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REVIEW

Spheroid construction strategies and application in 3D bioprinting

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Abstract

Tissue engineering has been striving toward designing and producing natural and functional human tissues. Cells are the fundamental building blocks of tissues. Compared with traditional two-dimensional cultured cells, cell spheres are three-dimensional (3D) structures that can naturally form complex cell–cell and cell–matrix interactions. This structure is close to the natural environment of cells in living organisms. In addition to being used in disease modeling and drug screening, spheroids have significant potential in tissue regeneration. The 3D bioprinting is an advanced biofabrication technique. It accurately deposits bioinks into predesigned 3D shapes to create complex tissue structures. Although 3D bioprinting is efficient, the time required for cells to develop into complex tissue structures can be lengthy. The 3D bioprinting of spheroids significantly reduces the time required for their development into large tissues/organs during later cultivation stages by printing them with high cell density. Combining spheroid fabrication and bioprinting technology should provide a new solution to many problems in regenerative medicine. This paper systematically elaborates and analyzes the spheroid fabrication methods and 3D bioprinting strategies by introducing spheroids as building blocks. Finally, we present the primary challenges faced by spheroid fabrication and 3D bioprinting with future requirements and some recommendations.

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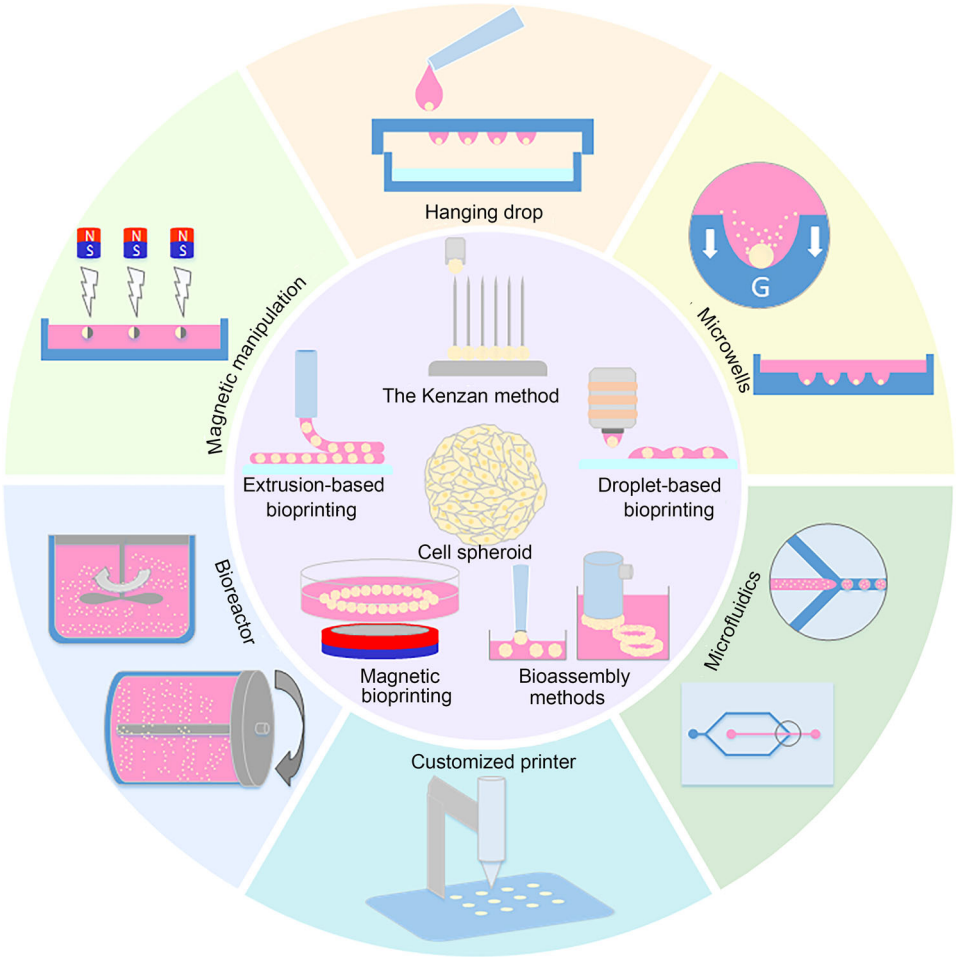
Graphic abstract



Keywords Spheroids · Strategies · 3D bioprinting · Biofabrication

Introduction

Spheroids are typically aggregated from the same or different types of cells, including cancer, stem, and normal cells. These cells self-assemble into three-dimensional (3D) spherical structures in an in vitro culture medium, resembling a tiny tissue [1]. In traditional two-dimensional (2D) cell culture, the growth conditions of cells are relatively uniform, which does not truly reflect their growth environment of in vivo [2]. However, the internal environment of spheroids differs from the traditional cell culture environment. Cells are exposed to different gradients of oxygen, nutrients, and metabolites, which closely mimic the in vivo environment and conditions [3, 4]. In a 3D structure, cells can naturally form complex cell–cell and cell–matrix interactions, and this structure is close to the natural environment of cells in organisms, carrying substantial biological significance [5]. Spheroids are becoming increasingly popular for studying the physiological state of cells in a 3D environment in vitro. Spheroids can be used as models in histology, oncology [6, 7], pharmacology [8–10], and bioengineering [11, 12] owing to their ability to simulate tissue or tumor characteristics in vitro, offering a close approximation to real physiological conditions. Spheroids are frequently used to study cell proliferation, differentiation, invasion, response to drugs, and the interaction between different cell types in a 3D environment [13, 14]. For example, in simulating tumor tissues [9, 15], cells aggregate into spherical clumps, forming a 3D structure called a tumor spheroid. This interaction of a tumor spheroid with surrounding tissues affects tumor cell proliferation, invasion, and therapeutic responses [16]. Therefore, the mechanisms of tumorigenesis and progression can be better understood by studying the behavior of tumor cells in spheroids [6, 17].

By studying the structure and function of spheroids, scientists can better understand how cells and tissues behave in physiological and pathological situations, and develop more effective treatments and methods. Coculture systems and in vitro pretreatment strategies contribute to the continuous improvement of spheroid functions and regenerative capacities [2], increasingly developing spheroids as building blocks toward directed incorporation into larger macrotissues.

The 3D printing (additive manufacturing) is a layer-by-layer digital manufacturing technology [18, 19]. The 3D bioprinting is similar to traditional 3D printing but differs in material selection and printing conditions. As a crucial branch of 3D printing technology, 3D bioprinting involves the precise layer-by-layer positioning of biomaterials, biochemicals, and living cells to construct highly customized and intricate structures, thus manufacturing tissues or organs with highly biomimetic architectures [20–23]. The bioink of 3D bioprinting is hydrogel and cell suspension. After printing the hydrogel fiber, the cells proliferate and differentiate in the fiber [24]. However, scaffold-based 3D bioprinting has some problems. The nature of the interaction between cells and solid scaffolds at the cellular level is two-dimensional, limiting intercellular and intracellular interactions [11].

Spheroids as bioinks are a promising direction in bioprinting. Unlike the typically used cell-laden hydrogel method, spheroid 3D bioprinting involves spatially arranging preprepared high-cell-density spheroids to relatively quickly expedite tissue construction [25, 26]. Although spheroid 3D bioprinting is still in the early stages of research and development, it has shown considerable potential. It can provide many innovative solutions for the biomedical field and should become a promising medical technology. Many methods have been developed and expanded to realize the combination of the advantages of spheroids and bioprinting. Combining spheroids and bioprinting in tissue engineering and regenerative medicine and technical features with different manufacturing and manipulation capabilities should provide a new solution to this series of problems [27]. This paper emphasizes the strategy of spheroid 3D bioprinting, including spheroid fabrication and its application in 3D bioprinting. Finally, the primary challenges, future requirements, and some suggestions for the current spheroid fabrication and 3D bioprinting are presented.

Fabrication of tissues/organs

The goal of tissue engineering and organ fabrication is to design and produce natural and functional human tissues and organs [28–30]. These tissues and organs can repair, regenerate, and replace damaged or lost body parts [20, 31]. They are not only technically artificial but also natural living tissues and organs. Therefore, one of the fundamental challenges that should be overcome is biocompatibility. Any biomaterial-based approach for fabricating artificial organs, devices, or prostheses must significantly reduce, eliminate, or modify biocompatibility issues. Without tissue engineering, functional living human organs can only be produced through natural embryonic development processes. Owing to economic constraints, ethics, and the inherent limitations of producing living tissues and organs, biofabrication technologies can still fail to create completely functional replicas of living organs.

