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A Review On 3D Bioprinting

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- Abstract :- Three-dimensional (3D) bioprinting is an innovative technology that has emerged recently and holds significant potential to revolutionize medicine. This article reviews the most commonly utilized techniques and biomaterials in 3D bioprinting. We will examine the advantages and limitations of various techniques and biomaterials, providing a comparative overview. Additionally, we will explore the recent applications of these techniques across different industries. The aim of this article is to provide a foundational understanding of the techniques and biomaterials used in 3D bioprinting, their benefits and drawbacks, and their recent applications in various fields.

Introduction :- Three-dimensional (3D) printing of biological material is an innovative technology that enables the printing of various materials, from simple muscle and neural tissues to cartilage and entire organs. The process begins with creating a 3D model of the desired structure using patient scans such as X-ray, CT, or MRI. This model is then printed layer by layer, ensuring both microscopic and macroscopic details are accurately represented. The printed model is further processed to function cohesively as a single unit. When printing a specific structure, it is crucial to consider the properties of the biomaterials used, such as biocompatibility, strength, stability, and immunogenicity, to select the appropriate material.

Bioprinting involves multiple complex steps to produce customized 3D structures for patients. This includes designing the structure using computer-aided tools based on the patient's radiological imaging reports and then prototyping with a technique known as solid free form fabrication, which accounts for every detail of the tissue. Advances in bioprinting technology and biomaterials can lead to numerous long-term benefits. Although the idea of having a printed organ might seem daunting to some, if successful, this technology could save many lives by reducing the wait time for organ transplants.

Additional applications of 3D bioprinting include treating burn wounds with artificial skin, bioprinting bones and cartilage, drug testing, creating diseased tissue models to assess treatment efficacy before patient administration, bladder implants, and heart valve implants. Despite these potential benefits, there are significant challenges, such as the high cost of the technology, which may limit its accessibility. The technology's current level of advancement also poses risks, as the full range of potential complications is not yet understood. Extensive research is required to make this procedure safe and effective.

The primary objective of 3D bioprinting is to replace malfunctioning or defective tissue or organs with newly bioprinted ones that mimic the native organ's structural and functional properties. The bioprinted tissue must be capable of regeneration and differentiation when implanted in the patient. With the appropriate use of technology and biomaterials, it is possible to print tissue that performs these necessary functions. Therefore, significant research in biomaterials is essential to identify the right material that can function like native tissue. This review article discusses commonly used bioprinting technologies, their applications, advantages, and limitations, as well as the types of biomaterials (both natural and synthetic) used in 3D printing and their applications across various industries.

