Bioink: a 3D-bioprinting tool for anticancer drug discovery and cancer management

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'Bioinks' are important tools for the fabrication of artificial living-tissue constructs that are able to mimic all properties of native tissues via 3D bioprinting technologies. Bioinks are most commonly made by incorporating live cells of interest within a natural or synthetic biocompatible polymeric matrix. In oncology research, the ability to recreate a tumor microenvironment (TME) using by 3D bioprinting constitutes a promising approach for drug development, screening, and *in vitro* cancer modeling. Here, we review the different types of bioink used for 3D bioprinting, with a focus on its application in cancer management. In addition, we consider the fabrication of bioink using customized materials/cells and their properties in the field of cancer drug discovery.

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Introduction

Finding the most efficient and effective anticancer drug, either by improving existing therapeutics, drug repurposing, or *de novo* discovery of new active agents, is the daily quest for millions of scientists worldwide. However, translating a molecule 'from the bench to the bedside' is a costly and time-consuming process. Indeed, once the lead compound has been identified, it must enter preclinical testing (both *in vitro* and *in vivo*) and human clinical trials before the new chemical entity can undergo any regulatory agency approval process [1–5].

Additive manufacturing (i.e., 3D printing), a remarkable technological advancement fostered by the analog/digital process transition, exploits computer-assisted methodologies to deposit material(s), layer by layer, according to precise space and time indications to ultimately generate a

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3D object. In the biomedical arena, the utilization of 3D printing-like techniques (3D bioprinting) is being adopted to combine different biological entities (e.g., living cells, hormones/growth factors, and a plethora of other bio-based materials, collectively known as bioinks) to produce tissue-like structures able to mimic corresponding living constructs. In oncology research, 3D bioprinting is increasingly gaining attention because it offers further advantages, such as the potential to recreate reliable 'out-of-patient' *in vivo* TME. Thus, 3D-bioprinted cancer models are expected to quickly bridge the gap between conventional cell culture testing and animal trials both in anticancer drug discovery and cancer management [6–8].

In routine practice, cancer is studied and modeled by exploiting data originated from monolayer cell cultures and small animal species (mainly mice). Although these animals are physiologically competent, they often fail to predict human behaviors and responses, whereas planar cell models, because they are overly simplified, are often poorly representative (if at all) of *in vivo* performance. One of the many reasons for these failures can be ascribed to the TME. *In vivo*, the TME is an exceedingly complex dynamic and time-evolving ensemble of proliferating tumor cells/stroma, blood vessels, infiltrating inflammatory cells, and a variety of other tissue-associated cells. In the TME, cells not only interact with other cells of the same/different origin, but are also entrenched in an dysregulated extracellular matrix (ECM) [6,7,9]. Thus, 3D cell cultures are considered to offer a more realistic emulation of TMEs compared with monolayer (2D) cell models; however, most current 3D biomanufacturing techniques have not yet been able to recapitulate the TME complexity to a satisfactory level. However, by exploiting robotized manufacturing processes able to deploy/assemble various types of cell/biomaterial according to highly reproducible time- and space-ordered arrays, the obstacles faced by other bio-manufacturing techniques in the generation of tightly controlled, and well-defined structures required for integration into realistic *in vitro* cancer models can be effectively overcome [10–13].

3D bioprinting offers other substantial advantages. For instance, it (i) efficiently decreases the occurrence of cross-contamination during, for example, the generation of precisely organized co-culture models, a risk intrinsically connected to cells handling in limited spaces; (ii) permits fine control over genes, growth factors, and drug delivery; and (iii) allows high-throughput generation of constructs with pores of customized size(s) to fit the heterogeneity of a specific tissue architecture. Remarkably, although bioprinting still lacks in efficiency in reproducing highly vascularized tumor constructs, it remains the mainstay among others methods [14–20]. Other significant benefits include construct fabrication under conditions that are physiologically relevant (e.g., different pHs, temperatures, and degrees of hydration) and the fundamental ability to bioprint cells along with genetic and protein material to regulate cell functions [21]. These advantages have contributed to the rapid movement of bioprinted entities from initial prototype levels to the commercial stage [17,18]. However, bioprinting-based manufacturing of 3D constructs is faces several obstacles, such as fully reliable high-throughput proficiency and error-free production of models with dimensions $<$50 mm [19].

Currently, the resolution of the arm-stage movement of bioprinters determines the resolution of the corresponding 3D-bioprinted models [20]. Nevertheless, new advances based on magnetic levitation (maglev) technologies in the bioprinting of tissue spheroids have enhanced the flow and resolution characteristics of bioprinted structures. There is an ever-increasing number of publications concerning the adoption of bioprinted models in a range applications, from disease modeling [22,23], drug development and pharmaceutical manufacturing [24,25], to the engineering of complex tissue constructs [26]; however, the application of bioprinting in drug design development across a variety of tissues has not yet been addressed in depth in the literature [2,27].

Components of bioprinting

3D bioprinting technology relies on three major pillars, the bioink, bioprinter, and associated bioprinting procedure, from the specific tissue design to bioink deposition during target tissue development.

In general terms, a bioink comprises a biocompatible hydrogel in which the living cells of interest are embedded. Note that the term 'bioink' should not be confused with so-called 'biomaterial inks', which are generally conceived as synthetic or biomaterials that must be first printed, sterilized if needed, and then seeded with cells to realize scaffold components/implants or to generate hybrid supports to improve the mechanical resistance of 3D printed specimens [28] (Fig. 1). Bioinks are the materials used in the preparation of processed (artificial) living tissues using 3D-printing technology [29]. They can comprise only cells, but an extra carrier substance (usually either a biocompatible synthetic or a natural polymer gel or a gel based on the combination of both), which surrounds the cells and acts as a 3D molecular scaffold, is often included. The purpose of this gel is manifold because it can: (i) serve as a platform upon which cells can adhere, spread, grow, and proliferate; (ii) can be chemically/physically crosslinked/stabilized during or immediately after bioprinting to create the desired construct; and (iii) prevents cells from being damaged during the printing process.

An ideal bioink should in principle be endowed with the same rheo/mechanical and biological properties displayed by the targeted tissues (or organisms). If realized, these bioink characteristics in turn guarantee matching of the mechanical behavior between the replicated construct and the original tissue/organ, replica shape preservation via easy mastering of the underlying cross-linked network structure, biocompatibility/biodegradability, and accessibility to chemical modification when required. The optimal bioink should also be amenable to good manufacturing processes (GMP), upscaling production to large volumes and minimization of batch-to-batch variability, to be consistently produced and controlled according to quality standards at the commercial level [30].

Currently, bioinks are broadly classified into six major categories: (i) protein- or peptide-based bioinks; (ii) polysaccharide-based bioinks; (iii) ECM-based bioinks; (iv) synthetic polymer-based and commercial bioinks; (v) cell-aggregate or pellet-based bioinks; and (vi) composite bioinks or bioinks based on bioactive molecules (Table 1).

Peptide-based bioinks have been designed with the purpose of maximizing their ability to regenerate the ECM by, for example,

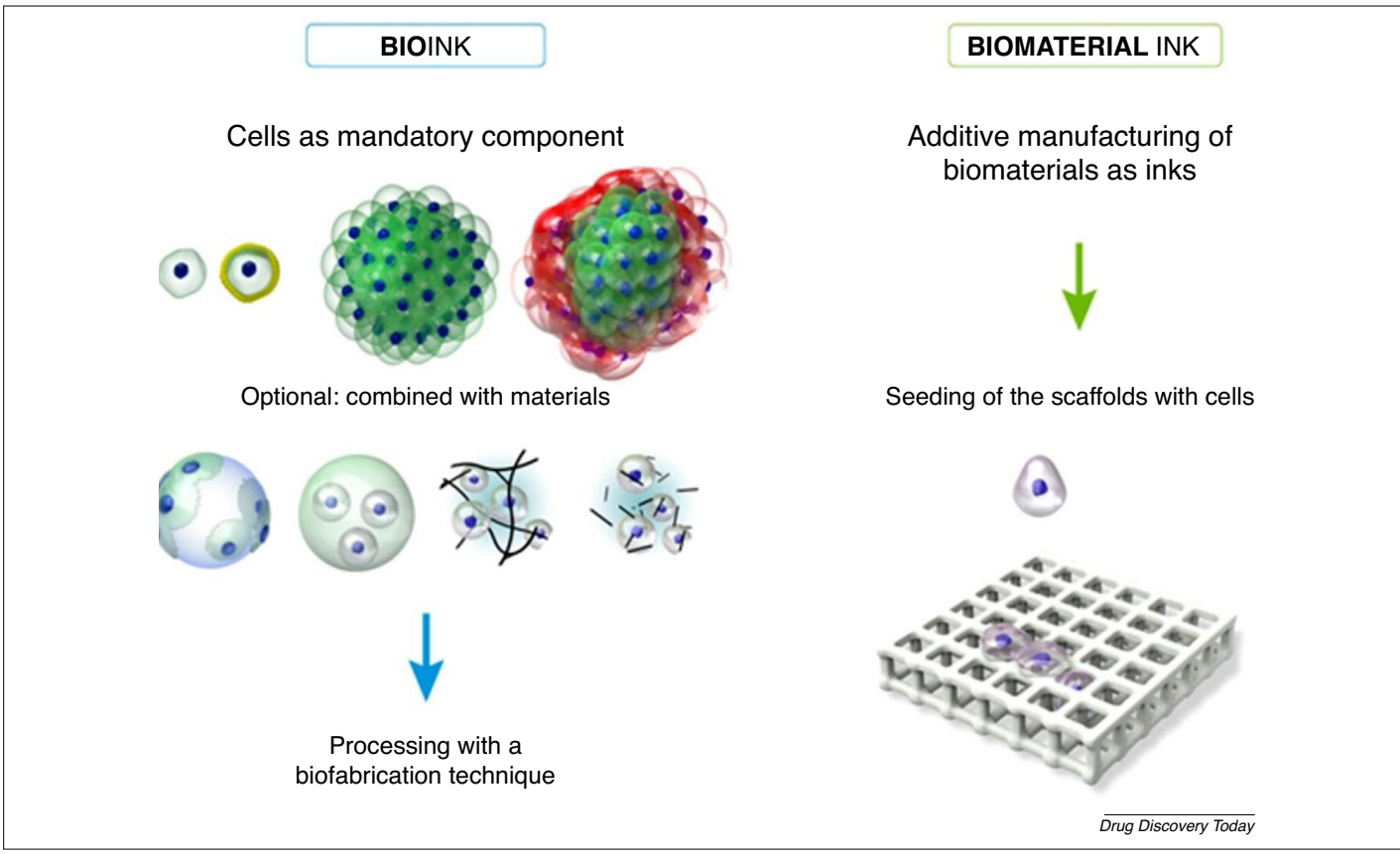

FIGURE 1

Differences between the concepts of bioinks **(a)** and biomaterial inks **(b)**. In a bioink, cells in different forms (single cells, cell aggregates, coated cells etc.) are the main components of the printing formulation. Cells can also be loaded onto microcarriers or embedded into physical or chemical hydrogels, before printing. By contrast, biomaterial inks can be comprised by any synthetic or natural biocompatible material amenable to 3D printing; cell seeding occurs only post fabrication [28].