The established tissue construction methods are primarily divided into two categories: bottom-up and top-down. The former is a biological structure constructed by the direct or indirect controllable assembly of cells, growth factors, and matrix materials. A typical example is the combination of micro–nanofabrication, microfluidics, and micro–nanomanipulation to construct biological tissues containing multicellular aggregates. The latter is achieved by biofabrication represented by scaffold bioprinting. In 3D bioprinting, scaffold-based methods involve fabricating porous or nonporous templates, usually made of ceramics, metals, or polymers [32–34]. Scaffolds provide a platform that facilitates cell attachment and tissue remodeling. The porous scaffold structure promotes cell attachment and provides a favorable tissue growth environment [35, 36]. The scaffolds have suitable biodegradability and mechanical properties. The biodegradability of the scaffold material can be regulated so that it degrades within an appropriate time and supports the nascent tissue [37]. The scaffold must have sufficient mechanical strength to maintain its shape and rigidity. The porous structure of the scaffold can promote blood vessel formation and tissue growth. Proper porosity provides good oxygenation and nutrient delivery and promotes vascularization and growth of new tissues. However, scaffold-based approaches expose living cells to shear stress, heat, or other toxic chemicals, which are inherently stressful for the cells [25]. The scaffold method relies on suitable hydrogel materials or other biopolymers that should have suitable mechanical strength and meet the expected degradation rate [38].

However, the development cycle of scaffold materials is lengthy and costly. Users are limited to a finite selection of mature materials for research. The vascularization of thicker tissue structures remains problematic, especially in engineering scaffolds with completely developed vascular networks to induce angiogenesis [39]. Researchers are committed to studying the vascularization of tissues because it is a realistic technical goal [40]. The long-term survival of tissues can be ensured only by having blood vessels for nutrient transport [41]. Accomplishing organ-specific cellular density levels within tissue constructs remains a significant challenge, particularly at the micro–nanoscale.

However, despite the significant potential of bioprinting, some challenges and drawbacks exist. Biological microorganisms have moved from 2D to 3D and four-dimensional. The composition has changed from a single cell to a controllable distribution of multiple cells and matrix materials. However, limited by the environmental adaptability, flexibility, controllability, repeatability, and assembly efficiency of assembly technology, artificial tissues and organs from the bottom-up assembly of microtissues frequently cannot break through the size limit. Building complex biological structures involves the precise positioning of cells and the accurate assembly of tissues. Researchers face several technical difficulties when dealing with these complex biological structures. Therefore, scientists pay attention to the application of spheroids. In tissue engineering, bioprinting with self-assembling spheroids is a potential alternative to traditional biodegradable scaffolding methods [42]. Spheroids are preformed structures that can be further assembled into large structures (tissues). Owing to their strong self-assembly capability, spheroids can be used for constructing intricate biological structures, addressing specific challenges encountered in traditional bioprinting. By taking advantage of the self-assembly properties of spheroids, researchers can precisely position cells, making bioprinting technology flexible and efficient. Therefore, spheroids are crucial in bioprinting, providing a new way to construct complex biological structures and promoting the development and application of bioprinting technology.

Fabrication of spheroids

Spheroids fabrication is a critical technology in preparing spheroid 3D bioprinting to construct 3D cell aggregates with specific functions and structures. Spheroids are typically spontaneously assembled into spherical structures by cells of one or different kinds passing through a surface to which cells cannot adhere, forcing the cells to interact with each other [43, 44]. Researchers have been dedicated to various methods of producing spheroids, including hanging drops, microwells, microfluidics, magnetic manipulation, bioreactors, and customized printers [45]. Spheroids typically range in diameter from tens to hundreds of microns. The size and shape of spheroids can be adjusted and controlled according to different research needs [46]. Ward and King investigated the relationship between cell viability and spheroid size when exposed to drugs, indicating that small spheroids with a diameter <50 μm respond rapidly, similar to monolayer-cultured cells. However, large spheroids with a diameter >50 μm exhibit high survival rates and subtle differences in drug response [47]. However, owing to the need for diffuse oxygen supply throughout the spheroid structure, efficient oxygen transfer was observed for cell aggregates below 200 μm in diameter. Therefore, it is recommended to produce spheroids with 200–400 μm diameters in tissue engineering to ensure nonlimiting mass transfer conditions and prevent spheroid core cell necrosis and damage [44]. Owing to the small size of spheroids, thousands to millions spheroids are typically required to generate clinically relevant volumes of tissue substitutes. Table 1 demonstrates an overview of spheroids fabrication techniques.

Hanging drop

The hanging drop method is common for generating spheroids. Its primary technical principle is to quantitatively absorb 10–30 μL of cell suspension and inject it into a culture plate with bottomless or bottomed wells. The culture plate is inverted; therefore, the droplets form hanging drops under gravity conditions. Cells also gather at the bottom of the hanging drop owing to gravity and aggregate to form spheroids. The advantage of the hanging drop method is that the preparation is straightforward and cost-effective and basic petri dishes can be prepared. The disadvantage is that the hanging drop size is small and requires high personnel operation. It is easy to lose the spheroids when changing the culture medium and transferring them. However, increasing research focuses on improving the throughput and stability of the hanging drop method. Tung et al. developed a robot-assisted hanging droplet culture and studied the appropriate pore size by customizing a 384-well plate to create spheroids in a high-throughput manner [50, 51]. Fu et al. proposed a fast and low-cost fabrication process for 3D spheroid-cultured flexible hanging drop chips. The Parafilm® hanging drop chip (PHDC) was made by cutting and bonding paraffin films with two pore sizes and bonding a layer of polyethylene terephthalate film to increase the mechanical strength. The influence of pore size and sample volume on suspended droplet formation was discussed. The application of spheroids formed on the PHDC in drug screening was demonstrated [77]. Sun et al. proposed a medium-reservoir-integrated superhydrophobic platform. The integrated chamber designed within the device provides several cultured mediums, allowing the spheroids to maintain high viability for up to 30 days [78] (Fig. 1a). Tang et al. used a circular motion to produce an inertial focusing effect on suspended droplets, promoting cell aggregation and accelerating spheroid formation [79].

Microwells

The microwell method loads the cell suspension above the microwells. The cells are confined in micron-sized pits of a specific size under the action of gravity and aggregate to form spheroids. The microwell surface generally requires a low-adhesion coating to prevent the cells from clinging to the microwell walls. Compared with the hanging drop method,

Table 1 Biofabrication of spheroids and their comparison

Strategy	Spheroids throughput	Dimensional accuracy	Labor intensity	Spheroids loss or risk of injury	Characteristics	Reference
Hanging drop	Low	Medium	High	High	Low difficulty Cost-effective	[48–52]
Microwells	High	High	Medium	Medium	High throughput	[53–59]
Microfluidics	High	High	Medium	Low	High degree of automation Strong scalability	[52, 60–63]
Magnetic manipulation	Medium	Medium	Low	Low	High controllability Quick response	[64–68]
Bioreactor	High	Low	Low	Medium	Large processing capacity	[69–73]
Customized printer	High	High	Low	Low	High degree of automation High cost	[74–76]

the microwell method does not require separate quantitative delivery of cell suspension, reduces labor intensity, has high throughput, and has controllable size. In addition, researchers must be careful when changing the medium to prevent the spheroids from falling out of the microwells. Typical commercially available platforms use 96- and 384-well plates with U-shaped bottoms and low-adhesion treatments to better control the spheroid size and improve spheroid formation efficiency. However, some cell suspension must be injected through each well to form a spheroid at the bottom. Therefore, some studies have used 3D-printed stamps to manufacture agarose microwell arrays in standard well plates to simplify operations and increase throughput so that multiple microwells are formed in a single well [57]. Fukuda et al. synthesized photocrosslinkable chitosan to prepare hydrogel microstructures through micromolding. Spheroid microarrays were prepared alone or cocultured on hydrogel microstructures [58]. Tu et al. proposed a fast and low-cost method for manufacturing microwells. The respective characteristics were observed and analyzed using laser ablation of polymethylmethacrylate (PMMA), polydimethylsiloxane (PDMS), and polystyrene (PS). Finally, concave microwells for generating multicellular aggregates were created on a petri dish. The spheroids were successfully generated, and the size could be controlled [59] (Fig. 1b).