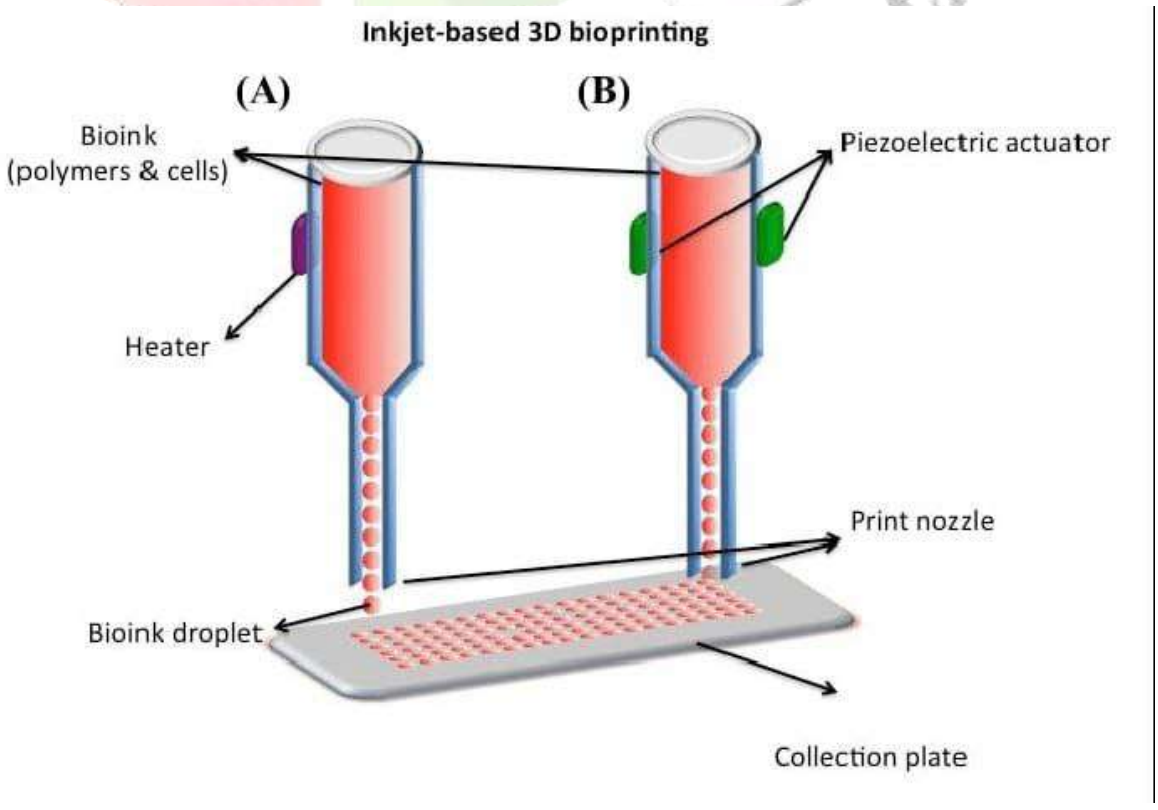
- Keywords :- 3D Bioprinting ,3D Bioprinting of Organ , 3D Bioprinting of Skeletal Muscle tissue engineering , 3D Bioprinting of Cells , 3D Bioprinting in Neuronal Engineering , 3D Bioprinting for cardiovascular regeneration and pharmacology , orthopaedic translation research, Bioink

- **3D BIOPRINTING**

- **Bioprinting Strategies**

  The success of tissue engineering largely hinges on the ability to create complex, cell-laden 3D structures that closely mimic original living tissues. Thus, the strategies employed to design and develop the architecture and topography of biomaterial scaffolds are crucial for effective tissue engineering. Functional scaffolds can be prepared using either top-down or bottom-up approaches. Various bioprinting strategies, based on their fundamental principles, are utilized for fabricating functional tissue constructs. These include inkjet-based bioprinting, laser-assisted bioprinting (LAB), pressure-assisted (extrusion) bioprinting, acoustic bioprinting, stereolithography (SLA)-based bioprinting, and magnetic bioprinting. These bioprinting strategies can be used independently or in combination to achieve the desired objectives in additive manufacturing and tissue fabrication.

- **Inkjet-based bioprinting**



utilizes a well-understood printing technology adapted from traditional 2D desktop inkjet printers. This non-contact bioprinting method involves creating and precisely positioning picoliter volume (1-100 pL) droplets of "bioink" onto a substrate under computer control . Each droplet of bioink typically contains between 10^4 to 10^6 cells . The ink droplets used in bioprinting are generated through two main strategies: continuous inkjet printing (CIJ) and drop-on-demand printing (DOD).

Continuous inkjet printing operates based on the natural tendency of a liquid stream to break up into drops due to Rayleigh-Plateau instability, forming continuous-discrete drops of ink. These drops, which range in diameter from 10-150 µm, can be directed to specific locations using electric or magnetic fields due to their electrically conductive nature. In contrast, drop-on-demand printing generates ink droplets only when needed. CIJ bioprinters generally produce drops at a higher speed compared to DOD systems. However, CIJ printers require conductive fluid inks and may pose contamination risks during fluid recycling, limiting their suitability for biomedical applications. DOD bioprinters are preferred for their precision in material deposition and minimal wastage of bioink.