TABLE 1

Examples of bioinks used for cancer and drug screening

Bioink	3D models	Type of action	Refs
Protein	3D model of ovarian cancer	Cell responses to different chemotherapeutics	[33]
Polysaccharides	Triple-negative breast cancer and lung adenocarcinoma	High-throughput drug screening	[34]
ECM	Human lung (human airway epithelial progenitor and smooth muscle cells)	Generating human functional tissues	[36]
Human skin derived natural bioink (Dermamatix)	Human skin	Bone formation	[51]
Thermoresponsive hydrogel (Novogel)	AORTA	Tissue regeneration	[52]
Antibody-drug conjugate based bioink (Kite ,a Gilead Company,and Oxford Biotherapeutics Ltd.)	Solid tumors	Cell therapy for treatment of blood cancers and solid tumors	[53]
Biomolecule-loaded bioink	Complex cell-laden constructs	*In vitro* research for clinical applications	[41]

allowing coordination, fine control of the rheomechanical behavior, and the integration of protein-mimetic peptides for angiogenesis. Since its inception, this technique has been used to co-assemble peptides with different biologics, such as polysaccharides [glycosaminoglycans, including heparin and hyaluronic acid (HA) in particular] [31] and proteins to grow *in vivo* mimicking 3D cell cultures. For example, Ma *et al*. reported the development of a 3D-bioprinted model mimicking the brain matrix microenvironment in which the bioink comprised normal glial cells (HEB) embedded in a HA hydrogel with different amounts of added gelatin [32].

Different bioink formulations have been tested in terms of their biological and rheomechanical responses in compared with *in vivo* brain tissues. It was reported that bioinks containing a specific amount of gelatin were able to recapitulate relatively closely both structural and flexibility features of human brain specimens; this, in turn, supported several normal cell functions, including prolif-

eration, aggregation, and motility. Ultimately, this study provided reliable experimental models for further exploration of multiple factors involved in the brain microenvironment and as scaffolds for the study of malignant glioma invasion (e.g., glioblastoma multiforme; GBM) [32]. Within the same class of bioinks, other sophisticated and tunable hydrogel-based 3D models have been reported based on the co-assembly/organization of different peptide amphiphiles (PAs) with ECM proteins from different sources (i.e., ovarian cancer, endothelial cells, and mesenchymal stem cells) [33]. These complex 3D constructs were able to promote the rapid formation of F-actin-decorated 3D tumor spheroids characterized by cell–cell/cell–matrix communication/interactions and encapsulation responses comparable to those observed using standard Matrigel. The functionality of these models was further validated by analyzing their responses to treatments based on clinically approved chemotherapeutics and, although 3D bioprinting was not utilized in this study, the overall characteristics of these constructs speak in favor of their adoption as bioinks for the preparation of complex, reproducible, and reliable 3D ovarian cancer models [33].

In the quest for easy-to-handle hydrogels to generate stable and shape-preserving 3D-printed tumor models mimicking TME for chemotherapeutic drug screening, Gebeyehu and coworkers developed polysaccharide-based bioinks endowed with good printability, rheological, and biocompatible properties36. This bioink allowed for fast (7 days) and large (500 μm) tumor spheroid growth and TME formation from xenografts derived from patients with nonsmall cell lung cancer (NSLC). Moreover, these 3D bioprinted cell constructs exhibited increased drug resistance toward three anticancer drugs [docetaxel, doxorubicin (DOX), and erlotinib] with respect to the relevant 2D cell cultures [i.e., monolayers of NSCLC derived from patient xenografts, from wild-type triple-negative breast cancer (MDA-MB-231 WT) cells and from lung adenocarcinoma (HCC-827) cells'. Overall, these results support the validity of this bioink for 3D cell bioprinting of constructs amenable to *in vitro* TME development for the high-throughput screening of anticancer therapeutics [34].

In the arena of ECM-based bioinks, technological progress in the extraction/purification methods of decellularized ECM (dECM) from both healthy and malignant tissues paved the way to the manufacturing of *in vitro* 3D cancer models exhibiting all the essential characteristics and reproducing the dynamical nature of malignant cell–ECM crosstalk [35]. For example, De Santis *et al*. produced a tissue-specific dECM/alginate hybrid bioink featuring an optimal combination of rheomechanical properties (essential for 3D printing) and biological activity (required to promote *ex vivo*/*in vivo* tissue regeneration) [36]. Furthermore, the fast-gelation mechanisms of alginate endowed the resulting bioink with the ability to overcome one of the major challenges faced during printing of complex structures: resisting cell sedimentation. Importantly, the resulting constructs did not elicit the end-stage response of the inflammatory response (i.e., a foreign body response), stimulated angiogenesis and, once grafted in a mouse model of transplant immunosuppression, promoted recipient-derived *de novo* blood vessel formation [36].

As alternative to biopolymers, synthetic macromolecules are also major players in both the formulation of bioinks and their protection during all stages of 3D printing. These polymers are preferable candidates for bioartificial organ manufacturing provided they comply with a series of crucial properties including, among others, excellent biocompatibility/biodegradability (including well-defined and controllable degradation mechanisms/rates and the production of nontoxic degradation products), versatile chemistry and optimal rheological/mechanical behavior [37]. Commercial bioinks is the term used to define custom-made, application-specific, on-demand, ready-to-use bioinks. One representative of this class of materials is Dermamatrix, a market-available bioink comprising a human acellular dermis allograft derived from skin and mainly used as bioprinting biopaper [38]. CELLINK is the collective name for a group of customizable bioinks based on functionalized gelatin (i.e., gelatin methacryloyl or GelMA) and a plethora of other components, including polysaccharides in the form of alginate and cellulose nanofibrils, synthetic polymers, such as polycaprolactone and Pluronic [a block copolymer comprising poly(ethylene oxide) and poly(propylene oxide) arranged in an A-B-A triblock structure], and inorganic ions. CELLINK products can be tailor-made for different 3D bioprinting applications, enabling convenient 3D tissue engineering with almost any cell type. Cell or pellet-based bioinks are also being utilized in several studies. One remarkably complex application of these systems is represented by human induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) embedded in a fibrin-based bioink also containing microspheres loaded with guggulsterone (a small-molecule morphogen essential to promote hiPSCs differentiation into mature phenotypes) [39,40].

The last class of bioinks gathers those materials for 3D bioprinting in which the bioink is combined with additional components, such as (bio)nanomaterials, including carbon-based nanoparticles (NPs) (carbon nanotubes and graphene), clay NPs, ceramic NPs, nanofibers/nanocrystals, or biomolecules to generate hybrid and/or multi-composite inks [41]. By exploiting the so-called nano effect (i.e., the synergistic effect brought about by the presence of the nano-additive on the embedding matrix), bioinks leading to constructs with enhanced properties can be ultimately achieved. For instance, a bioink made from human platelet derivatives and nano-engineered via the addition of cellulose nanocrystals led to 3D printed structures with exceptional cell-sustaining properties. Interestingly, stem cells encapsulated in this new nanocomposite bioink during printing were shown to grow and proliferate also in the absence of serum supplements [7,42].

Bioprinters are the second important component of bioprinting. A 3D bioprinting process relies on three main printing technologies: inkjet-based, extrusion-based, and light-assisted bioprinting (Fig. 2). Given that a specific bioinks influences its embedded living cells in the final bioprinted product, the adoption of a printing technique dictates not only the choice of suitable bioink, but also the degree of architectural complexity characterizing the final bioprinted object.

Inkjet-based bioprinting (Fig. 2a) exploits two methods to deploy small bioink drops: (i) air-pressure pulses through a thermal heater; or (ii) mechanical pulses via a piezoelectric actuator. In the latter, acoustic waves generated by piezoelectric crystals guide the bioink to, and force it through, the printer nozzle. By contrast, in thermal inkjet printers, air pressure pulses the bioink to a firing chamber, where electronic resistors vaporize the fluid into a bubble; as the bubble further expands, the bioink droplet is propelled

