MEDICINE

Enhancing regenerative cell therapy with 3D bioprinting

In-situ additive biomanufacturing of cell-dense structures may boosts regenerative medicine

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Regenerative medicine uses viable cells that are injected, grafted, or implanted into a patient to exert a desired therapeutic effect, such as healing of traumatic wounds. However, achieving efficient delivery of therapeutic cells to the target site, without loss of survival and function, and tissue integration , are among the key challenges to success. Therefore, to fully unleash the potential of cell therapy in regenerative medicine, it is beneficial to adopt, adapt, or develop better tools to produce high-quality cell-based products via various biofabrication techniques.

Regenerative cell therapy traditionally involves a collection of cells suspended in a fluid that is injected *in vivo* directly at the target site through a syringe needle or a catheter. Despite the method's simplicity, the clinical outcomes have been unsatisfactory, partly because the injected cells are not maintained in the target site. To address this limitation, larger cell-laden modules that are much less prone to disintegration once delivered, have become an attractive substitute. Amorphous injectable hydrogel biomaterials are arguably the most common formulation to serve as carriers for therapeutic cells (*1*). Alternatives are pre-shaped modular structures that include microgels and porous polymer microspheres that are loaded with cells (*2*).

These approaches face several shortcomings including the inevitably reduced cell density compared to native tissues and the different composition and spatial organization of artificially introduced biomaterials compared with the native extracellular matrix (ECM) and tissue structure. Such disadvantages have propagated the use of biomaterial-free cell-dense constructs. For example, cells delivered in flat sheets (*3*) or in 3D arrangements, such as spheroids (*4*), have had varying levels of success in regenerating target tissues in preclinical studies and clinical trials.

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Nevertheless, whether biomaterial-enriched or biomaterial-free modular constructs are adopted, they are almost always randomly packed into irregular architectures in a post-injection scenario. This might be acceptable for many cell therapy scenarios, but the lack of cellular organization could prove insufficient for a number of applications in which conformation to the native geometries of therapeutic sites is crucial. For example, in the case of skin damage or muscle loss, it is important to match the native tissue architecture to achieve structural, functional, and aesthetic outcomes.

To this end, biofabrication approaches that feature precise spatiotemporal patterning of cells and biomaterials have emerged. Along these lines, additive biomanufacturing has the capacity to generate both microscale and macroscale cell-laden constructs bearing close geometric resemblance to their native target tissues (*5*). More commonly known as 3D bioprinting, this approach has a collection of modalities.

A widely used modality is extrusion bioprinting, which utilizes a nozzle to deposit a bioink (cells plus other necessary structural and active components), and often multiple bioinks, onto a collecting plate point-by-point and then layer-by-layer (*6*). Additional bioprinting modalities that are frequently adopted are inkjet bioprinting (*7*) and vat-polymerization bioprinting (*8*). Inkjet bioprinting ejects tiny droplets of bioinks into precise patterns, whereas vat polymerization utilizes one or several vats pre-loaded with bioink(s) to produce 3D constructs using photochemistry (both single-photon and multi-photon) (*8*) or sonochemistry (*9*).

So far, additive biomanufacturing has largely relied on the use of biomaterials to realize volumetric structuring and shaping of tissue architectures. The initial demonstrations of combining bioprinting with biomaterial-free cell-dense modules to ensure native-like cell-cell interactions focused on skewing multicellular spheroids onto arrays of patterned needles. In subsequent iterations of this approach, aspirating micronozzles were used to transfer the spheroids one-by-one to overlay them into volumetric patterns without need for skewing needles (*6*).

Major developments have recently allowed for precise structuring of biomaterial-free cell aggregates into tissue-relevant patterns at improved resolutions and complexities compared with those attainable by simply adding spheroids together (*6*). For example, bioinks solely comprised of somatic cell pellets can be loaded into bioprinting nozzles and extruded to become cell-only strands. The patterning capacity is then endowed by extruding cell-only bioinks into a supportive hydrogel bath. Notably, when combining stem cell-only biomaterial-free bioinks with extrusion bioprinting, organoids that feature well-defined spatiotemporal arrangements can arise. These bioprinted organoids display improved consistencies, differentiation efficiencies, and tissue-forming performances *in vitro* compared with conventional spherical organoids generated purely by cell self-assembly.

Most human solid organs exhibit high cell densities of a few billion cells per cubic centimeter. This arrangement is critical for their sophisticated functions and structural integrity. For instance, the human liver has a dense cellular arrangement that is necessary for its metabolic and detoxification activities. Similarly, cardiac tissues exhibit tightly packed cells that in turn ensure efficient electrical conductivity and contractile force to regulate pumping. For this reason, bioprinted tissues that mimic these high cell densities are anticipated to substantially enhance regenerative cell therapy outcomes by providing synergistic cell interactions, signaling, and tissue integration upon transplantation.

Until now, the integration of additive biomanufacturing with biomaterial-free, cell-dense bioinks has relied on the use of benchtop bioprinters, meaning that the tissues must be manufactured first, matured to differentiated phenotypes, and then transplanted. Such a process could be improved further by switching the conventional additive biomanufacturing method to *in-situ* bioprinting. This method involves direct patterning of bioinks onto a patient's body at the target site, allowing for precise construction of a site-matching tissue structure within the actual physiological location where regeneration or repair is needed (*10*). As such, *in-situ* bioprinting is likely to allow high adaptability, reduced risk of contamination, more streamlined procedures, as well as improved cell viability, function, host integration, and aesthetic appearance. Therefore, *in-*

situ bioprinting could not only enhance wound regeneration in hospital settings but also be critically beneficial in emergency scenarios, such as on battlefields, where rapid dressing is urgently needed.