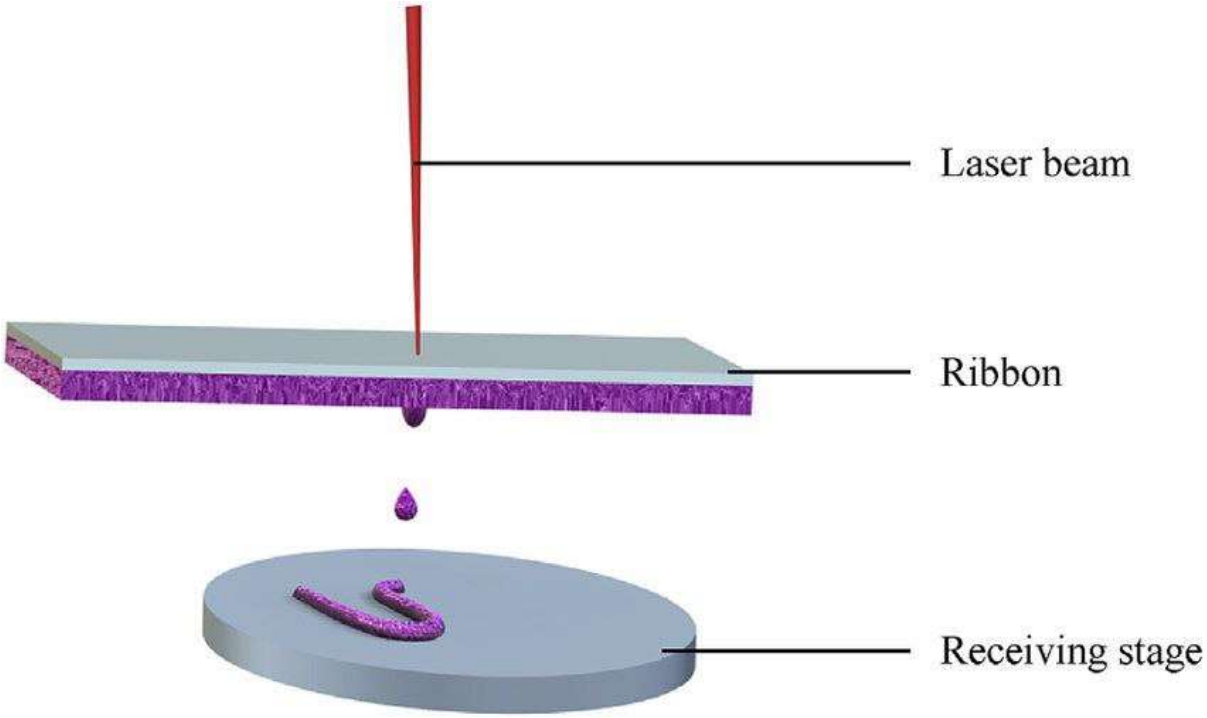
Drop-on-demand (DOD) technology can be achieved through thermal, piezoelectric, or acoustic methods . In commonly used inkjet bioprinters, drops are created and ejected using heat or mechanical compression. These ejected drops typically have a diameter of less than 30 µm, providing high resolution.

A thermal inkjet bioprinter consists of an ink chamber with a nozzle and a heating element, typically a thin film resistor . To generate a drop, a brief electric pulse is applied to the resistor, generating heat that forms a small vapor pocket or bubble. The bubble expands or collapses when the heat is removed, propelling ink drops of various volumes out of the nozzle . Hence, thermal inkjet bioprinters are sometimes referred to as "bubble jet bioprinters."

In piezoelectric inkjet technology, a pressure pulse is generated by the mechanical movement of piezoelectric crystals positioned at the back of the ink chamber, causing them to vibrate. These internal vibrations force bioink droplets out of the nozzle. Thermal inkjet technology is favored for its simplicity, efficiency, and cost-effectiveness. However, challenges such as nozzle clogging due to bioink gelation and the production of unevenly sized drops can disrupt the printing process . Moreover, thermal and shear stresses involved in drop formation may impact cell viability. Studies indicate that short exposures (2 µs) to temperatures up to 300 ºC during printing have minimal effects on cell viability .