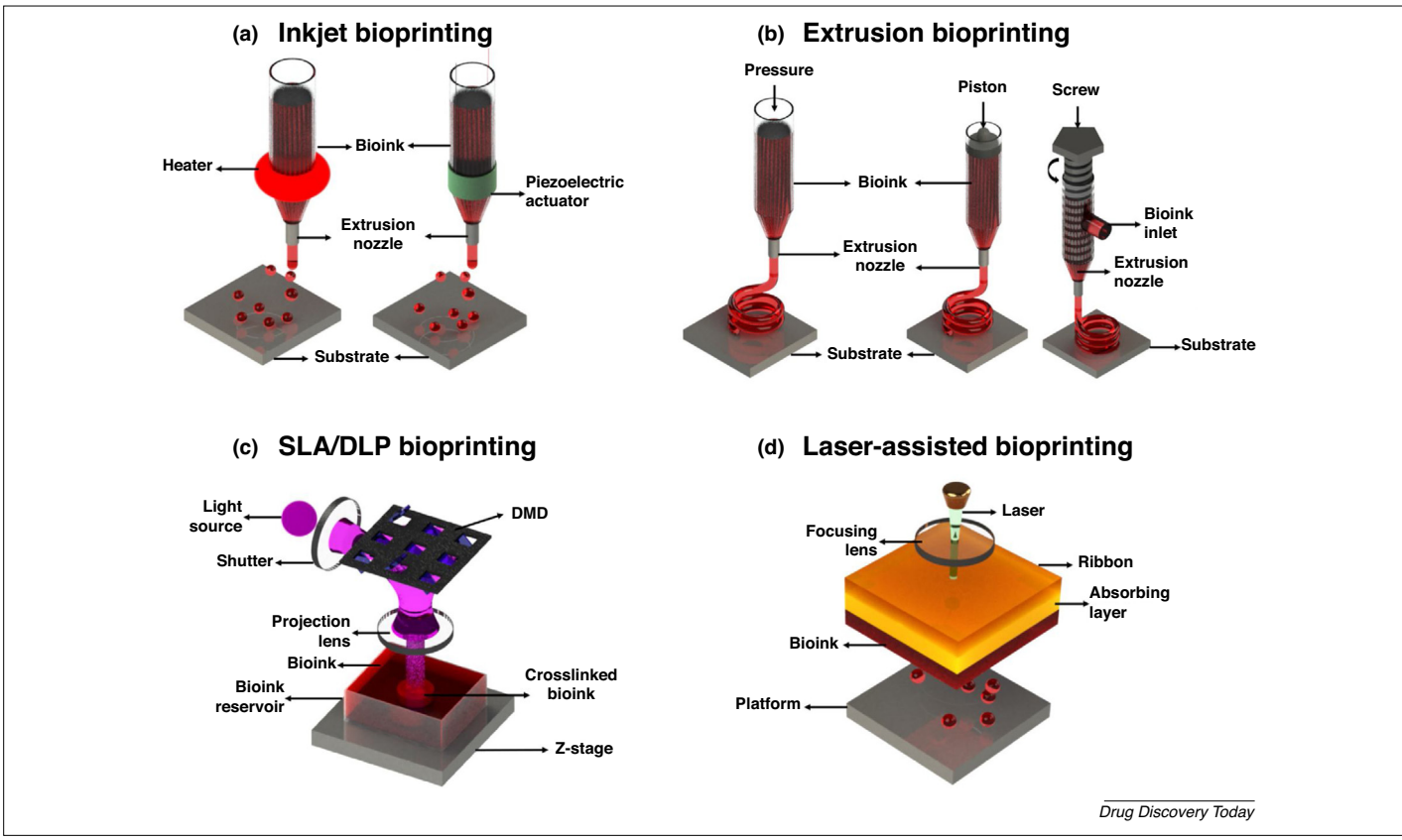

FIGURE 2

Schematic view of the most common 3D bioprinting techniques. **(a)** Inkjet-based bioprinting exploits two methods to deploy small bioink drops: air-pressure pulses through a thermal heater (i) or mechanical pulses via a piezoelectric actuator (ii). **(b)** Extrusion bioprinting relies on pneumatic or mechanical pressure to force the (bio)ink through the printer nozzle. **(c)** Stereolithography (SLA) or digital light processing (DLP) bioprinting is based on ultraviolet (UV)-vis light curing of photoresponsive bioink in a layer-by-layer fashion. Laser-assisted bioprinting (LAB) is a contactless and nozzle-less bioprinting approach in which a laser pulse is focused on the donor (absorbing) ribbon and converted into a shockwave to activate the bioink layer underneath. Then, the bioink travels to the recipient slide from the donor slide. Adapted, with permission, from [93].

from the chamber and expelled from the nozzle. Generally, this 3D-printing methodology presents several advantages, including precise cell positioning onto the substrate and high preservation (>85%) of cell viability. In most commercially available thermal inkjet printers, the living cell-containing bioinks are subjected to a thermal jump from 4–10 °C to 300 °C in ~2 μs. Although this short processing time interval has a marginal impact on nucleic acid stability, cells and tissues might experience substantially higher negative effects related to the applied temperature and stress fields (remarkably, malignant tumor cells have shown to sustain shear stress more efficiently compared with normal cells). High printing speed, low cost, and wide availability are other positive aspects of inkjet bioprinting. However, this printing technique requires low-viscosity (bio)inks to fully achieve droplet deposition and this might result in flawed cell encapsulation and/or limited droplet volumetric patterns [43].

Extrusion bioprinting (Fig. 2b) relies on pneumatic or mechanical pressure to force the (bio)ink through the printer nozzle. This should be the methodology of choice when printing bioinks containing cells embedded at high densities; however, the cell viability in the relevant 3D printed constructs is 40–80%. To produce multimaterial extrusion bioprinters, more nozzles can be added to alternate printing between rigid materials and softer-conforming layers for encapsulated cells. Adoption of this methodology can also assist in the optimal spatial patterning of various cell types within the same cancer model. These benefits reflect in the improved differentiation of various cell types, as testified by the expression of numerous relevant markers.

The discovery/evaluation of chemical entities and/or mechanical behavior associated with metastasis growth would significantly support cancer research. In particular, the identification of new cancer-related druggable targets to prevent/limit such malignant interactions would be beneficial. Freeform constructs can be produced via extrusion based on a technique known as embedded bioprinting. This method enables antigravity bioprinting of 3D freeform products within a supporting hydrogel bath, which is removed at the end of the printing process, allowing the retrieval of the final constructs featuring the requested shape. Although the range of bioinks and support materials is comparatively small, embedded bioprinting is still a technology with vast potential. Light-assisted (i.e., photocuring-based) bioprinting is based on the adoption of photoactive polymers that undergo liquid–solid transition to generate constructs printed to high precision and at speed via precisely controlled lighting. Given the various light-scanning types, this printing category can be further subdivided into stereo-

lithography (SLA) and digital light processing (DLP) (or projection-based printing) (Fig. 2c).

Compared with other 3D bioprinting technologies, SLA/DLP offers a relatively fast bioprinting speed, and nozzle-free and high-resolution printing. Furthermore, inter-log spacing in the same sample has been reported to be 100 μm, whereas the logs the supporting transverse logs were 50 μm wide and ~1730 μm long. SLA/DLP can also increase bioprinting resolution for cell-laden structures, while also shielding cells by limiting their susceptibility to nonphysiological environments. Also, SLA/DLP enables constructs with mechanical stiffness patterned according to gradients, and this can be adopted as a method to analyze local neoplastic cell invasion and its interaction with the EMC rheomechanical features. However, the small choice of biocompatible photosensitive polymers coupled with nonideal density and uniformity of loaded cells, ultraviolet (UV) exposure, and the presence of toxic photoinitiators might ultimately negatively impact cell vitality/activity. Although living cells are not subjected to high process-related shear stress because of the underlying methodology, specimens designed to feature hollow parts (e.g., human blood vessels and heart components) are hampered by product imperfections, mainly obstructed parts ascribable to the residual hydrogel. Elimination of these flaws will unavoidably require, besides time, rigorous, highly engineered formulations of both bioinks and light-activable polymers. Constructs produced using SLA/DLP bioprinting with light sources in UV–vis range usually yield constructs characterized by cell viability ≥90%; unfortunately, however, most of the light sources currently adopted in these bioprinting techniques for photoinitiator activation cause cell damage. Accordingly, the search for visible light-activated photoinitiators or photo initiator-free alternative SLA/DLP printing systems is an active research field. Laser-assisted bioprinting (LAB) is a contactless and nozzle-less bioprinting approach that enables precise depositions of the bioink. The technology has three main components: a donor slide (or ribbon); a laser pulse; and a receiver slide (Fig. 2d) [22,44]. Initially, a laser pulse is focused on the donor (absorbing) ribbon and converted into a shockwave to activate the bioink layer underneath. Then, the bioink travels to the recipient slide from the donor slide. While preserving precision, LAB can place one cell per droplet or can deploy multiple cell types [40], but with a higher economical cost [41]. Remarkably, cell viability after LAB bioprinting is >95%. Another advantage of this method is that, by using several ribbons covered with different bioinks, concentric structures characterized by different cell types can be realized [40].

4D bioprinting techniques, in which time constitutes the fourth dimension, have been proposed recently and are currently a focus of further development. These methods aim to create dynamic, patterned, 3D biological structures that can undergo shape transformation and/or behavioral change when exposed to various stimuli [45]. For instance, these techniques can master the time- and space-controlled release of, for example, growth factors from the surrounding matrix; create constructs with regions characterized by stiffer and softer mechanical behavior distributed within the sample according to variegated patterns; and realized controlled perfusion into vasculature [50]. 4D bioprinting enables monitoring/testing of individual variables to identify cancer cell modulators or key environmental factors that affect tumor activity.

One of current progress relies on a bioprinting technology mix (i.e., different combinations of the consolidated printing modes described earlier) to achieve optimal 3D bioprinting of different healthy and disease-related tissues. In particular, the generation of reliable 3D cancer constructs via bioprinting constitutes a cornerstone in the study of cancer cell signaling pathways and the related development of targeted/personalized cancer chemotherapeutics. Contextually, the wide adoption of 3D bioprinting techniques is hampered by the nonavailability of standardized 3D printers. To progress in this respect, and to maximize data reproducibility, standardized machines and protocols along with consistent on-the-shelf bioinks and toolkits should be rapidly made available [46]. The still high costs of the overall 3D bioprinting process is another deterrent for the wide adoption of these methodologies; to compensate for this, low-cost open-access 3D bioprinting projects are currently underway in which scientists can build their own machines. The ongoing research and technological advances in this sector will undoubtedly continue to expand its boundaries by capitalizing on the jointed, interdisciplinary efforts from experts in engineering, biology, chemistry, and medicine.

With the goal of overcoming some of the limitations posed by the currently available 3D printing machines, an off-the-shelf 3D desktop printer was re-engineered to produce reliable results using bioinks based on alginate and alginate/gelatin hydrogels. This low-cost machine (with dimensions compatible with installation under standard laboratory laminar flow hoods) was successfully tested for performance by producing both 2D and 3D cell-free and recombinant HEKYFP cell-containing constructs (stably expressing YFP) as model systems and for high-content imaging [47].

In analogy with other biofabrication practices, 3D bioprinting can be carried out in either the presence or the absence of a predesigned scaffold. Either choice will be endowed with the relevant constructs with different characteristics likely required for specific applications. Scaffold-based bioprinting provides stronger commercial appeal, ease of use, and lower economic efforts. Minimal cell migration and dissemination across the whole bioink constitute major characteristics of scaffold-based approaches; yet, the presence of additional components, such as low-acyl gellan gum, poloxamer, or fluoro surfactants, might be necessary because cell-containing bioinks must undergo prolonged bioprinting processes [48]. Indeed, living cell cultures embedded in hydrogels for long time intervals have shown reduced vitality/survival, limited functionality, and phenotypic conformability. By contrast, *in vitro* models can achieve high cell density by use of the alternative, scaffold-free bioprinting approach. Indeed, when seeded at high density, cells deposit their own EMC while dynamically self-assembling and organizing into microtissues during proliferation; moreover, the absence of a scaffolding hydrogel also supports preservation of cellular phenotype and greater intercellular connectivity, prerequisites for normally active cell signaling.

