D in vitro tumor models -Advances and prospects. *Acta Biomater.* 75, 11–34
75 Yamada, M. *et al.* (2015) Cell-sized condensed collagen microparticles for preparing microengineered composite spheroids of primary hepatocytes. *Lab Chip* 15, 3941–3951
76 Jakob, P.H. *et al.* (2016) A 3-D cell culture system to study epithelia functions using microcarriers. *Cytotechnology* 68, 1813–25
77 Skardal, A. *et al.* (2010) The generation of 3-D tissue models based on hyaluronan hydrogel-coated microcarriers within a rotating wall vessel bioreactor. *Biomaterials* 31, 8426–8435

78 Bratt-Leal, A.M. *et al.* (2013) A microparticle approach to morphogen delivery within pluripotent stem cell aggregates. *Biomaterials* 34, 7227–7235
79 Zhang, Y. *et al.* (2016) Enhanced Biological Functions of Human Mesenchymal Stem-Cell Aggregates Incorporating E-Cadherin-Modified PLGA Microparticles. *Adv. Healthc. Mater.* 5, 1949–1959
80 Ahmad, T. *et al.* (2017) Hybrid-spheroids incorporating ECM like engineered fragmented fibers potentiate stem cell function by improved cell/cell and cell/ECM interactions. *Acta Biomater.* 64, 161–175
81 Bratt-Leal, A.M. *et al.* (2011) Incorporation of biomaterials in multicellular aggregates modulates pluripotent stem cell differentiation. *Biomaterials* 32, 48–56
82 Baraniak, P.R. *et al.* (2012) Stiffening of human mesenchymal stem cell spheroid microenvironments induced by incorporation of gelatin microparticles. *J. Mech. Behav. Biomed. Mater.* 11, 63–71
83 Abbasi, F. *et al.* (2018) Engineering mesenchymal stem cell spheroids by incorporation of mechanoregulator microparticles. *J. Mech. Behav. Biomed. Mater.* 84, 74–87
84 Yue, S. *et al.* (2017) SERS–Fluorescence Dual-Mode pH-Sensing Method Based on Janus Microparticles. *ACS Appl. Mater. Interfaces* 9, 39699–39707
85 Shin, D.S. *et al.* (2018) Synthesis of Microgel Sensors for Spatial and Temporal Monitoring of Protease Activity. *ACS Biomater. Sci. Eng.* 4, 378–387
86 Barrila, J. *et al.* (2017) Three-dimensional organotypic co-culture model of intestinal epithelial cells and macrophages to study Salmonella enterica colonization patterns. *npj Microgravity* 3, 10
87 Kolesky, D.B. *et al.* (2014) 3D Bioprinting of Vascularized, Heterogeneous Cell-Laden Tissue Constructs. *Adv. Mater.* 26, 3124–3130
88 Neufurth, M. *et al.* (2017) 3D printing of hybrid biomaterials for bone tissue engineering: Calcium-polyphosphate microparticles encapsulated by polycaprolactone. *Acta Biomater.* 64, 377–388
89 Zhong, M. *et al.* (2014) Establishing a cell-affinitive interface and spreading space in a 3D hydrogel by introduction of microcarriers and an enzyme. *J. Mater. Chem. B* 2, 6601–6610
90 Tan, Y.J. *et al.* (2016) Hybrid microscaffold-based 3D bioprinting of multi-cellular constructs with high compressive strength: A new biofabrication strategy. *Sci. Rep.* 6, 39140
91 Roh, S. *et al.* (2017) 3D Printing by Multiphase Silicone/Water Capillary Inks. *Adv. Mater.* 29, 1701554
92 Highley, C.B. *et al.* (2018) Jammed Microgel Inks for 3D Printing Applications. *Adv. Sci.* 6, 1801076
93 Wang, C. *et al.* (2009) The control of anchorage-dependent cell behavior within a hydrogel/microcarrier system in an osteogenic model. *Biomaterials* 30, 2259–2269
94 Neufurth, M. *et al.* (2017) 3D printing of hybrid biomaterials for bone tissue engineering: Calcium-polyphosphate microparticles encapsulated by polycaprolactone. *Acta Biomater.* 64, 377–388
95 Mekhileri, N. V *et al.* (2018) Automated 3D bioassembly of micro-tissues for biofabrication of hybrid tissue engineered constructs. *Biofabrication* 10, 024103

96 Hospodiuk, M. *et al.* (2017) The bioink: A comprehensive review on bioprintable materials. *Biotechnol. Adv.* 35, 217–239
97 Schmidt, J.J. *et al.* (2011) The Interplay Between Cell Adhesion Cues and Curvature of Cell Adherent Alginate Microgels in Multipotent Stem Cell Culture. *Tissue Eng. Part A* 17, 2687–2694
98 Griffin, D.R. *et al.* (2015) Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks. *Nat. Mater.* 14, 737–744
99 Li, F. *et al.* (2018) Cartilage tissue formation through assembly of microgels containing mesenchymal stem cells. *Acta Biomater.* 77, 48–62
100 Kamperman, T. *et al.* (2017) Single Cell Microgel Based Modular Bioinks for Uncoupled Cellular Micro- and Macroenvironments. *Adv. Healthc. Mater.* 6, 1600913
101 Graham, A.D. *et al.* (2017) High-Resolution Patterned Cellular Constructs by Droplet-Based 3D Printing. *Sci. Rep.* 7, 7004
102 Zhong, M. *et al.* (2017) Vascularization in Engineered Tissue Construct by Assembly of Cellular Patterned Micromodules and Degradable Microspheres. *ACS Appl. Mater. Interfaces* 9, 3524–3534
103 Matsunaga, Y.T. *et al.* (2011) Molding Cell Beads for Rapid Construction of Macroscopic 3D Tissue Architecture. *Adv. Mater.* 23, H90–H94
104 Sheikhi, A. *et al.* (2019) Microfluidic-enabled bottom-up hydrogels from annealable naturally-derived protein microbeads. *Biomaterials* 192, 560–568
105 Lee, B.W. *et al.* (2016) Modular Assembly Approach to Engineer Geometrically Precise Cardiovascular Tissue. *Adv. Healthc. Mater.* 5, 900–906
106 Sun, T. *et al.* (2018) Magnetic alginate microfibers as scaffolding elements for the fabrication of microvascular-like structures. *Acta Biomater.* 66, 272–281
107 Ma, S. *et al.* (2017) Gel Microrods for 3D Tissue Printing. *Adv. Biosyst.* 1, 1700075
108 Yang, W. *et al.* (2017) High-Throughput Fabrication and Modular Assembly of 3D Heterogeneous Microscale Tissues. *Small* 13, 1602769

Figure Captions:

Figure 1. Schematic representation of (bio)chemical/physical cues and architectural features as modulating moieties of microparticles.

Figure 2. Overview of the multidisciplinary nature of microparticles. (A) Microparticle conventional approach as microcarrier platforms within bioreactors for large-scale cell expansion and differentiation. (B) Microparticles as moldable and injectable systems, able to accurately fill and fit in irregularly-shaped defects and promote tissue regeneration. (C) Microparticles as structural supports and cue providers within multicellular aggregates. (D) New generation of modular bioinks composed of (i) solely and tightly-packed microparticles (granular inks/gels) and (ii) of particle embedded in hydrogel matrices.