Piezoelectric-based bioprinting, on the other hand, can affect cell membranes and biomolecule structures due to vibration frequencies and power levels, potentially leading to protein unfolding . Nonetheless, studies report high cell viability (>90%) for human fibroblasts printed using piezoelectric inkjet printers . Overall, thermal inkjet bioprinters are extensively utilized for printing biological materials.

- **Laser-assisted bioprinting**



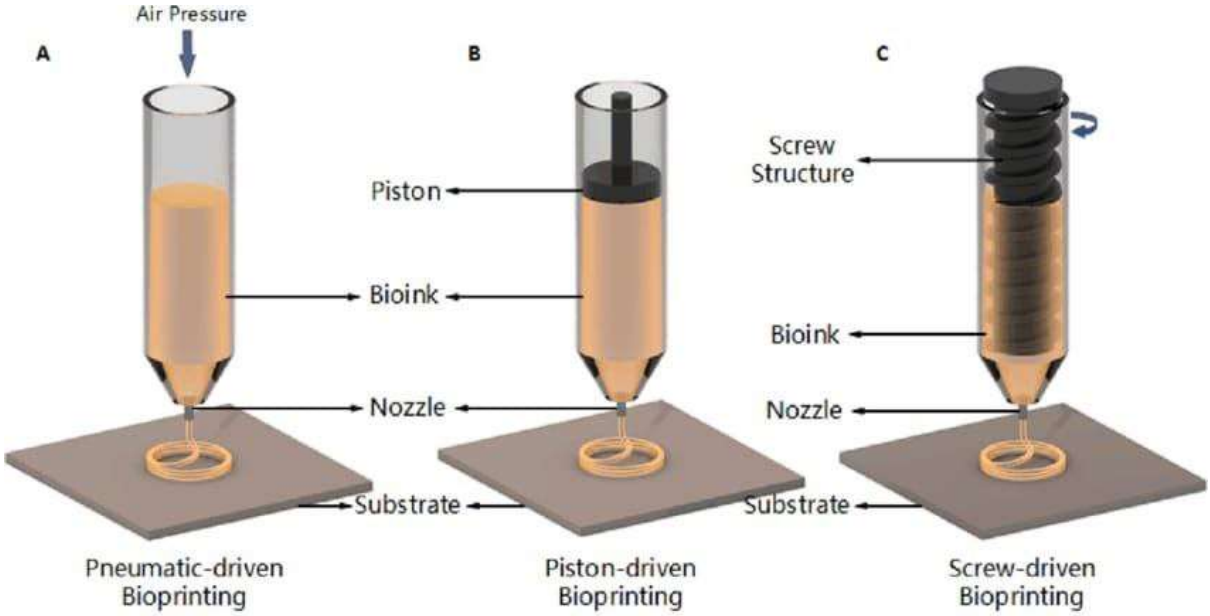
(LAB) is a bioprinting technique derived from the laser-induced forward transfer (LIFT) effect, enabling precise deposition of various living cells and biomaterials with micrometer-scale resolution . Originally developed for metals, LIFT has been adapted successfully to deposit biological materials such as cells, DNA, and peptides using a laser pulse repetition rate of 5 kHz . In 2004, Barron and colleagues introduced the biological laser printer (BioLP), achieving spatial accuracies of > 5 µm for biological patterning .

LAB systems typically include an energized pulsed laser (often infrared), a donor ribbon or target film containing the biological material, and a receiving substrate for material deposition. The ribbon consists of a laser-transparent substrate (e.g., quartz or glass) coated with a thin layer of laser-absorbing metal (e.g., gold or titanium). Bioinks containing cells or molecules in liquid or gel form (e.g., culture media, collagen) are applied over this metal-coated support. The incident laser vaporizes the metal film, ejecting bioink droplets onto the receiving substrate .