Scaffold-free bioprinting can be performed using tissue strands, tissue spheroids, or cell pellets [49,50]. Essentially, the selection of a scaffold-based or scaffold-free approach is application driven. Bioprinting procedures relying on the use of hydrogels are recommended, for example, for drug delivery studies because the biopolymeric material degradation rate can be a finely tunable

parameter in mastering controlled drug release. Similarly, scaffold-based bioprinting is considered to be more appropriate in stem cell research because the different degree of networking achievable by selecting various hydrogels can be exploited to obtain biologically relevant compositions of these peculiar cells [51]. By contrast, scaffold-free 3D bioprinting techniques are more effective for the assessment of active principle effectiveness during the early phase of drug discovery, one of the principal reasons being the hypoxic conditions experienced by cells in the construct core, which is considered a good TME mimic. Contextually, a scaffold-less bioprinting process is the method of choice when considering constructs embedding co-cultured cells to avoid hydrogel interference in cell–cell crosstalk.

The rheological properties of hydrogels have a prominent role in industrial scaffold-based 3D bioprinting for high-throughput fabrication. High viscosities are usually associated with negative technical problems (e.g., clogging of the printer nozzles) while, at the same time, a typical shar-thinning behavior is requested to allow: (i) smooth bioink flow during printing; (ii) shape regaining/preservation once the printing process is over; and (iii) high architectural/shape fidelity in complex anatomical constructs (e.g., blood vessels, heart, ear, etc.). Last but not least, optimized values of the hydrogel yield stress [52] and overall short processing time are also required to limit cell sedimentation in the relevant bioinks; this, in turn, contributes to limit batch-to-batch variability.

Thus, it appears clear that the selection of the most suitable hydrogel in scaffold-based 3D bioprinting is a crucial step. In particular, various physicochemical characteristics, including viscoelastic behavior and mechanical resistance, biocompatibility of the intact hydrogel and of its eventual degradation products, and hydrogel/cell adhesion need to be optimized before proceeding with bioprinting. Biocompatibility can be more easily achieved by selecting hydrogels based on natural biopolymers. When feasible, tissue-specific hydrogels should also be adopted in bioprinting: for instance, collagen type I is ideal for bone constructs, collagen type II is the preferred option when dealing with cartilage, fibrin is preferable for angiogenesis, and Matrigel is the choice for 3D bioprinting of tumor and cardiac tissues. In some instance, hydrogel functionalization with specific cell-recognizing motives (e.g., the RGD peptide) led to improved 3D bioprinted constructs. The degree, mechanism, and kinetics of hydrogel crosslinking are another set of parameters that must be fine-tuned, because they directly reflect its rheological properties and mechanical behavior.

Chemical, physical, and enzymatic are the three principal mechanisms of crosslinking. Chemical crosslinking generally results in the formation of the strongest constructs because of the formation of strong covalent intermolecular bonds. However, such tight intermolecular links can result in 3D networks with mesh dimensions too narrow for the effective diffusion of nutrients and gases, ultimately resulting in compromised cell viability. Although high concentrations of crosslinkers might improve bioprinting process, they have also been shown to negatively affect cellular migration [53,54]. From a kinetic standpoint, crosslinking mechanisms endowed with fast kinetics are ideal to achieve quick cell immobilization in a stable state. The most popular physically crosslinked hydrogel for 3D printing is based on the natural anionic polysaccharide alginate, the gelation of which is achieved in water by the addition of divalent cations (mainly Ca2+). Photo-induced crosslinking is also commonly used in bioink formulations, resulting in fast intermolecular networking in the absence of any crosslinking bath; as such, this method is amenable to high-throughput and/or multilayered model fabrication. Although physical gelation is achieved faster and with reduced viscosity variation during bioprinting with respect to the chemical-based alternative, the resulting constructs are weaker and reversible in nature.

Cross-linking hydrogels via enzymes leads to highly biocompatibility bioinks; moreover, depending on the biopolymer (e.g., collagen, gelatin, fibrin, and HA) a variety of specific enzymes are available [e.g., transglutaminase, horseradish peroxidase (HRP) and thrombin] to crosslink hydrogels such as fibrin, gelatin, HA, and collagen. Although bioprinting hydrogel enzymatic crosslinking poses minimal toxicity issues, the technique *per se* is cost ineffective; accordingly, the application of this methodology remains limited [38,55]. Hydrogel photo-crosslinking is a rapidly emerging, alternative crosslinking strategy for bioprinting; with the aid of minimally invasive light sources, it is possible to exert a fine local control on photosensitive cell-embedded hydrogels with negligible effects on cellular viability. A recent study described the development and testing of an *in situ* photo-crosslinking approach in which a nonviscous photosensitive bioink was cured with light as it passed through a photo-permeable capillary before deposition [56].

3D bioprinting can better recapitulate the TME by accurately printing different types of TME in spatially localized regions. *In vivo*, tumors interact with ECM and surrounding cells to form the TME. Cells that form TME include immune cells [e.g., T cells, B cells, and natural killer (NK) cells], vascular cells, fibroblasts, pericytes, and adipocytes. The ECM allows cells to adhere and grow. Growth factors are other important components of TME. Fig. 3 depicts the elements of normal tumour microenvironment that can be recreated by 3D bioprinted bioinks.

Bioinks are useful for the creation of extracellular mechanics similar to that of TME. Also, there is an option to select polymers, so that the matrix stiffness can be designed and controlled as per requirements. In addition, the spatial distribution of chemical factors can also be mediated to mimic the native TME. 3D-printed stimuli-responsive capsules loaded with plasmonic gold nanorods have been fabricated to create TMEs [57–59].

Bioprinting as a novel strategy to develop anticancer drug-screening platforms

Drug optimization and disease modeling

Extensive *in vitro* investigations of novel anticancer drugs are an essential and obligatory stage before moving on to preclinical (i.e., animal) studies. During the initial, high-throughput, phases of drug discovery and screening, 2D cell cultures are adopted to rapidly verify/confirm drug effectiveness and to derive the relevant safety profiles required for animal trials. In a second stage (also known as lead selection and optimization step), only the most promising active principal compositions/formulations (lead compounds) are optimized, and biological pathways at the cellular/system level are explored to gain fundamental information about the mechanisms of action of the compound(s) in complex 2D or 3D disease models. This ‘bench’ portion of a drug translational

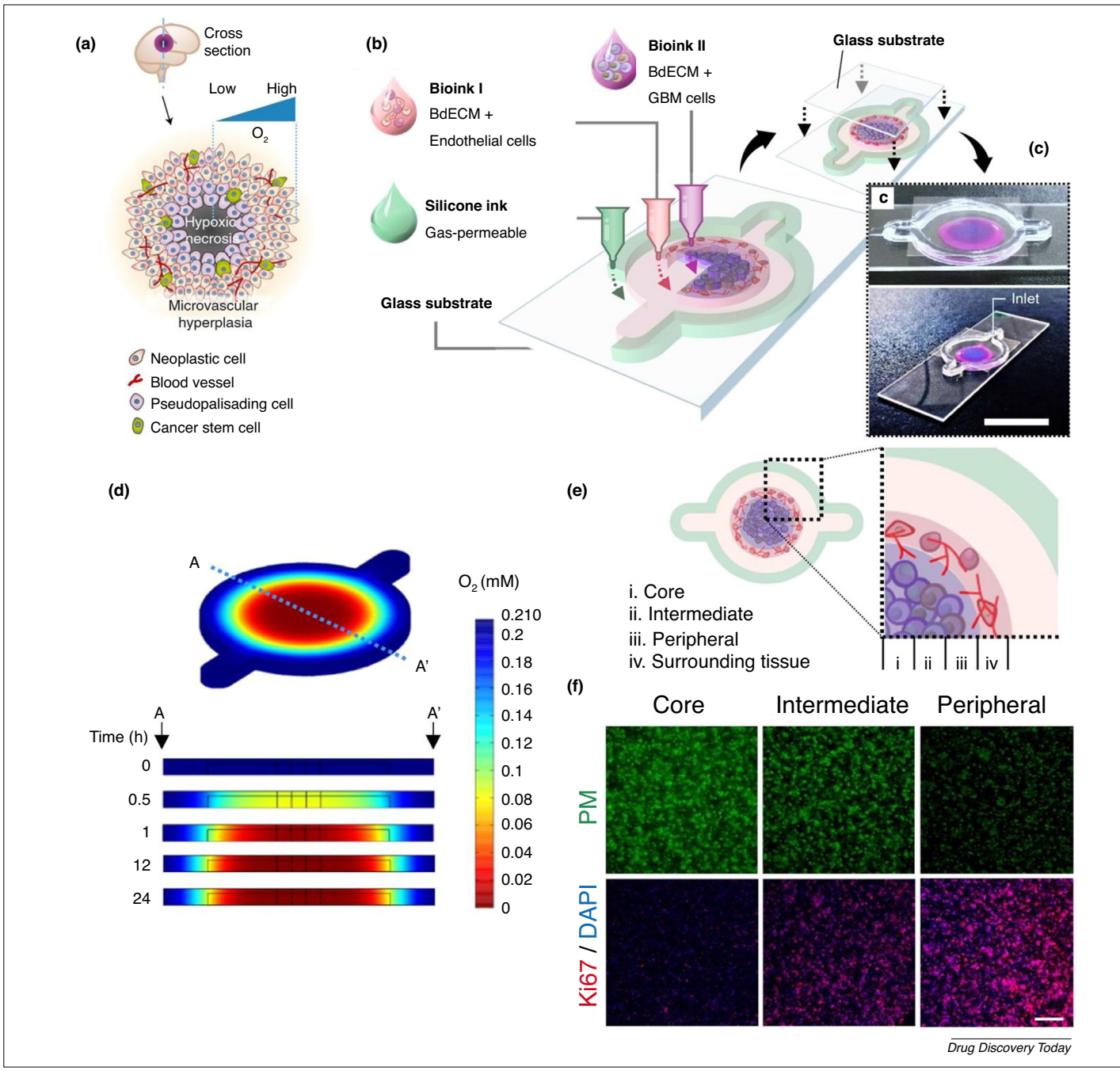

FIGURE 3

Biological elements found in tumour microenvironment. **(a)** Schematic diagram depicting a tumor cross-section revealing the hypoxic core and various biological elements usually found in a tumor microenvironment (TME). **(b)** Illustrative diagram of bioinks used in the fabrication of a glioblastoma multiforme (GMB)-on-a-chip model. **(c)** Simulated representation of compartmentalized bioink comprising brain decellularized extracellular matrix (dECM) embedded with human umbilical vein endothelial cell (HUVECs) (in magenta) and GBM cells (in blue): (i) top view; (ii) sideview (scale bar: 2 cm). **(d)** Finite-element calculation of oxygen concentration distribution along the system cross-section indicated by A–A′ highlighting the onset of an hypoxic core of central hypoxia in a chamber comprising selectively gas-permeable elements. **(e)** Schematic illustration of the different regions of the 3D printed GBM model. **(f)** Regional differences in hypoxia experimentally observed via tumor immunofluorescent staining (using pimonidazole for hypoxic cells, Ki67 for proliferating cells, and DAPI for cellular nuclei as markers) (scale bar: 200 μm). Adapted from [94].

pathway is both costly and time consuming and usually results in low-throughput results; thus, the availability of more reliable and effective disease models could be a boost to this phase.