Microfluidics

The microfluidic method designs flow channels of specific sizes, usually tens to hundreds of microns, and uses fluid shearing to divide the cell suspension into droplets, and the cells form spheroids within the droplets. Microfluidic methods allow excellent control of droplet size while supporting high throughput. The 3D monodisperse alginate hydrogel tumors were formed using a flow-focusing droplet generator for various omics studies and therapeutic efficiency screening [62]. The disadvantage is that the segmented droplets hinder the communication between the cell spheroids, limiting the proliferation and movement of the spheroids. Moreover, the microfluidic method requires the predesign and manufacturing of chips, with high equipment requirements and costs. Wang et al. described a microfluidic droplet-based method to easily generate concentrations of cell aggregates of different shapes in Ca-alginate microparticles by varying alginate and $CaCl_2$. They obtained human cervical carcinoma, hepatocellular liver carcinoma, and umbilical vein endothelial cell aggregates in a reproducible and controllable manner in the shape of spheres, spindles, and branches [80]. Microfluidic chips can also be designed to manufacture spheroids using microwells or hanging drop methods. Chen et al. designed a high-throughput chip using the microwell method, producing 1024 spheroids of uniform size in a core area of 2 cm × 2 cm. Photodynamic therapy (PDT) was used to compare traditional 2D monolayer cell cultures with 3D cellular spheres, demonstrating enhanced PDT resistance in spheroid cultures [63] (Fig. 1c). Frey et al. used a microfluidic chip to generate hanging drops to prepare spheroids and the chip could gradiently adjust the nutrient supply. Metabolic communication between spheres was realized through the chip, and the process can be analyzed [52].

Magnetic manipulation

Various 3D cell types, such as spheroids, cell fibers, and vascular networks, can be successfully formed using sonic or magnetic field-based 3D cell culture platforms [65]. Cells

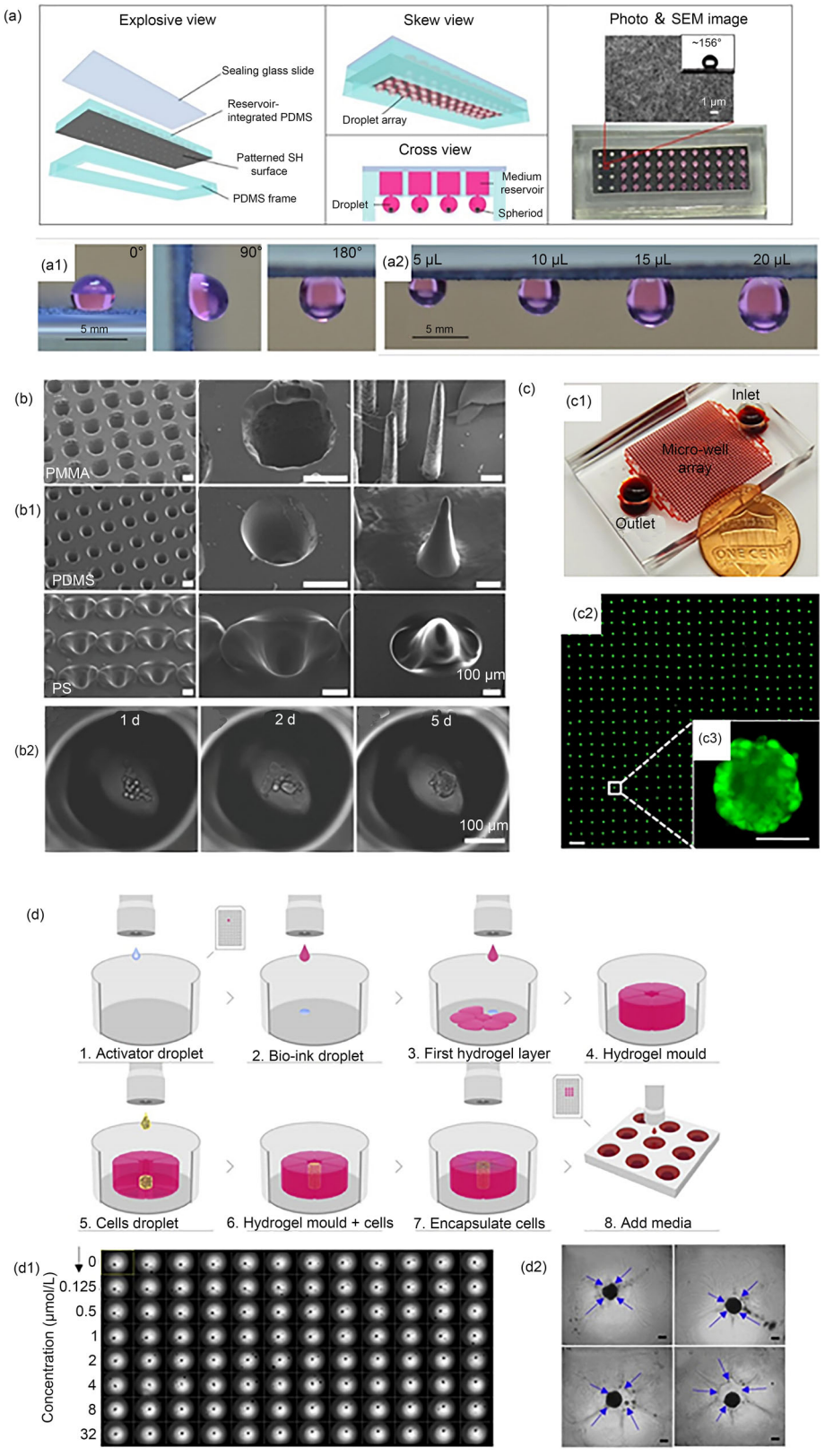


Fig. 1 **a** The medium-reservoir-integrated hanging drop device: **a1** images of a 10-μL culture medium droplet adhered on the laser-patterned superhydrophobic (SH) surface at different angles; **a2** images of cell culture medium droplets with different volumes (reproduced from Ref. [78], Copyright 2021, with permission from Acta Materialia Inc.). **b**, **b1** Isometric-view scanning electron microscopy images of microwells from three materials; **b2** multicellular cancer aggregates observed 1, 2, and 5 days after seeding (reproduced from Ref. [59], Copyright 2013, with permission from WILEY–VCH Verlag GmbH & Co. KGaA, Weinheim). **c**, **c1** The high-throughput sphere formation platform comprising an array of 1024 nonadherent microwells connected by a single inlet and outlet; **c2** an array of spheres formed in the device (scale bar: 1 mm); **c3** an enlarged view of a sphere in a microwell (scale bar: 100 μm) (reproduced from Ref. [63], Copyright 2015, with permission from the authors, licensed under a Creative Commons Attribution 4.0 International License). **d** The 3D bioprinting of tumor spheroids inside the hydrogel matrix cup structure: **d1** Operetta image of a full 96-well plate of spheroids; **d2** spheroids encapsulated inside varying cup sizes (scale bar: 200 μm) (reproduced from Ref. [83], Copyright 2020, with permission from the authors, licensed under CC BY-NC-ND)

are magnetized with paramagnetic nanoparticles and rapidly aggregated using a magnet to form spheroids [66, 67]. Magnetic manipulation is a promising spheroid fabrication method, with high spheroid forming efficiency, good size control, and high automation. However, the biocompatibility of the magnetic material is a crucial consideration. Kim et al. customized the magnetic needle array system, which can be placed above the standard well plate, and the cells are magnetically driven to aggregate into spheroids, ensuring high throughput and efficiency. Simultaneously, it can manufacture multifunctional spheroids, such as random mixing, core–shell, and fused spheroids [66]. The latest research shows that the magnetic manipulation method is not necessarily limited to acting on the spheroid but can also act on the manufacturing system of the spheroid. Zhang et al. established a simple magnetic system comprising magnetic microwell arrays and blocks for efficiently producting functional hepatocyte spheroids. The magnetic microwell array was prepared by mixing magnetic nanoparticles (MNPs) into low-attachment PDMS. Cells were captured with magnetic array micropores adsorbed by magnets, and the low-adhesion micropores promoted cell aggregation to form spheroids. Finally, the therapeutic effect of the prepared functional hepatocyte spheroids in treating acute liver failure was proved [68]. The cell aggregates formed by magnetic manipulation are not limited to simple spheroids; they provide a basis for the subsequent production of large tissues.