During this process, interactions between the laser and cells, as well as between cells and the substrate, can influence cellular integrity . However, LAB, being a nozzle-free technology, can accommodate bioinks with varying viscosities (1-300 mPa.s) and high cell concentrations (~10^8 cells mL^-1) [2]. Thus, LAB is highly adaptable for creating complex tissue constructs with high cell densities, precise resolution (10-100 µm), and diverse sizes to closely mimic native physiological structures.

LAB offers advantages such as automation, reproducibility, and high throughput, making it an appealing approach for 3D tissue fabrication. However, selecting appropriate biomaterials is crucial; these materials should exhibit rapid gelation kinetics (quick cross-linking) and be compatible with the laser wavelengths to preserve cellular arrangement and resolution in printed constructs, which remains a challenge. Issues such as gravitational settling of cells in solution and lengthy fabrication times are also significant considerations in LAB bioprinting.

- **Extrusion-based bioprinting**



Source: Figure courtesy of Gu *et al.* (2020)

Extrusion-based bioprinting, also known as pressure-assisted bioprinting, is widely utilized in both research and commercial sectors for creating 3D constructs laden with cells. Unlike thermal inkjet bioprinters, which are limited to low-viscosity bioinks with air bubbles below 10 mPa.s, extrusion bioprinting can handle highly viscous bioinks that cannot be dispensed through small nozzles. In this method, bioinks are loaded into disposable medical-grade plastic syringes and then extruded pneumatically or mechanically (using a piston or rotating screw) onto sterile substrates.

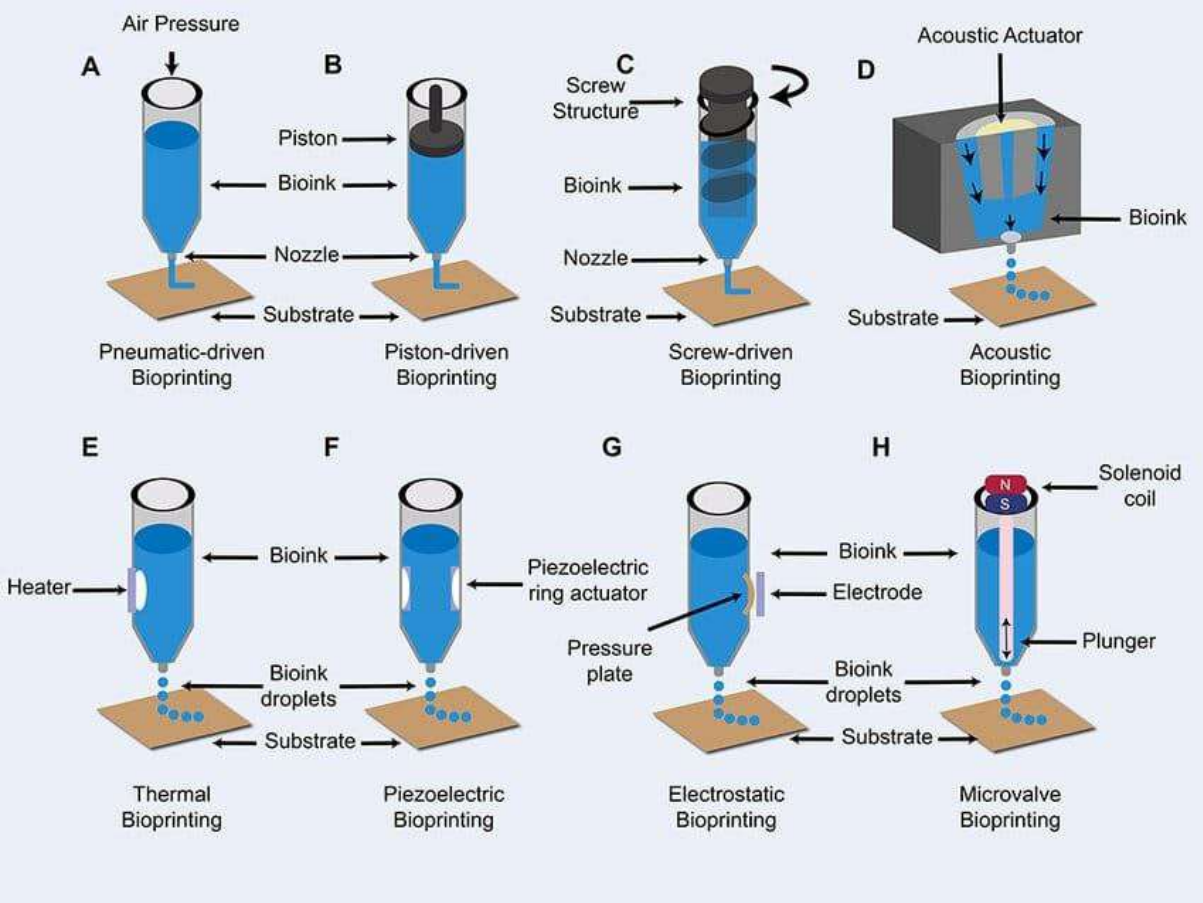
Piston-driven systems offer extended control over bioink dispensing, while screw-driven systems provide precise spatial control and are effective for depositing highly viscous bioinks. Pneumatic systems are advantageous for their ability to deposit bioinks of varying types and viscosities by adjusting pressure and valve gating times . Under pressure, highly viscous bioinks flow out as continuous cylindrical filaments (approximately 150-350 µm in diameter) . These filaments are then cross-linked using light (often UV), enzymes, chemicals, or heat to form mechanically robust structures.