Current pharmaceutical industry models are frequently driven by academic discoveries, with genetically modified cell lines (or animals) expressing disease-related genotypes or phenotypes being prime examples [60]. Although these 'surrogate models' often deliver impressive results, they often also often fail to recapitulate complex human behavior. This is particularly true for single cell-type models, where a patient-like microenvironment is unavoidably absent. In an attempt to circumvent these limitations, brain-like tissue constructs providing microenvironment conditions

compatible with neural cell proliferation were recently obtained via 3D bioprinting. Interestingly, the slow formation of neural circuits within these constructs was observed, which ultimately endowed these models with the ability to respond to external stimuli [61]. Similarly, a scaffold-less 3D bone model for the study of osteochondral defect repair and drug development has been produced. Starting from bone marrow-derived mesenchymal stem cells, an *in vitro* drug-testing platform was derived and used to investigate the effects of administered drugs on proto or impaired osteogenic differentiation in 3D and 2D cultures [62].

In general, 3D cell cultures represent better the natural organism environment compared with their 2D counterparts; moreover, and most importantly, 3D cell cultures derived both from primary tissues or stem cells can aggregate to originate organoids (i.e., miniaturized and simplified 3D *in vitro* version of an organ). As such, the composition and architecture of organoids are similar to primary tissue, and organoids are endowed with self-renewal/organization abilities, and exhibit organ functionality. Accordingly, they are not only biologically relevant, but allow for local microenvironment modification and gene editing. Finally, organoids represent extremely stable systems that can be easily cryopreserved for extended cultivation. However, advanced technologies are required to produce organoids that are both reproducible and complex enough in design to faithfully replicate disease [63]; yet, most of the challenges posed by drug development/screening can be addressed by exploiting these models. In cancer therapeutics, two distinct classes of these 3D cellular aggregates are distinguished: organoids and spheroids. The latter are generally grown as aggregates obtained by seeding cancer cells (either from cell lines or from *in vivo*-derived cancer tissues) on low-adhesive plates (e.g., agarose), whereas organoids are obtained by embedding somatic (adult) stem cells in an hydrogel with EMC features (usually Matrigel). Therefore, organoids are more complex and representative of an *in vivo* situation compared with spheroids.

As an example, organoids were developed via high-throughput 3D bioprinting technologies using patient-derived pancreatic and colorectal carcinoma cell lines along with cancer-associated fibroblasts. These models were extensively challenged against different known anticancer drugs and the results were directly compared with those produced with the corresponding 2D cellular cultures. Where expected, organoids successfully exhibited ECM-related cytotoxicity resistance, whereas no such evidence was inferred from the relevant simpler models. Based on these results, the authors proposed the introduction of a 'resistance factor' to account for the observed IC_{50} differences between 2D and 3D models [64].

In another interesting study, cancer spheroids with vascularized TME with a controllable size were obtained via 3D bioprinting and used to investigate their response to chemotherapeutics. Initially, a blood vessel layer was obtained by culturing bioprinted human umbilical vein endothelial cell (HUVECs) and lung fibroblasts in a hydrogel comprising a mixture of gelatin, alginate, and fibrinogen. Once the blood vessel lumen was formed, multicellular tumor spheroids (MCTSs) were laden into the blood vessel layer. Upon incubation, the endothelial cells in the blood vessel layer began their migration into the MCTSs and started to show angiogenesis; contextually, some cancer cells began their invasion of the blood vessel layer. The feasibility and reliability of this 3D model in drug discovery/testing was further verified through experiments involving two well-known, clinically approved anticancer drugs: temozolomide (a DNA-damaging agent used as first-line treatment for GBM and as second-line treatment for astrocytoma) and sunitinib [an oral, multitargeted receptor tyrosine kinase inhibitor approved by the US Food and Drug Administration FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors (GIST)]. Specifically, the two drugs were challenged against MCTSs with and without the blood vessel layer; the results obtained with the vascularized 3D cancer models were analogous to those observed *in vivo*. Given that such realistic 3D tumor constructs can be bioprinted in microwell plates, they constitute an excellent platform for the high-throughput screening of anticancer drugs [5].

In summary, different 3D bioprinted models can be efficiently and effectively exploited along the drug discovery pipeline. During the initial stage of drug development, simple 3D bioprinted models could replace monolayer cell cultures to increase the number of hit-to-lead compounds from high-throughput screening [65]. Once the most promising drug candidates have been identified, more complex disease models can be created in replicate by 3D bioprinting techniques, allowing for in-depth investigations and understanding of the drug mechanism of action and potential dose response. Lead toxicity can then be evaluated in 3D bioprinted organ-specific models (e.g., heart or liver) to capture the organ response in a more extensive and reliable way. Ultimately, the adoption of 3D bioprinted models should allow for safer and more effective drugs to reach the next steps in the relevant translational pathway.

Drug toxicity

Identification of both systemic response (toxicity) and disease behavior before *in vivo* testing of any drug candidate is a crucial issue. The safety profiles for any new drug treatments must first be well characterized *in vitro* and then closely monitored during animal studies. The opportunity to derive *in vitro* drug toxicity profiles as accurately as possible has several, fundamental implications. From an economic standpoint, they might suggest drug modifications or even withdrawal before animal testing, when adverse effects are also noted. This, in turn, has a profound ethical implication, in that animals would be subjected to trials with only those drugs showing potential significance after these advanced screening techniques. Moreover, the results from animal testing could further serve to not only highlight *in vitro*/*in vivo* discrepancies, but also eventually refine the 3D *in vitro* models and to indicate specific areas of focus when moving into advanced clinical settings.

3D bioprinting was used to construct a kidney-like organoid for evaluation of toxicity of aminoglycosides in a proof-of-concept drug-testing study. Also, the ability of bioprinted organ-on-a-chips and 3D-bioprinted models to mimic tissue/living organ physiopathological ME [a fundamental characteristic required to investigate absorption, distribution, metabolism and excretion (ADME) of new active molecules] was reported. When considering toxicity, the liver is an organ of primary concern given its prominent role in drug metabolism. Liver drug degradation can result in molecular entities or fragments (metabolites) that might, in turn, be endowed with their own activity and/or toxicity (toxins). Accordingly, liver can *in primis* be subjected to toxin overexposure, and

this can ultimately generate organ inflammation, cirrhosis, and eventually hepatocellular carcinoma if exposure persists long term. These adverse effects, collectively known as drug-induced liver injury, all result in a life-threatening condition.

For these fundamental reasons, liver drug pharmacokinetics and toxicity must be intensively investigated during the development of new therapeutic compounds. At present, both 2D culture and 3D spheroids are already used for introductory drug development and toxicity examination followed by animal testing, although it is widely recognized that laboratory animals can be misrepresentative of human metabolism because of differences in cytochrome p450 enzymes across species [66]. Liver pathology and its complicated microenvironment are a significant barrier in drug metabolism, generation an increasing need for physiologically upgraded, scalable liver models for HTS adoption. In bioprinting, several innovative techniques have been proposed and tested to refine/integrate HTS liver models. *In vitro* 3D cell cultures are developed from cells that are isolated from a patient, and then further refined to yield unique or individual cell separation and ECM recovery. Usually, cell isolation is conducted by utilizing diseased tissue resections or biopsies, thereby ensuring the presence of different cell types and ECM components.

When patient-derived tissues are not available, human induced pluripotent stem cell (hiPSC) isolation methods can be adopted; these involve directly patient-derived easy-to-isolate cell platforms containing cells that, once isolated, dedifferentiate into hiPSCs, which then differentiate into the desired diseased cell type for investigation [67–69]. The nature of hiPSCs, such as the variability within their differentiation process, is a challenge for culturing patient cells and, thus, experimental outcomes are unpredictable and/or an exception to the disease state being investigated. For high-throughput 3D-bioprinted precision medicine applications, reproducible 3D culture systems are needed. To achieve multiple reproducibility, patient-derived cell expansion must be optimized, so that experiments can be performed only once. When patient-derived diseased tissues or biopsy samples are available, single cells can be isolated from biopsy samples, grown in 2D cultures to reach a cell volume that can then be expanded for 3D experimentation. Furthermore, patient-derived tissue samples present many technical hurdles, such as limited cell expansion yield because the plastic substrate used for cell growth acts as barrier to cell adhesion, which limits the cell population size. This can be bypassed exploiting 3D hiPSC cultures to yield millions of cells for subsequent differentiation of the desired diseased tissues. Precision medicine is a recent but rapidly expanding field of interdisciplinary science and research. 3D bioprinting technology providing both predictive disease modeling and drug toxicity screening capabilities and, thus, could be advantageous for the future of precision medicine [70,71].

3D bioprinting and tumoroids as new 'avatars' for drug screening

Current state-of-the art drug discovery and development pipelines require ~15 years and US$2.6 billion to move a new chemical entity 'from the bench to the bedside'. However, the use of bioengineered human cell-based organoids could help decrease the probability of failure during human trials by providing human-specific preclinical data. Organoids can also be used for designing personalized medicine diagnostics to optimize therapies in many diseases, including cancer. Bioprinting can be adopted to scale up the deposition of such organoids and tissue constructs, and to help address the goal of drug screening in a large number of homogeneous organoids characterized by form factors compatible with high-throughput screening. However, the 3D deposition of hydrogel bioinks into small-sized wells is another technical obstacle because of possible bioink spreading and consequent well-surface wetting instead of the formation of the expected organoid. In a recent report, a solution to this problem was proposed whereby a hydrogel bioink comprising HA and collagen was bioprinted into a viscous gelatin bath, which both prevented bioink/well–wall interactions and provided a mold for the construct to maintain an aspherical form. This method was validated using different cancer cell lines, and applied to patient-derived GBM and sarcoma biospecimens for drug screening [72].