Bioreactor

Using a bioreactor to stir a large cell suspension, the cells remain in the dynamic fluid, cannot adhere to the wall, and aggregate to form spheroids [69]. Many efforts have been devoted to generating spheroids in large quantities using bioreactors. Spinner flasks [70], rotary shakers [71], and microgravity bioreactors [72] are three typically used bioreactor systems for high-throughput spheroid generation [46]. Gallegos-Martínez et al. developed miniaturized continuous stirred-tank reactors (mCSTRs) based on 3D printing with an eccentric stirring function. They created a homogeneous and dynamic environment that promotes adequate access to nutrients for the spheroids in the culture. The continuous perfusion of culture mediums in these mCSTRs continuously supplies nutrients, removes byproducts, and maintains a sufficient dynamic environment for spheroid formation, maturation, and expansion cultures [73]. Theoretically, although bioreactors efficiently generate spheroids with minimal handling requirements, large cell volumes cannot precisely control their spheroid size, reducing batch-to-batch reproducibility [81]. Moreover, multiple variables, such as bioreactor design and stirring speed, require optimization to obtain spheroids with the desired properties [82].

Customized printer

Although many methods for manufacturing spheroids have been developed, some highly labor-intensive approaches are not conducive to automation. With the continuous development of automation equipment and three-axis robots, customized printers with high controllability and intelligence can produce high-quality, high-precision, and high-throughput spheroids. Faulkner-Jones et al. designed a multi-nozzle droplet printer to achieve on-demand array spraying of multigradient spheroids onto a culture dish. The gradients of cells and bioinks can be achieved using dual nozzles, resulting in the gradients of cell aggregates. The valve-based printing process is gentle enough to preserve stem cell viability and precise enough to produce uniformly sized spheroids [76]. Utama et al. built a custom bioprinter that uses a solenoid valve microvalve print head to produce the hydrogel matrix cup structure, and prints cell suspensions to form spheroids within the structure. During printing, the size of the hydrogel matrix cup and the number of cells can be adjusted to control the final spheroid size. Completely automated spheroids were similar to manually prepared spheroids regarding cell proliferation, apoptosis, cytoskeletal structure, hypoxia, and stem cell presentation. Moreover, the equipment is compatible with commercially available standardized orifice plates and has scalability [83] (Fig. 1d). Although the cost of custom printers for producing spheroids is higher than traditional methods, the advantages are less labor intensity and standard product quality, a crucial prerequisite for advancing the commercialization and clinical development of spheroids. The automated manufacture of standard-sized spheroids is a preparation for the further use of spheroids in printing large tissues.

Application of spheroids in 3D bioprinting

The primary purpose of 3D bioprinting is to assemble cells according to a planned path. Hydrogel is the primary material for 3D bioprinting. However, communication between cells in hydrogels is limited. Each cell is relatively scattered and has low cell density [25, 84–86]. Traditional 3D bioprinting relies on biodegradable solid scaffolds and the mechanical properties of the scaffold. Research continues on manufacturing porous scaffolds and adjusting their biodegradability [11, 32, 87]. However, scaffold-free technologies, such as spheroid 3D bioprinting, take advantage of the self-assembly of spheroids to form tissues [88, 89]. Therefore, controlling the spatial distribution of spheroids and the functions required for their formation has led to various methods of spheroid 3D bioprinting [90]. This section will describe the current spheroid 3D bioprinting and its advantages and

Table 2 Spheroids in 3D bioprinting and their comparison

Strategy	Print efficiency	Resolution	Spheroids damage risk	Scalability	Multicellular spheroids	Three-dimensional complexity	Characteristics	Reference
Extrusion-based bioprinting	Medium	Medium	Medium	Medium	Yes	High	Cost effective Spheroids are susceptible to mechanical damage	[91–93]
Droplet-based bioprinting	Low–medium	High	Medium	No	Yes	Low	High-resolution Easy to jam	[94, 95]
The Kenzan method	Medium	Low	High	Medium	No	Low	High degree of automation	[96–98]
Bioassembly method	Low–medium	High	Low	High	Yes	Low–medium	Accurate operation	[99, 100]
Magnetic bioprinting	High	Low	Low	High	Yes	Medium	High degree of freedom High cost	[101, 102]

disadvantages. Table 2 demonstrates an overview of spheroid 3D bioprinting techniques.

Extrusion-based bioprinting

The most commonly used technology in 3D bioprinting is extrusion-based bioprinting (EBB), combined with cell-laden bioinks owing to its cost-effectiveness, reliability, scalability, and ability to replicate tissue complexity [103]. Common power systems in EBB systems are powered by pneumatic, piston, screw, or microfluidic mechanisms [104]. The EBB extrudes or distributes a continuous bioink chain primarily comprising biological materials, living cells, or bioactive molecules from a nozzle to a designated platform position through a computer-generated path using a predesigned 2D or 3D model. Compared with other 3D bioprinting technologies, the EBB can manage various biological materials or cell types [91, 105–107].

Jakab et al. were the first to research spheroid bioprinting. They used computer simulations to demonstrate how to use tissue liquidity to construct tissue structures with prescribed geometric shapes in vitro. The eventual printing of a ring of spherical aggregates demonstrates the potential for future vascularization [92]. Bulanova et al. developed a novel and original multifunctional 3D bioprinter, Fabion, equipped with a turnstile system that dispenses a single spheroid at a time. The printer includes a cooling and heating system to control the polymerization of the collagen hydrogel better. The system releases spheroids onto a receiving substrate along a preset path via three pistons [108]. Mekhileri et al. developed an automated 3D bioassembly platform to assemble human chondrocyte microtissues with thermoplastic frameworks. The singularization system collects the microtissues collected during the high-throughput manufacturing into the reservoir hopper to control the extrusion of individual microtissues and avoid clogging the extrusion head with many microtissues [93] (Figs. 2c and 2d).

In theory, the EBB extrudes bioink using a nozzle to deposit continuous microfilaments to form the desired geometry. For spheroid 3D bioprinting, the bioink is based on spheroids, loaded into a syringe, and extruded onto the substrate by depositing one after another to allow fusion to form a tissue patch. Although the EBB can support many hydrogel materials, controlling the fusion of spheroids, preventing the spheroids from blocking the nozzle, and reducing the mechanical damage of spheroids are fundamental issues that must be urgently solved. When spheroids are far apart during extrusion, they will affect the communication and fusion between them. The size of spheroids should be focused on during fabrication. If the size of spheroids is smaller than that of the nozzle, the spheroids loaded into the bioink will be deposited randomly, resulting in uncontrollable spheroid density and distribution in the bioink. However, if the size