Maintaining controlled temperatures of the ink container and receiving platform is crucial for dispensing thermo-sensitive and light-sensitive polymers, controlling bioink viscosity, and inducing in situ gelation [59]. Other parameters such as air pressure, extrusion speed, platform positioning, and type can also be adjusted to directly impact printing fidelity and resolution. However, high pressure and rapid speed can induce shear stress, potentially reducing cell viability, which is a significant concern. Additional challenges include nozzle clogging and lower resolution (ranging from 200-1000 µm) . Therefore, optimizing printing parameters is essential to ensure stable printed structures without compromising cell viability, thereby maximizing the benefits and ease of extrusion printing.

To create customized 3D structures, the desired shape is first designed using computer-aided design/manufacturing (CAD/CAM) software in STL (standard template library) file format, which is then printed layer-by-layer (LbL) to achieve the desired thickness . Modern extrusion bioprinters are equipped with multiple printer heads, enabling simultaneous deposition of different bioinks with minimal cross-contamination . This capability allows for precise control over porosity, shape, and cell distribution within the printed construct. A wide range of cell types and designs have been successfully printed as tissue substitutes using this technology . Additionally, other biomolecules

such as DNA, RNA, and peptide fibrils have been 3D printed using extrusion bioprinting . Due to its versatility, this method is particularly well-suited for fabricating scaffolds and prosthetic implants for tissue engineering applications.