Hepatorganoids (i.e., organoids based on liver tissues) were also successfully produced via 3D bioprinting of fully functional, adult-phenotype, human hepatic cells (HepaRG); their liver-like functions were investigated *in vitro* and, upon transplantation into two liver injury mouse models, also *in vivo* [73]. Female patients who undergo partial or complete mastectomy as a consequence of breast cancer are often advised to undertake breast reconstruction; however, given the psychologically negative consequences this invasive surgery might have, in particular in young patients, tissue-engineering approaches combining different biomaterials and stem cells have recently been studied for the purpose of regenerating breast structures [74]. Yet, the reconstruction of advanced personalized structures by conventional techniques of tissue engineering remains difficult. Given that 3D bioprinting involves microstructure computer-assisted design, precise spatiotemporal regulation of bioink, and specific integration of advantageous seed cells and related factors, it might offer new and alternative approaches to bypass such shortcomings (Fig. 4).

Extensive experimental data have revealed that, during cancer development, the tissues involved stiffen and the rigidity of the EMC affects cancer cell growth and activity. This could be another drawback in 3D bioprinted cancer models because most traditionally adopted 3D scaffolds (e.g., collagen or Matrigel) are endowed with too low rigidity to mimic effectively the naturally stiff cancer environment. Other recently available alternatives, such as hydrogels based on poly(ethylene glycol) or other synthetic biocompatible materials, are also not able to comply with this function, because they generate scaffolds with mechanical moduli as low as 2 kPa. A notable exception is a new set of commercial products, globally called Biogelx materials, which can form gels with a range of stiffness values (0.5–100 kPa), thereby offering better mimics of the stiff ECMs of solid tumors. Moreover, by bioprinting different constructs characterized by diverse matrix stiffness values, tumors at various stages of disease progression can also be investigated. Biogelx hydrogels are peptide-based materials that can be biochemically fine-tuned to provide biomimetic sequences that endow the material with tumor matrix characteristics. Several native molecular components of the EMC (including fibronectins, laminins, and collagens) are replicated in Biogelx hydrogels as functional peptide units; these proteins provide cell–cell and integrin-binding sites, creating a suitable synthetic matrix for reproducible research in cancer biology and drug discovery [75].

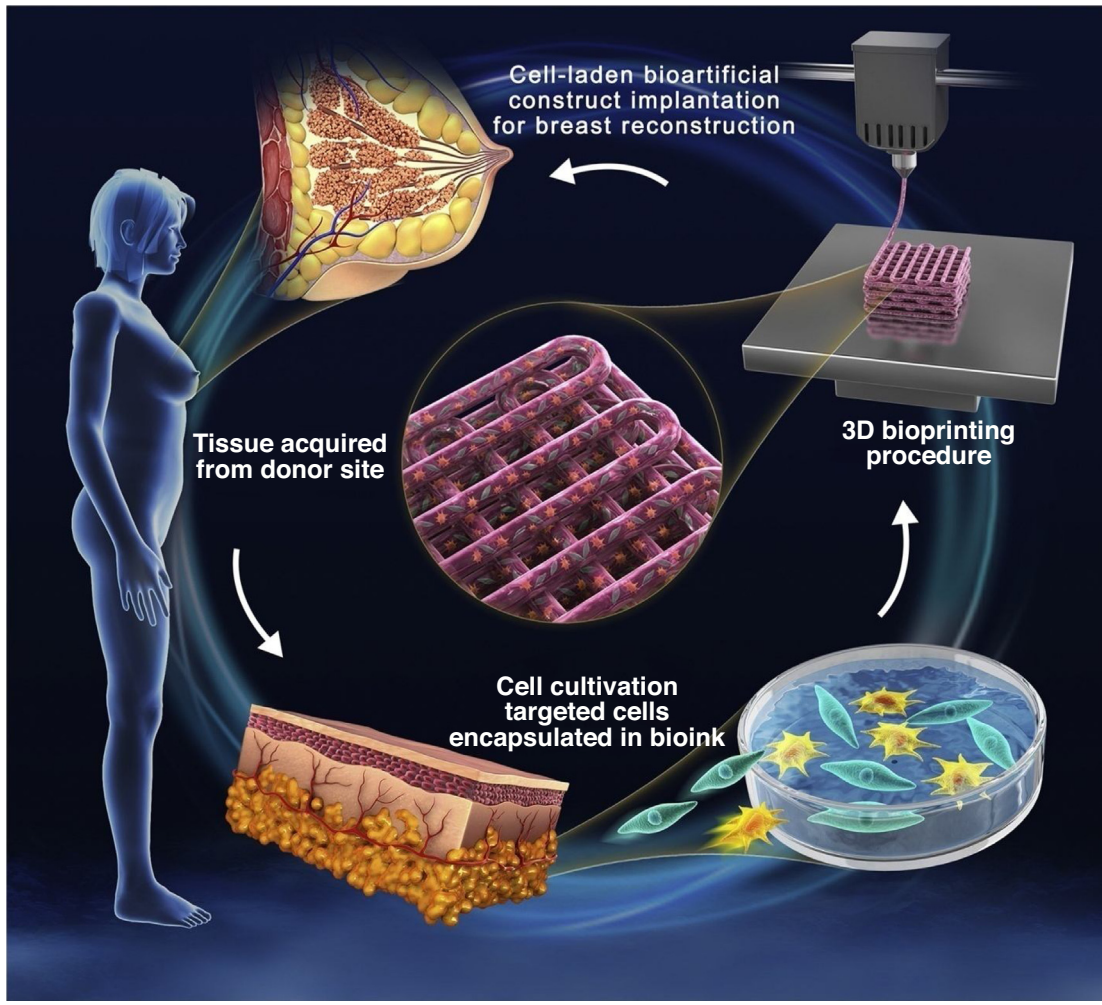

FIGURE 4

Cartoon showing a strategy involved in female breast reconstruction based on a 3D bioprinted adipose tissue construct personalized using autologous tissue-derived stem cells [74].

Osteosarcoma is the most common type of bone cancer. It ranges from low-grade tumors (usually treated with surgery) to high-grade tumors, which require aggressive treatment regimens. Chemotherapy resistance and bone defects resulting from osteosarcoma surgery continue to pose challenges to the clinical treatment of this disease. One reason for this is the combined insufficient concentration of intrinsic hydrogen peroxide (H_2O_2, known to stimulate osteoclast formation) and low intratumoral penetrability of this molecule within chemotherapeutic-insensitive tumors. As a contribution to therapeutic treatments of this important cancer, a multifunctional biomaterial was recently proposed, which was obtained by co-embedding calcium peroxide (CaO_2) and iron oxide (Fe_3O_4) NPs into a 3D-printing scaffold based on akermanite (AKT), a Mg/Ca-containing mineral [7]. In this complex system, the magnetite NPs catalyze the formation of H_2O_2 from CaO_2, which also acts as a source of Ca2+ ion pools to foster bone regeneration. In addition, further anticancer effects can be realized via magnetic hyperthermia generated by the Fe_3O_4 NPs under alternative magnetic fields (Fig. 5).

A variety of design criteria should be borne in mind when designing new *in vitro* phenotype-dependent tumor-invasion models. Although evidence supports the increased predictive powers of such models when different elements of complexity (such as vasculature and stromal/immune cell components) are included, such complexity can hamper their practical, large-scale implementation. To become widely adopted by both industrial and university researchers, these models must be simple to use, yield accurate and reproducible results on a short timescale, and possibly be subjected to standardization and automation for HT applications. Finally, to exploit such systems to their maximum potential, their use should not be limited to a single outcome evaluation (e.g., cytotoxicity), but they should be used in more complex analyses, where different cellular pathways/processes are contextually investigated [76].

Light-induced forward transfer phenomenon has been used to design 3D bioinks (Fig. 6) for fabrication of miniaturised spheroid based array model. Both acinar cells and ductal cells were used for recreation of 3D Construct similar to original tumour environ-