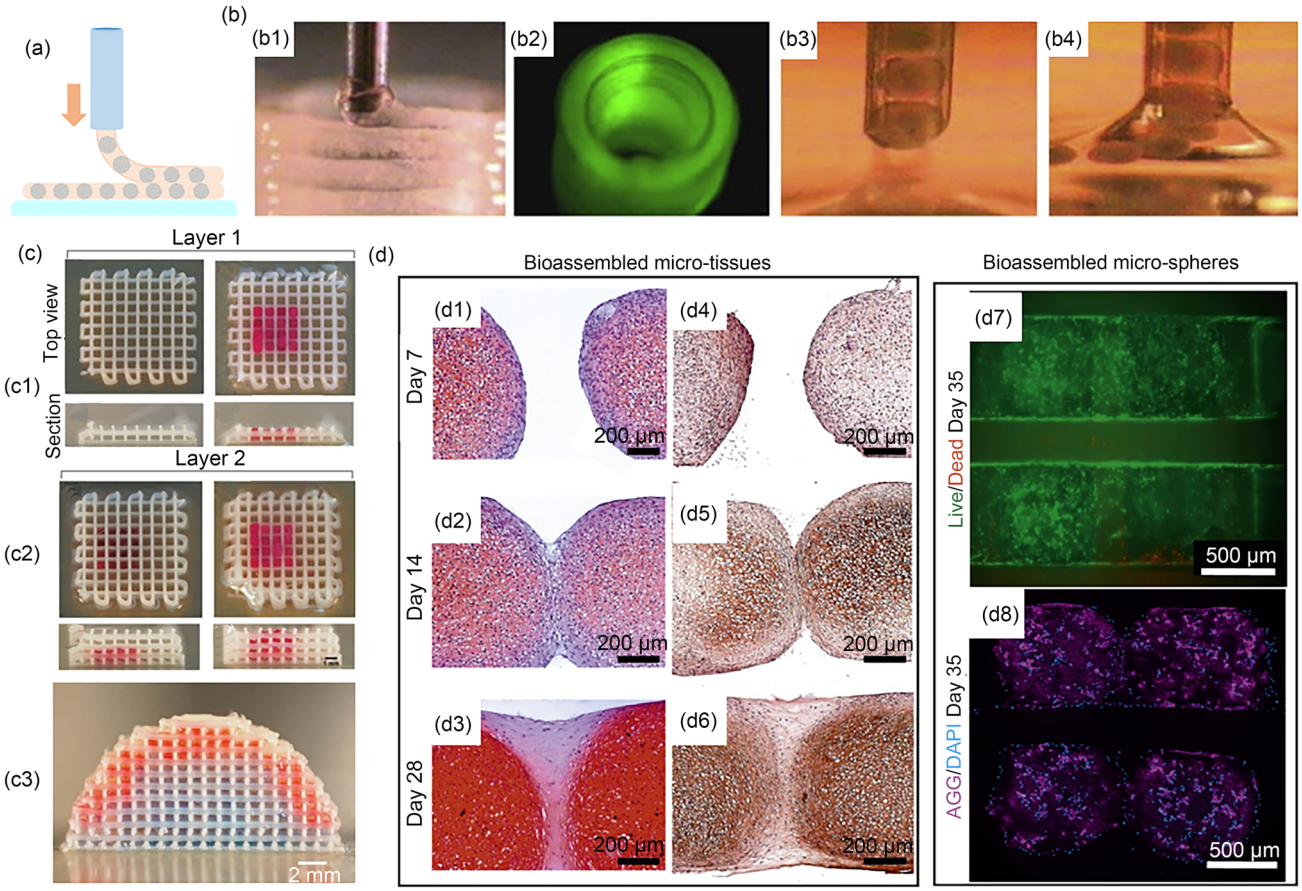


Fig. 2 Extrusion-based bioprinting (EBB). **a** EBB of spheroids one by one. **b**, **b1** bioprinter (general view); **b2** multiple bioprinter nozzles; **b3** tissue spheroids before dispensing; **b4** tissue spheroids during dispensing (reproduced from Ref. [11], Copyright 2009, with permission from Elsevier Ltd.). **c**, **c1**, **c2** The top and sectional front view photographs of the bioassembled construct; **c3** a biphasic hemispherical construct with stained gelatin methacryloyl (GelMA) hydrogel microspheres representing chondrogenic (red) and osteogenic (blue) phases of an osteochondral construct fabricated by applying the bottom-up automated tissue bioassembly strategy. **d**, **d1**–**d6** Sections of assembled microtissues and associated tissue fusion in an adjacent culture over 28 days. **d7**, **d8** Bioassembled microspheres after 35 days culture in chondrogenic differentiation media (reproduced from Ref. [93], Copyright 2018, with permission from IOP Publishing Ltd., licensed under the Creative Commons Attribution 3.0 license)

of spheroids is larger than the nozzle diameter, it will easily congest the spheroid at the outlet. If the extrusion pressure is increased, it will damage the spheroid. Many problems can affect the continuity of the printing process. As existing bioprinters are open loops, researchers must constantly observe and judge the printing process. Therefore, realizing a closed-loop biofabrication method that can be controlled in real time is a vital research direction in the future.

Droplet-based bioprinting

Droplet-based bioprinting originated from the traditional inkjet printing technologies, including inkjet [109, 110], acoustic droplet ejection [111, 112], and microvalve bioprinting [113, 114]. The droplet-based bioprinting offers simplicity, agility, versatility and precise deposition control. It can achieve highly precise cell localization and resolution. This high-resolution cell localization helps precisely control the spatial layout and arrangement of cells when constructing tissue structures. The ability to print many types of cells simultaneously opens the possibility of constructing complex tissue structures and organs [115]. The droplet-based bioprinting can be combined with automated systems to achieve large-scale and high-throughput cell and tissue construction. It can meet the high printing speed and print many cells and biological materials in a short time. This efficient printing speed can help realize large-scale tissue engineering and biomanufacturing. Combining robotics and intelligent control systems can realize the standardization and customization of biological manufacturing.

Gutzweiler et al. proposed a large-scale and rapid production of human umbilical vein endothelial cell spheroids based

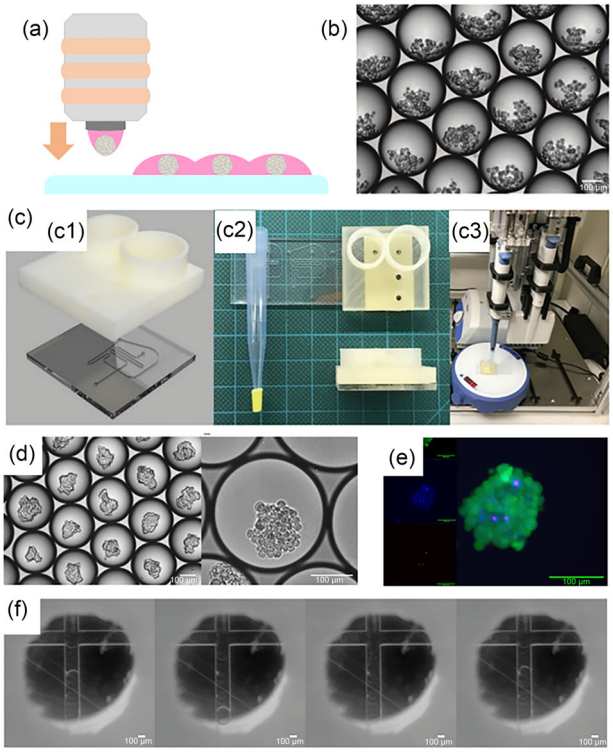


Fig. 3 Droplet-based bioprinting. **a** Printing droplets using various driving forces, such as heat, piezoelectricity, electrostatics, gravity, and atmospheric pressure. **b** The robotized droplet microfluidic platform produces highly monodisperse 290-μm droplets. **c**, **c1** The droplet-generating chip comprising 3D-printed components and polymethyl methacrylate (PMMA); **c2**, **c3** connecting the pipette tip with the droplet-generating chip with an automated system. **d** Human embryonic kidney cells in droplets after 16 h. **e** A fluorescent image of the viability-stained spheroids. **f** The process of droplet generation actuated by the sequential movement of the computer-controlled pipette observed in the droplet-generating chip. Scale bar: 100 μm. Reproduced from Ref. [95], Copyright 2019, with permission from Society for Laboratory Automation and Screening

on the hanging drop method in an array form by installing magnetic valves on a three-axis system. They investigated a single spheroid deposition setup by building a transmitted light setup enabled by cameras and light-emitting diodes to image the nozzle interior close to the orifice. If a single spheroid is not detected, the vacuum shutter system sucked away the generated droplets, ensuring the reliability of spheroid deposition [94]. Langer and Joensson provided a robotic automated droplet microfluidic platform to generate spheroids by encapsulating the cells in these droplets. The system has a maximum throughput of 85,000 spheroids per hour. They also proposed a scalable method for sample recovery from droplets that do not require chemicals to disrupt the emulsion or support process automation [95] (Fig. 3).

Although droplet-based bioprinting has been widely used in several applications including tissue engineering and regenerative medicine, drug testing, cancer research, and high-throughput screening, the technology faces several limitations. First, biomaterials and carriers suitable for printing must be used. Selecting the correct material and adjusting its properties to meet the requirements of the printing process is challenging. The range of available bioink materials is narrow, and most bioink materials used in EBB are unsuitable for droplet-based bioprinting. As the pore diameter of the nozzle ranges from 10 to 150 μm [116], extremely small fragments of the bioink can accumulate inside the pores and impede the flow, eventually leading to complete clogging. Some cell types might be more sensitive to pressure, shear, and environmental changes during printing, which could result in cell damage or death. Droplet-printed tissue and organ constructs have weak structural and mechanical properties, and there might be limitations in creating tissues with fine structures, cavities, or complex shapes. Building complex vascular networks and ensuring tight connections between cells and surrounding tissues remain challenging [117].