In-situ bioprinting encompasses several different methodologies (*10*). One is the use of a conventional bioprinter, such as direct application during the surgical procedure (*8, 11*). Another method involves miniaturization of the printhead so that it can be fitted into a handheld device, to enable surgeon-directed hand-plotting across the therapeutic site (*12*). This method will enable a rapidly adjustable and user-defined 3D pattern-formation. Alternatively, programming a robotically actuated catheter-based bioprinter could also result in a well-structured delivery of therapeutic cellular bioinks (*13*). Clinical studies using some of these approaches are already in the planning.

Notably, prior demonstrations of *in-situ* bioprinting have entirely focused on the utilization of cell-laden biomaterial-enriched bioinks. However, the integration of bioinks that comprise native tissue-like cell densities with *in-situ* bioprinting could boost performance. Some initial examples of such a combination have adopted microgels as building blocks that are populated with high densities of cells. These microgels are pre-cultured to form target cell-dense mini-tissues, before loading them into *in-situ* bioprinters as bioink aggregates. Using a robotic arm-enabled *in-situ* extrusion method enabled healing of rat cranial defects *in vivo* (*14*) and a jetting (*15*) bioprinter repaired muscle and skin defects in mice *in vivo*. These studies also showed that high-cell-density mini-tissue blocks delivered *in-situ* with pre-defined patterns can expedite better healing than simple, unstructured deployment of the same cell-dense microgels.

Looking forward, it is anticipated that when *in-situ* bioprinting will start to encompass truly biomaterial-free bioinks containing only individual cells or their aggregates (spheroids or organoids), it will unleash the full potential of regenerative medicine. This is because when the use of biomaterials (whose compositions and fine structures are frequently to some extent different to native tissues) is minimized, the high densities of cells within the bioprinted constructs would have the chance to assume more robust intercellular interactions through tissue maturation. This maturation process also allows the cells to form their own tissue-specific, hierarchically composed, and fine-structured ECM that otherwise is not precisely mimicked with artificially introduced biomaterials. Moreover, when stem cells will be bioprinted *in-situ* at required densities to mimic the morphology of early developmental-stage tissue, highly functional replacement tissues that are amenable to enhanced regeneration may be within reach. Importantly, comparing to random architectures previously achieved only with simple injections, cell-dense aggregates that fulfill the exact structural, functional, and aesthetic needs of the local tissues will become a possibility.

Integrating *in-situ* bioprinting with biomaterial-free, cell-dense bioinks for use in cell therapy for regenerative medicine does not come without challenges. Unlike patterning biomaterial-rich bioinks, bioprinting of cell-dense modules requires proper adhesion with surrounding tissue microenvironments. Additionally, the internal cohesiveness within the modules needs to be maintained, which may not be straightforward and requires additional design considerations such as incorporating growth factors that promote cellular interactions to form integral tissue pieces. Moreover, although needle injection is minimally invasive, the use of an *in-situ* bioprinter could result in a slightly more invasive procedure, depending on the specific method used. As such, meticulous engineering of the instrumentation is necessary, possibly by taking advantage of state-of-art microsurgical devices and medical imaging platforms.

The patterns to be bioprinted *in-situ* will need to match the specific tissue needs, which, for example, would be unidirectional alignment for muscles and for liver is repeating lobular units. To this end, achieving precise structuring of cell-dense bioinks with *in-situ* techniques may not be trivial and could require additional improvement of the instrumentation such as possible integration with artificial intelligence-driven imaging. Another interesting area for exploration may be the inclusion of suitable cues such as chemokines, gene-editing tools, therapeutic moieties, or other bioactive agents to enable directed cellular modification or differentiation at the therapeutic site post *in-situ* bioprinting, which otherwise can post risks *in vivo* without pre-maturation steps. Such an approach might be realized using polymeric nanoparticles, synthetic artificial stem cells, or extracellular vesicles for controlled release of these agents while still retaining minimal presence of exogenous biomaterials. Lastly, given the early stage of the technology, extensive preclinical and clinical studies are warranted to address these challenges and safety concerns prior to translational application.

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Variety of *in-situ* bioprinting methods and bioinks

A multitude of *in-situ* bioprinting techniques have been developed, ranging from the adaptation of traditional benchtop bioprinters with nozzles, such as extrusion and inkjet models, to those utilizing vat-polymerization methods with light or ultrasound as the shaping energy source, for direct application in surgical settings. Additionally, there has been a move toward miniaturizing bioprinters for handheld use or combining them with interventional tools like robotically controlled catheters, allowing for swift, surgeon-directed modifications. These technologies are capable of depositing single or multiple bioink components. While the majority of existing examples have employed bioinks enriched with biomaterials—resulting in a dilute cell concentration and diminished physiological relevance—recent research is shifting toward the use of denser cellular bioinks encapsulated in biomaterial-based microcarriers. The anticipated progression to biomaterial-free, cell-rich bioinks composed of single-cell pellets, spheroids, or organoid and organoid-forming stem cells, could significantly enhance the therapeutic impact and clinical applicability of *in-situ* bioprinting in regenerative medicine.