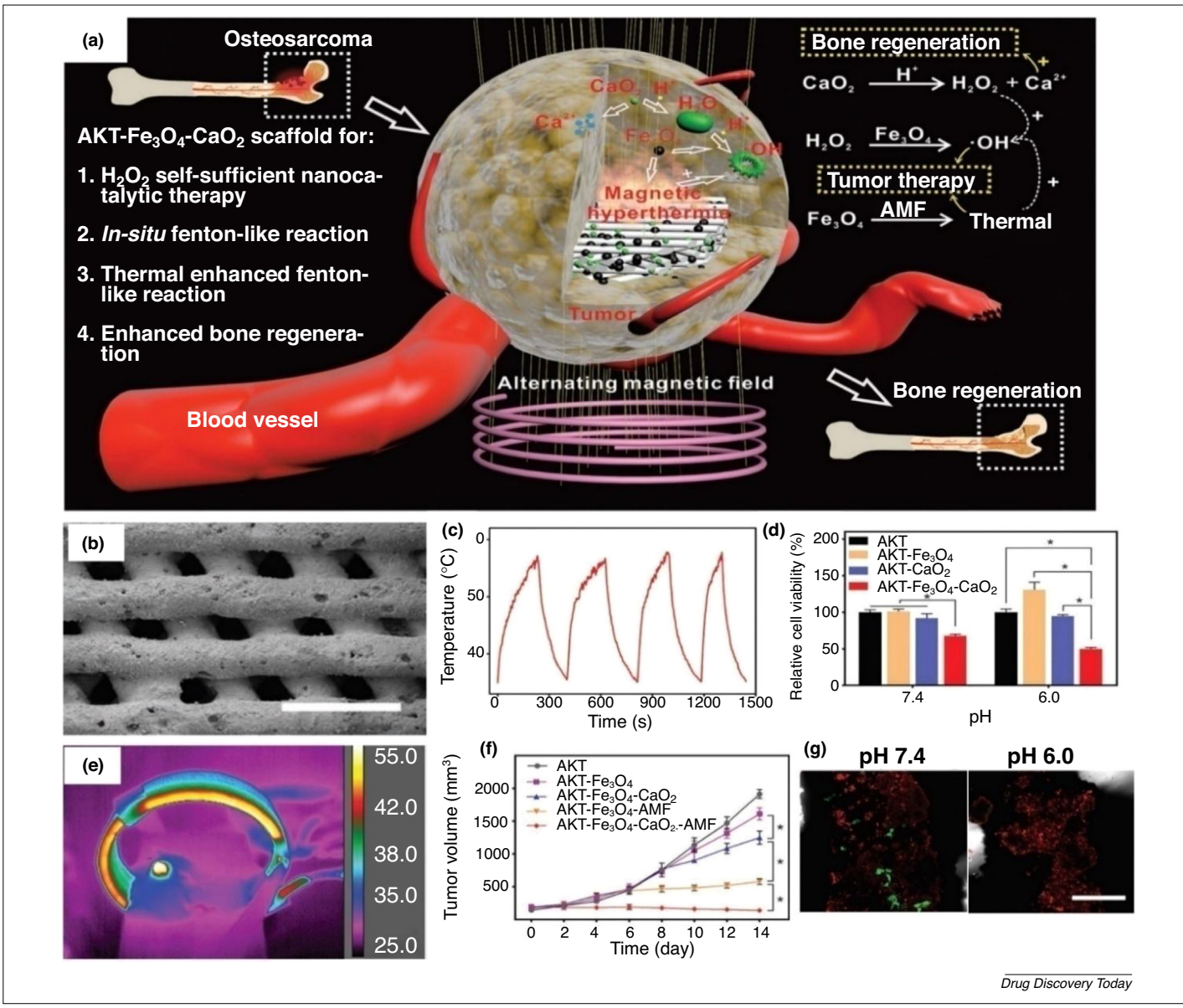

FIGURE 5

3D bioprinted scaffolds for cancer management Enable. **(a)** Cartoon showing the concept of the 3D-printing scaffolds based on akermanite (AKT) and coloaded with Fe_3O_4 and CaO_2 nanoparticles for osteosarcoma treatment and performance and bone-regeneration bioactivity. **(b)** Scanning electron microscopy (SEM) imaging of the multifunctional AKT/Fe_3O_4/CaO_2 scaffold. **(c)** Example of a cyclic-heating profile of the 3D AKT/Fe_3O_4/CaO_2 scaffold. **(d)** Comparison of cytotoxicity data obtained for the AKT/Fe_3O_4/CaO_2 scaffold under neutral and acidic culture media. **(e)** Infrared bitmap obtained upon magnetic heating of the 3D AKT/Fe_3O_4/CaO_2 scaffold once implanted in a tumor and after exposure to an alternating magnetic field. **(f)** *In vivo* anticancer effect achieved with the 3D AKT/Fe_3O_4/CaO_2 scaffold as a result of the combined chemical effect, H_2O_2, and magnetic hyperthermia. **(g)** Fluorescence microscopy images of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine transformed human osteosarcoma (MNNG/HOS) cells after treatment with magnetic hyperthermia generated by the 3D AKT/Fe_3O_4/CaO_2 scaffold under neutral and acidic conditions (scale bar: 200 μm). Adapted, with permission, from [95].

ment. The 3D spheroid model was used for study of external and internal factors that contribute to formations of pancreatic ductal adenocarcinoma. Such kind of minaiturised array vased model can also provide a good drug develoment platform for therpeutic applications.

Bioprinting as novel strategy to develop 3D organ-on-a-chip models for drug screening

Organ-on-a-chip systems (or organ chips) are microfluidic devices lined with living human cells/tissues for drug development, disease modeling, and personalized medicine [77], and constitute both a more realistic approach to emulate a 3D environment compared with 2D cell plating-type systems6 and a potential alternative to animal testing. In each organ chip, hollow microfluidic channels, in which living human organ-specific cells are interfaced with a human artificial vasculature, are embedded in a transparent and flexible polymeric matrix. The whole system, characterized by overall dimensions comparable to those of a computer memory stick, can be subjected to different mechanical solicitations to mimic different organ physiological functions,

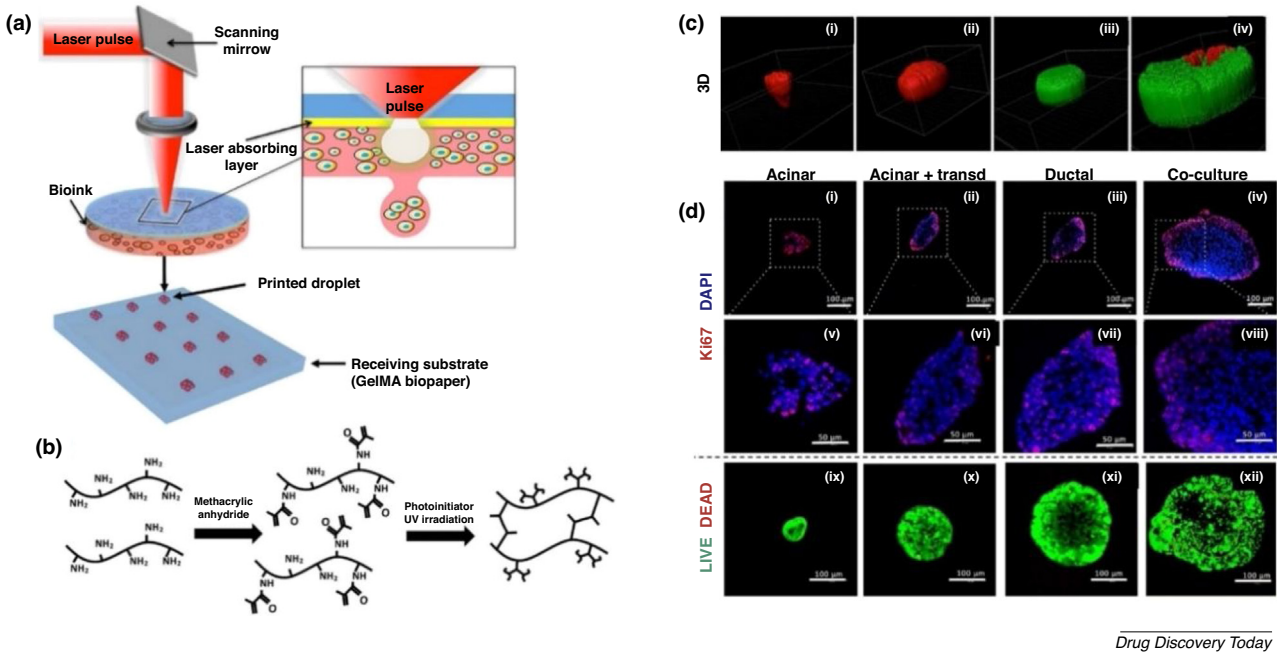

FIGURE 6

Schematics of the development of 3D bioinks by light-induced forward transfer phenomenon in laser-assisted bioprinting (LAB). **(a)** A thin layer of bioink is deposited on a transparent donor glass slide (coated with a laser-absorbing layer of gold). **(b)** Bioprinting along with simultaneous gel photopolymerization was achieved by gelatin methacrylation. **(c)** Confocal imaging and 3D reconstructions using Imaris of spheroids comprising acinar and ductal cells (i–iv). **(d)** Immunolabelling of Ki67-positive cells in spheroids comprising acinar cells [96].

such as intestine deformation under peristalsis and lung expansion/contraction. In essence, organ chips are a kind of living, 3D cross-section of the main functional units of the whole organ they are meant to represent. Given that they are translucent, they provide a window into the inner workings of human cells in living tissues within an organ-relevant context.

3D bioprinting techniques have substantially contributed to the engineering of many highly complex natural tissue structures, including heart-on-a-chip, lung-on-a-chip, liver-on-a-chip, and tumor-on-a-chip devices. However, the fabrication of these organ chips progressively becomes more sophisticated and labor-intensive, because they involve multistep lithographic processes, alignment, and integration. Thus, their production costs and times are still too high to be amenable for large-scale industrialization and the consequent biomedical market penetration, for which more automatic, robust and cheaper methods are needed [78,79]. The combination of cell-laden bioinks and their 3D bioprinting already offer the possibility to originate functional tissues and organs with complex architectures starting from accurately designed digital models. Given that 3D bioprinting has the potential to be automatized, the adoption of this technology in the production of organ chips can allow for tissue fabrication scale-up to the production level required for drug development. In addition, and as mentioned earlier, tissue spatial heterogeneity can be more easily and reliably reproduced via 3D bioprinting compared with traditional microfabrication techniques [80–82]. The multifaceted aspects and features involved in the fabrication of an organ-on-a-chip system by 3D bioprinting are illustrated in Fig. 7.

Research and development efforts in the production of organs-on-a-chip by 3D bioprinting have been mainly focused on two systems: liver chips and heart chips. An effective and efficient liver-on-a-chip system can have a dominant role in one of the most important aspects of drug screening: liver toxicity testing under 'living-like' conditions [78,83,84]. A heart-on-a-chip was printed using six functional bioinks. By way of embedded sensors in the cell incubators, electronic readouts of muscle contractile stresses were obtained via a non-invasive method. Drug dose-response data of the construct structures were collected, and the 4-week maturation of contractile growth was also investigated. The potential of such heart chips was validated through specific studies aimed at testing flexible drug-induced cardiovascular toxicity; however, more data are needed to determine the fundamental mechanisms of cell–drug interactions and intercellular activities in more detail. Furthermore, to achieve better replication of cardiac muscle contractility and cell–cell communication, the specific function of cardiac cell alignment and the related mechanism of force generation can be explored. Several techniques are available to quantify the contractile force exerted by cardiomyocytes; these include, among others, strain sensors, micro-spring devices, and microcantilevers. Nonetheless, these methods can be adopted only when dealing with bulk cardiac tissues or monolayer patches of aligned cardiomyocytes (CMs). However, an integrated heart chip able to solve this issue has yet to be developed.