The Kenzan method

The word Kenzan comes from Japanese and means sword-like mountain. Kenzan is an array comprising microneedles fixed on a base. It was initially a fixture for flower arrangements; however, Japanese researchers placed spheroids one by one on the microneedle array for tissue construction [118]. To allow the spheroids to interact and secrete matrix, they were robotically pierced in microneedles that served as temporary supports, relying solely on cells to construct complex tissue analogs of almost any composition [96].

In the Kenzan method, the arrangement and distribution of needle arrays are the primary selection parameters. The arrays are divided into squares and circles, such as 9×9 and 26×26 square arrays and 9×9 and 13×13 circle arrays [96]. Commercial printers have been used in rat bladder reconstruction [119], blood vessel-like structures [120], peripheral nerve reconstruction [121], trachea-like structures [122], esophagus-like structures [123], heart-like structures [124], liver-like structures [125], and diaphragm reconstruction [126]. Nakamura et al. used the Kenzan method to fabricate cartilage constructs to repair large cartilage defects, improving the mechanical strength of the construct and formation of the extracellular matrix (ECM) [97] (Fig. 4). Arai et al. used a bio-3D printer to fabricate a scaffold-free cardiac tube structure and evaluated it as a cardiac pump [88]. LaBarge et al. improved the nozzle of the Kenzan device to bioprint human induced pluripotent stem cell (iPSC)-derived spheroids onto a 4×4 needle array to print the entire tissue layer and improve printing efficiency [98].

Although the Kenzan method is an ideal solution for spheroids to be used as tissue building blocks and printed, it cannot completely realize 3D printing. The microneedle is completely vertical on the z axis, limiting the angle change

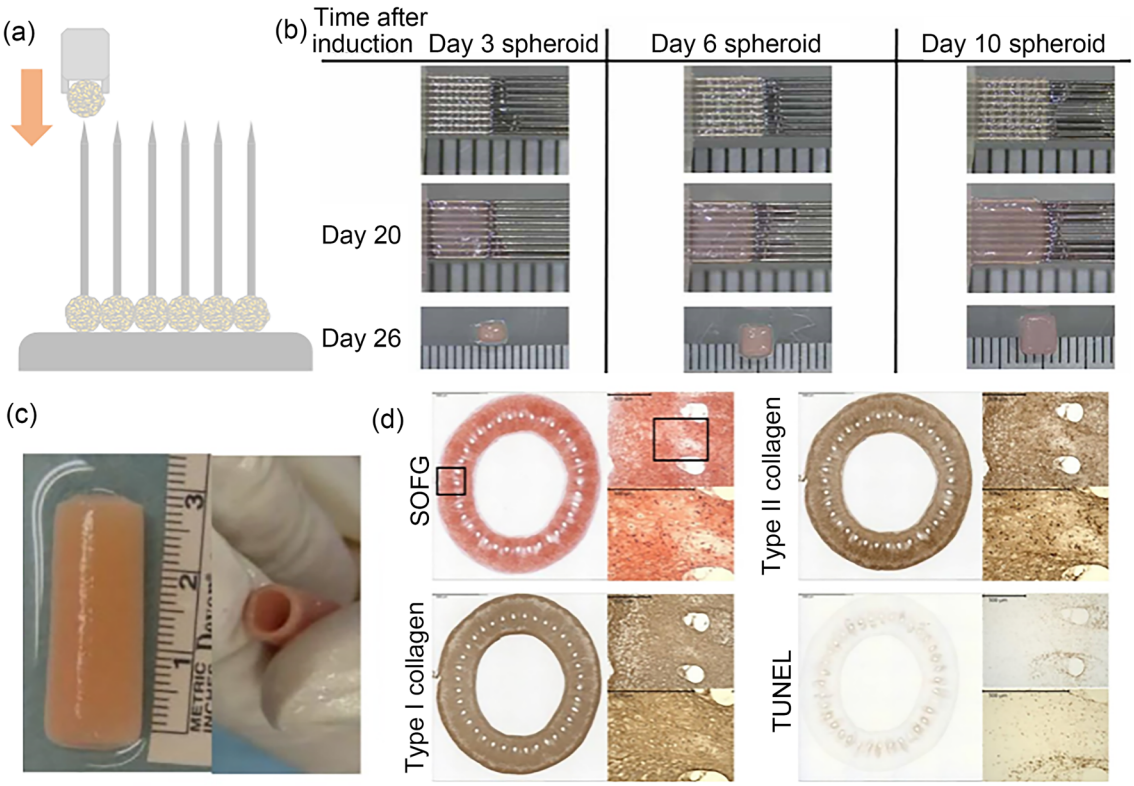


Fig. 4 The Kenzan method. **a** Spheroids are employed to fabricate scaffold-free cell constructs. **b** Gross images of constructs on the Kenzan and 6 d after removal from the Kenzan. **c** Bio-3D-printer design data and gross images of the construct. **d** Overview images and representative area of the construct stained with safranin o-fast green, types I and II collagen, and TdT-mediated dUTP nick-end labeling; the histological evaluation images were taken at ×4, ×10, and ×20 magnification. Scale bars represent 1000 μm on ×4 and 500 μm on ×10 and ×20 magnification. Reproduced from Ref. [97], Copyright 2021, with permission from the authors, licensed under the Creative Commons Attribution 4.0 license

on the z axis and making it impossible to print multidimensional complex tissue structures. Besides, spheroids can only be threaded on microneedles, meaning that the arrangement of microneedles limits its crossscale performance. The single method also leads to large printing limitations and low scalability. Moreover, the spacing between the microneedles defines the overall printing resolution. The requirement for the spheroid size is high. If the spheroids are too small, gaps will appear between the spheroids worn on the microneedles, hindering fusion and communication. Excessively large spheroids might result in impaired spheroid extrusion. Therefore, it is necessary to increase screening work before printing, affecting printing efficiency. Although the current Kenzan method has a relatively single function, biological researchers can directly use commercial bioprinters which have advantages regarding stability and reproducibility [118]. It is hoped that the manufactured tissues will not only be used for experimental research, but also can gradually move toward clinical applications.

Bioassembly methods

Cell aggregates as building blocks offer considerable possibilities for tissue engineering but are not limited to spherical shapes. Placing cells into molds triggers their aggregation in mold shapes, such as rings, tissue strands, and honeycombs [127–130]. Bioassembled spheroids are an exemplary means of rapidly creating tissues in vitro. Therefore, capturing these building blocks while minimizing damage to cells, assembling them in an orderly and controllable manner, and finally forming the required 3D structure is an urgent problem for researchers to solve.

Blakely et al. developed a system called Bio-Pick, Place and Perfuse (Bio-P3) [131] to fabricate large constructs with high densities of living cells. Bio-P3 can pick and place these multicellular microtissue parts (spheroids, rings, and honeycombs) from nonadherent molds. Later, they also improved the bio-gripper, which can be used to manipulate living microtissues of different sizes and shapes in aqueous environments [132]. Recently, the same research group verified the versatility of Bio-P3. The macrotissues constructed from