Other organs-on-a-chip, including a lung-on-a-chip and a kidney-on-a-chip, have also been fabricated by nonprinting approaches. In the former, an alveolar-capillary barrier was designed to attain human lung functionality, and the related data showed that this lung chip was able to recapitulate the toxic and inflammatory responses of the real organ. Using the same protocol, a kidney chip was also produced that reconstituted the glo-

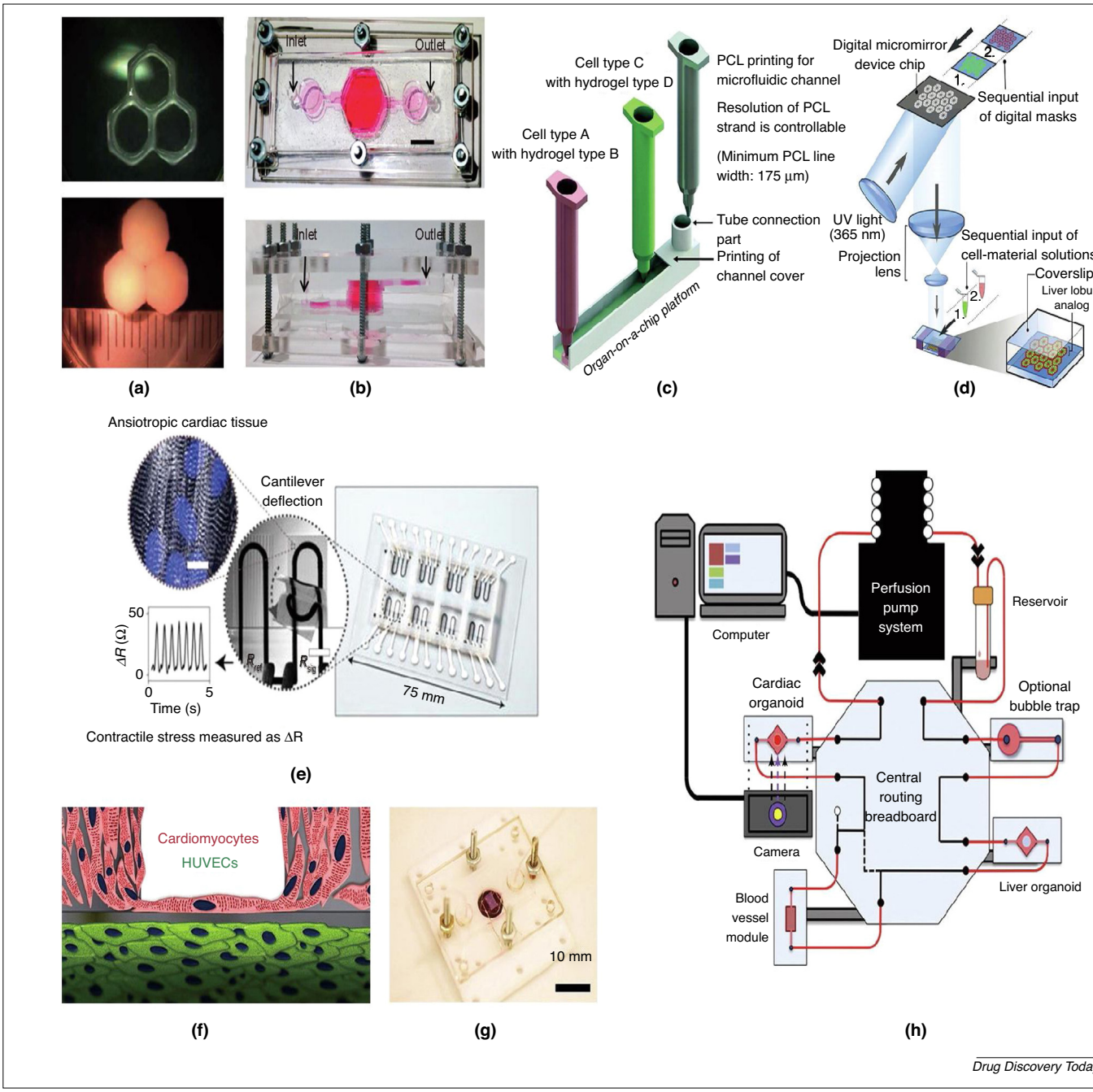

FIGURE 7

Application of 3D bioprinting for the fabrication of different organs-on-a-chip. **(a–d)** Liver-on-a-chip: (a) A multilayer heterogeneous hepatic tissue [97]; (b) a hepatic construct produced by 3D bioprinting for long-term culture of HepG2/C3A spheroids [98]; (c) one-step fabrication of liver-on-a-chip by multinozzle bioprinting [99]; (d) hepatic 3D triculture model containing induced pluripotent stem cell (hiPSC)-hepatic progenitor cells (HPCs) and human umbilical vein endothelial cells (HUVECs) and adipose-derived stem cells (ADSCs) in a microscale hexagonal architecture fabricated using digital light processing (DLP)-fabrication [100]. **(e–g)** Heart-on-a-chip: (e) 3D bioprinting for fabrication of a heart-on-a-chip using six different bioinks and embedding soft strain gauge sensors within a cell-laden microarchitecture to detect contraction of bioprinted cardiac tissues during drug toxicity evaluation [85]; concept (f) and realization (g) of an heart organoid for cardiotoxicity studies [101]. (h) Schematic representation of a multiorganoid body-on-a-chip [78].

merular capillary wall function. Finally, a body-on-a-chip was also recently reported [85–87]. Other examples of bioink-based tumor/organ-on-a-chip constructs for anticancer applications are provided in Table 2. Such platforms (Fig. 7h) are based on the integration of organoids with different physiological functions (e.g., a cardiac organoid, a liver organoid, blood vessel modules, etc.) connected through a microfluidic perfusion system. These systems could ultimately increase the efficiency while decreasing the cost of a drug-discovery pipeline; nonetheless, given the underlying technological complexity, formulating suitable culture media for, and high-throughput applications of, body-on-a-chip devices remains limited [88].

TABLE 2

Bioink-based tumor/organ-on-a-chip constructs for anticancer applications

Tumor type	Bioink-based tumor constructs	Refs
Tumor with vasculature	3D vascularized microtumor-on-a-chip without external pumps or valves; human endothelial colony-forming cell-derived endothelial cells (ECFC-ECs), human normal lung fibroblasts (NHLFs), HCT116 colorectal cancer cells; successful testing of FDA-approved drugs bortezomib, vincristine, CP-673451, linifanib, tamoxifen, axitinib, sorafenib, mitomycin C, vorinostat, and gemcitabine for tumor growth in presence of vasculature	[91]
Liver-on-a-chip	3D hepatic constructs of spheroids encapsulated within photocrosslinkable gelatin methacryloyl (GelMA) hydrogel; treatment with 15 mM acetaminophen induced toxic responses in hepatic construct	[87]
Skin	Human fibroblasts and human keratocytes; cell-suspended media for printing each cell with collagen solution for printing supporting layer in between each cell-printed layer	[92]
3D tumor array chip	Gelatin methacryloyl mixed with MDA-MB-231 breast tumor cells; drug screening of epirubicin and paclitaxel	[28]

3D bioprinting techniques have also been implemented in gene-based applications, including gene expression, gene-editing, and gene therapy techniques. Specifically, the applications of 3D bioprinting in this sector are extensive, ranging from gene modulation and expression in cancer, to tissue engineering, osteogenesis, and skin and vascular regeneration. Moreover, such techniques can be advantageously exploited for the real-time delivery or *in situ* production of nucleic acids within selected host cells [8]. The combination of nanotechnology and genomic-based3D bioprinting could contribute to overcoming limitations experienced in conventional fabrication methods and ultimately pave new ways for more effective treatments.

Concluding remarks and outlook

The science of 3D bioprinting has made valuable strides in clinical translation. Using an automated tissue organ printer, human-scaled mandible bones, ear-shaped cartilage, and skeletal muscle models are already available for clinical use. The successful establishment of Bio pen, an *in-situ* 3D bioprinting technology, is a clear indication that the field is quickly moving ahead, although a large-scale clinical translation of these fabrication technologies is not yet available. As a caveat, solid human organ biology is still unfeasible; current major progress is still being achieved only at the preclinical level, with only the areas of orthopedic medicine, maxillofacial surgery, and dentistry witnessing ongoing clinical trials. More steps need to be undertaken to foster large-scale 3D bioprinting applications, such as (i) new 3D bioprinting materials with outstanding biocompatibility, printing features, and mechanical behavior must be discovered and produced; (ii) (nano)composite materials endowed with heterogeneous/gradient characteristics and/or suitable to be processed via *in situ* bioprinting must be developed; (iii) the biological characteristics of 3D printing materials must be optimized via the incorporation of biomimetics and/or with bioactive factors; (iv) printed cell, tissue, and organ viability and functionality must be ensured, possibly by careful engineering and control of the relevant degradation performance and vascularization-promoting abilities; and, (v) standard guidelines and specifications for 3D bioprinting materials and related production process need to be issued [89].

3D-bioprinted tumor models can be used (at least in part) in place of xenografted animals, because they can preserve cancer–stromal cell interactions [90]. They can also be used as xenografts for Phase II and III clinical trials because they can more accurately recapitulate cancer–stroma cell and cancer–immune cell interactions. To constitute a breakthrough technology in cancer research, 3D-bioprinted tumors must be produced as low-cost, high-throughput systems (e.g., microwells) to enable 'at the bedside' drug testing in patient-derived cancer cells. Given that the latter can be collected in smaller numbers and their properties can be lost during cell passage compared with *in vitro* cultured cancer cell lines, dedicated 3D-bioprinting techniques able to be deploy constructs embedded with small cell numbers in confined spaces like a 96-well plate must be developed to definitively translate 3D bioprinting into therapeutics [91,92].

Cancer models based on organ-derived tissues can further help to decrease the high costs associated with drug discovery and, as an important added benefit, can contribute to refining, replacing, and/or reducing animal testing, in line with current ethical guidelines issued by regulatory bodies. Some hurdles remain to be overcome, mainly related to the scale-up engineering of these devices high throughput screening. 3D-bioprinted specimens have contributed to a deeper understanding and, hence, a subsequent refinement of *in vitro* tissue models; yet, because of their complexity, gaps to a full understanding of 3D culture systems persist, which hamper the easy reproduction and sequential evaluation of combination therapies. Indeed, the realization of 3D organoids exhibiting human physiology features still poses some challenges; nonetheless, currently available models have already established their potential as the most significant *in vitro* alternatives.

In the fast-evolving field of personalized medicine, next-generation techniques must strive to reveal patient-specific pathological mechanisms and reliably predict their response to different therapeutic treatments. With specific reference to cancer treatment, the requirements for scaling-up and fast processing also need to be satisfied to move 3D-bioprinted organoids based on patient-specific cancer tissues as drug testing, diagnostic, and prognostic tools from the bench to the bedside [26]. Given the major efforts and the vibrant research activity ongoing in the field, reaching all these goals really appears to be possible in the near future.

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