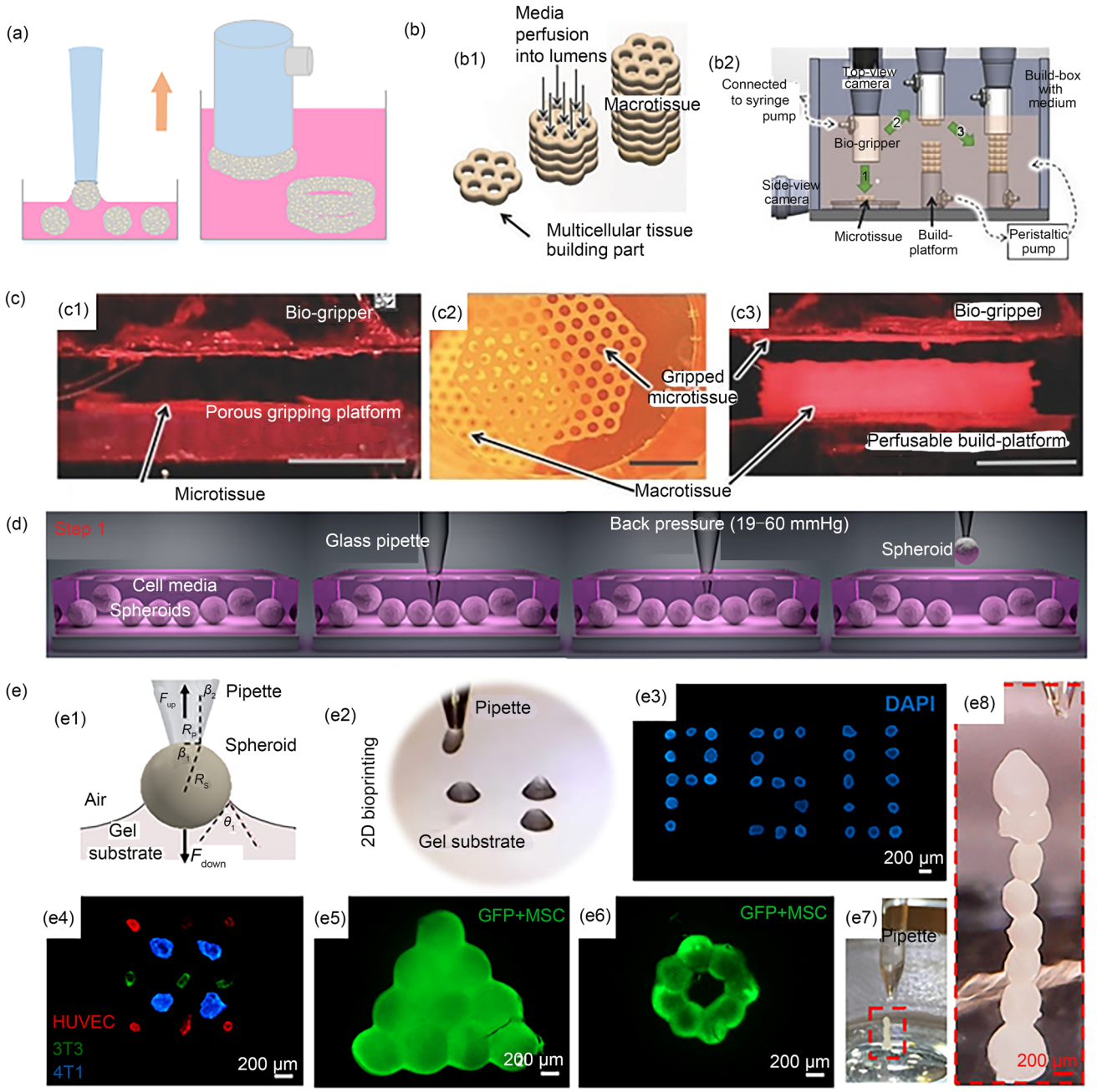


Fig. 5 Bioassembly methods. **a** Aspiration-assisted bioprinting (AAB) and the bio-gripper method. **b** Assembly using the bio-gripper. **c** Building a macrotissue from single six-orbital honeycomb-shaped tissues using the bio-gripper. Reproduced from Ref. [99], Copyright 2018, with permission from WILEY–VCH Verlag GmbH & Co. KGaA, Weinheim. **d** The spheroids are picked from the cell media by a glass pipette, where the required back pressure is set to lift the spheroids. **e**, **e1** A schematic showing critical parameters during bioprinting; **e2–e8** spheroids are printed at the desired locations. Reproduced from Ref. [100], Copyright 2020, with permission from the authors, licensed under CC BY-NC

human hepatocellular microtissues maintained geometry and function (albumin and urea secretion) over five days [99] (Figs. 5a–5c). Their Bio-P3 device is a crucial step toward large-scale biofabrication of organs with high cell densities.

Ayan et al. proposed aspiration-assisted bioprinting (AAB) using the suction power to pick up and precisely print/place cell aggregates onto the substrate [100] (Figs. 5d and 5e). The physical behavior of viscoelastic spheroids and their underlying mechanisms of interaction with physical control forces during uptake, lifting, and bioprinting were explained in detail. Compared with other spheroid bioprinting methods, AAB provides higher positioning accuracy and

reduces adverse effects on spheroid viability and tissue damage. AAB methods have been used to create bone tissue [133, 134] and hearts [135].

Bio-P3 cannot fully realize 3D printing because the simple vertical stacking of the same building blocks along the z axis cannot achieve complex 3D structures unless the building blocks of each layer are designed differently to meet the 3D complexity. AAB selects and accurately places spheroids one by one, which is better than Bio-P3 in resolution but is time-consuming and has a throughput disadvantage. In the future, when fabricated tissues are further scaled up, fabricating many spheroids and conducting long-term bioprinting in an automated manner are the biggest challenges. Maintaining sufficient spheroids or aggregates in storage within the repository while preserving their activity requires precise and efficient handling by the gripper head, which must accurately perform tasks such as capturing, assembling, and printing. Automation is essential throughout the manufacturing process; therefore, enabling the rapid selection of building blocks and developing clamping heads at high resolution and throughput promise to expand capture and assembly methods.

Magnetic bioprinting

Like the formation of spheroids by magnetic manipulation, the assembled spheroids can be reassembled through magnetic control to form larger tissue structures. Incorporating magnetic particles into the spheroids allows the magnetic manipulation of desired shapes, patterns, and 3D tissue structures using magnetic forces. Magnetic bioprinting offers high degrees of freedom and controllability. Magnetic bioprinting has an advantage in the speed of aggregate formation. 3D cultures can be formed in approximately 16 h [136]. Magnetic bioprinting can also create 3D cultures without artificial protein substrates and generate a robust endogenous ECM during their formation [137].

However, infiltrating magnetic particles such as iron oxide nanoparticles, which have direct and long-term interactions with cells and biological systems, can adversely affect cell viability, phenotype, and function and remains a critical issue. Mattix et al. incorporated magnetoferritin nanoparticles into spheroids without adversely affecting cell viability for up to one week. Finally, spheroids were magnetically patterned and fused into tissue rings, demonstrating their potential in tissue engineering applications [138] (Figs. 6a and 6b). Tseng et al. generated spheroids using magnetic bioprinting. They validated spheroid contraction as a cytotoxicity endpoint. Real-time toxicity monitoring was performed through mobile-based imaging, providing a straightforward, rapid, and reliable method for high-throughput cell toxicity screening in a 3D environment [101] (Fig. 6c).

Magnetic bioprinting costs are relatively high because unique accessories are required for magnetically generated spheroids and reassembly. Powerful magnetic fields (800–4000 G) have been shown to affect cell behavior [139]. Some nanoparticles might adhere to the bottom of the plate rather than remain suspended, leading to the incomplete attachment of cells to magnetic nanoparticles during incubation and the potential for cell loss. Furthermore, iron oxide in the magnetic nanoparticle will make the spheroid appear dark brown, which might affect imaging diagnosis and observation in some applications [137].

Discussion and perspectives

Fabrication of spheroid and 3D bioprinting are technologies that can create complex 3D biological structures, such as tissues, organs, and cell clusters. Although spheroids and 3D bioprinting are manufactured differently and with different materials, they can be used to study cell function, tissue engineering, drug development, and medical research. Combining spheroids with 3D bioprinting has advantages for creating large-scale, highly customized biological structures.

Although spheroids have shown considerable promise for tissue fabrication, much work remains. The first is the issue of consistency. During spheroid manufacturing, various factors frequently affect the self-assembly of cells, such as cell types, culture conditions, and the external environment, which might lead to inconsistencies in the size, shape, and tissue structure of spheroids. This inconsistency can affect the functionality and performance of the organization being built. Spheroids formed from commercially available spheroid culture plates can ensure stable sizes and have less surface variability. Spheroids are more consistent and easier to integrate into routine workflows. Since the size of spheroids is directly related to cellular function, it is crucial to maintain their size consistency in industrial applications, especially in drug development [8, 140]. The prerequisite for the success of spheroid bioprinting is to manufacture and collect spheroids with uniform and controllable sizes.

When manufacturing spheroids, precise control of the cell type, density, and environmental factors is required to achieve the desired tissue structure and function, which might require complex techniques and equipment, and time-consuming experimental optimization. When cells self-assemble to form spheroids, they will be affected by environmental changes, causing some cells to die or lose function, affecting the biological activity of the spheroids and the feasibility of tissue engineering applications. Some tissue structures, such as the heart, liver, nerve, and skin, require many spheroids to build complex tissue structures. While some spheroids can mimic the structural aspects of specific tissues, they

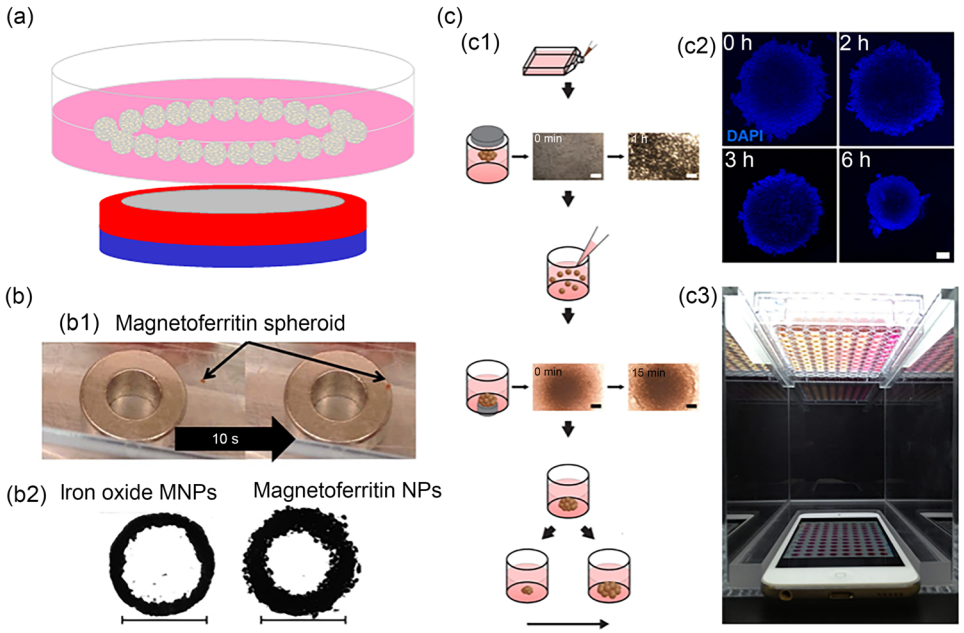


Fig. 6 Magnetic bioprinting. **a** Cells aggregate into larger tissues under the influence of magnetism. **b**, **b1** The ability of magnetoferritin cellular spheroids to magnetically attract to a permanent magnet was confirmed (magnet diameter: 10 mm); **b2** using magnetic force assembly, the results showed a fused homogeneous tissue. Reproduced from Ref. [138], Copyright 2013, with permission from Acta Materialia Inc. **c**, **c1** The cells mixing with magnetic nanoparticles were then printed for 15 min by placing the plate on top of a 96-well magnetic drive; **c2** spheroid contraction over 6 h as cells rearrange and compact (nuclei blue); **c3** the mobile device based imaging system. Reproduced from Ref. [101], Copyright 2015, with permission from the authors, licensed under a Creative Commons Attribution 4.0 International License

might be more in appearance than function. Cellular interactions and tissue function inside spheroids might still differ from real tissues. Therefore, the scalability of spheroid fabrication and controlled spheroid assembly should become fundamental research focuses in future tissue engineering developments. Powerful robotic manufacturing methods can be used to achieve crossscale manufacturing. With the continuous development of micro–nanomanufacturing and microfluidic technology, combined with automation, more new spheroids manufacturing platforms are continuously developed. Spheroids are constantly innovated and improved for high throughput, high precision, and multicells [61, 83]. The spheroid manufacturing platform developed in the future could be combined with artificial intelligence to reduce the labor intensity of scientific researchers significantly. Friendly human–computer interaction makes it easy to observe the growth of cell spheroids, reduce the cost and time of manufacturing, and improve reproducibility.

Currently, spheroid 3D bioprinting will still be affected by the following factors. (1) The choice of materials, such as bioink, is crucial in spheroid 3D bioprinting. Different bioinks can have different effects on the formation and biological activity of spheroids. However, few options exist for bioinks suitable for 3D bioprinting. (2) Some methods have limited ability to form complex 3D shapes. (3) The spatial distribution and density distribution of cell spheroids are uneven. Spheroid 3D bioprinting involves controlling multiple factors, including cell type, bioink, and printing parameters. This complexity can lead to parameters that are challenging to manipulate and optimize. (4) During printing, cells must undergo ejection, positioning, and solidification, which might damage or kill some cells, posing challenges for constructing biological structures with good bioactivity. (5) The stability of the tissue after printing is completed. The shape of the cell sphere might change due to fluid mechanics or force during printing, affecting the stability of the spheroid. (6) Blood vessels are formed in the tissue. Adequate vascularization is fundamental in tissue engineering. When the size of biological tissue is large, nutrients frequently cannot be effectively transported to the internal areas of the tissue. Printed tissue structures can only survive with sufficient nutrient supply [141]. Spheroids have been shown to be multifunctional vascularized units for future angiogenic and prevascularization approaches in tissue engineering and regenerative medicine [142]. However, it requires much effort to achieve vascularization and long-term culture of tissue engineering. (7) The cost is high. Spheroid 3D bioprinting involves specific equipment, bioink, and long-term culture of spheroids. It is still experimental, and the cost should be reduced when the subsequent process matures.

The function and mechanism of the existing spheroid manufacturing process and bioprinting equipment limit the

cell spheroid 3D bioprinting technology. While the Kenzan method can be fully automated, its equipment is not flexible regarding parameters the user can adjust. Each method has its own set of processes, but it is only suitable for laboratories and not for commercial mass production. Therefore, developing user-friendly, cost-effective, multifunctional, and highly flexible fabrication is crucial for developing spheroid 3D bioprinting.

Combining spheroid fabrication and 3D bioprinting should enable strict and robust process control to produce spheroids of consistent size and reproducible quality and create artificial tissues. Due to the varying results and the increasingly complex experimental requirements, the manufacturing process must be automated. Automation can reduce the influence of other instabilities, enhancing reproducibility and experimental quality. With the increasing choice of low-cost automated dispensing technology and the maturity and stability of three-axis system architecture, this transition has become easier economically. Future development of spheroid fabrication should focus on improving process control, standardization, scalability, monitoring, and advanced spheroid transfer and characterization methods. Developing a tissue engineering production line from the fabrication of basic building blocks to final printing or assembly entails developing an automated, intervention-free manufacturing process to enhance the quality and repeatability of experiments. Limited by space issues in laboratories, the size of the factory line system should be reduced to that of a desktop printer. Full automation is required to deliver safe and effective scalable products in the context of numerous diseases and organ/tissue injuries. Scalability and automation suitability using robotic bioprinters are perhaps the most attractive aspects of directed tissue self-assembly techniques.

Conclusions

Spheroid 3D bioprinting produces functional tissues by printing spheroids as building blocks. The hanging drop, microwell, microfluidic, magnetic manipulation, bioreactor, and customized printer methods are typically used to preproduce high-quality and controllable spheroids. Several printing strategies exist, such as EBB, droplet-based bioprinting, Kenzan, bioassembly, and magnetic bioprinting. Researchers are increasingly developing new methods to reconstruct tissue structure and function. However, technical challenges exist in current spheroid fabrication and 3D bioprinting, such as uneven spatial and density distribution of the spheroids after printing, mechanical damage to the spheroids, the stability of the tissue, and the formation of blood vessels in the tissue. Overcoming the above challenges requires a multidisciplinary team. We also need more insights into different methods of making and printing spheres and selecting effective means of spheroid production and application to achieve a high level of controllable spheroid distribution and density, and high-throughput manufacturing that considers high resolution, effective vascularization of thick 3D tissue constructs, standardized production of automated production lines, and intelligent robots.

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Author contributions CXL investigated and summarized the literature, and wrote the original draft. CG conducted deep review and editing. HQ and AXJ edited the images. YZ and HZL gave some advice. YYL helped revise the paper, supervised the work, and applied for funds. All authors have read and approved this manuscript for publication.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

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