- **Acoustic bioprinting**



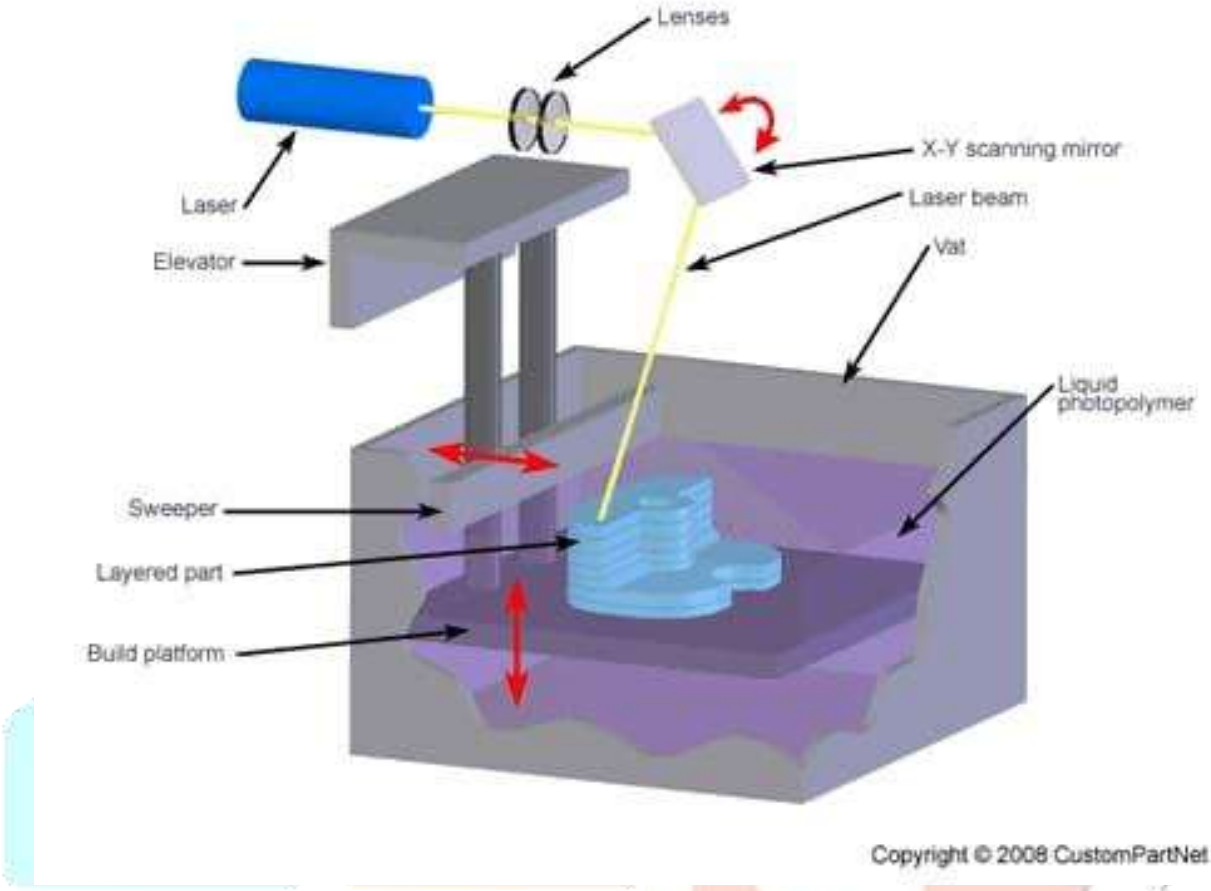
Acoustic bioprinting represents a groundbreaking approach in bioprinting by utilizing surface acoustic wave technology to manipulate single cells and construct intricate 3D patterns . Sound waves are employed to direct cells in various directions, facilitating the fabrication of complex structures. Unlike traditional bioprinters, acoustic bioprinters operate without nozzles, thereby avoiding issues like clogging and protecting cells from harmful shear stresses, heat, and pressure commonly associated with drop-on-demand printers .

Several years ago, researchers developed an acoustic bioprinter capable of encapsulating and printing multiple cell types (such as stem cells, fibroblasts, hepatocytes, and cardiomyocytes) in biological fluids at high throughput levels while maintaining cell viability (> 85%). This bioprinter utilized either a single or an array of 2D microfluidic channels to contain bioinks. The acoustic ejector, composed of a piezoelectric substrate (such as lithium niobate, tantalate, or quartz) with interdigitated gold rings, generated surface acoustic waves on demand. These waves converged to form an acoustic focal point at the air-fluid interface, causing bioink droplets to be ejected when the acoustic vibration exceeded the surface tension of the bioink. The size of the droplets varied with the acoustic frequency, and the ejection rate ranged from 1 to 10^4 droplets per second .

Recent advancements include the use of 3D acoustic tweezers based on standing surface acoustic wave (SSAW) technology. This technique enables the manipulation, translation, and precise arrangement of single cells or cell assemblies to create 2D or 3D patterns in a non-invasive, label-free, and contact-free manner . However, the potential of acoustic bioprinting to integrate multiple

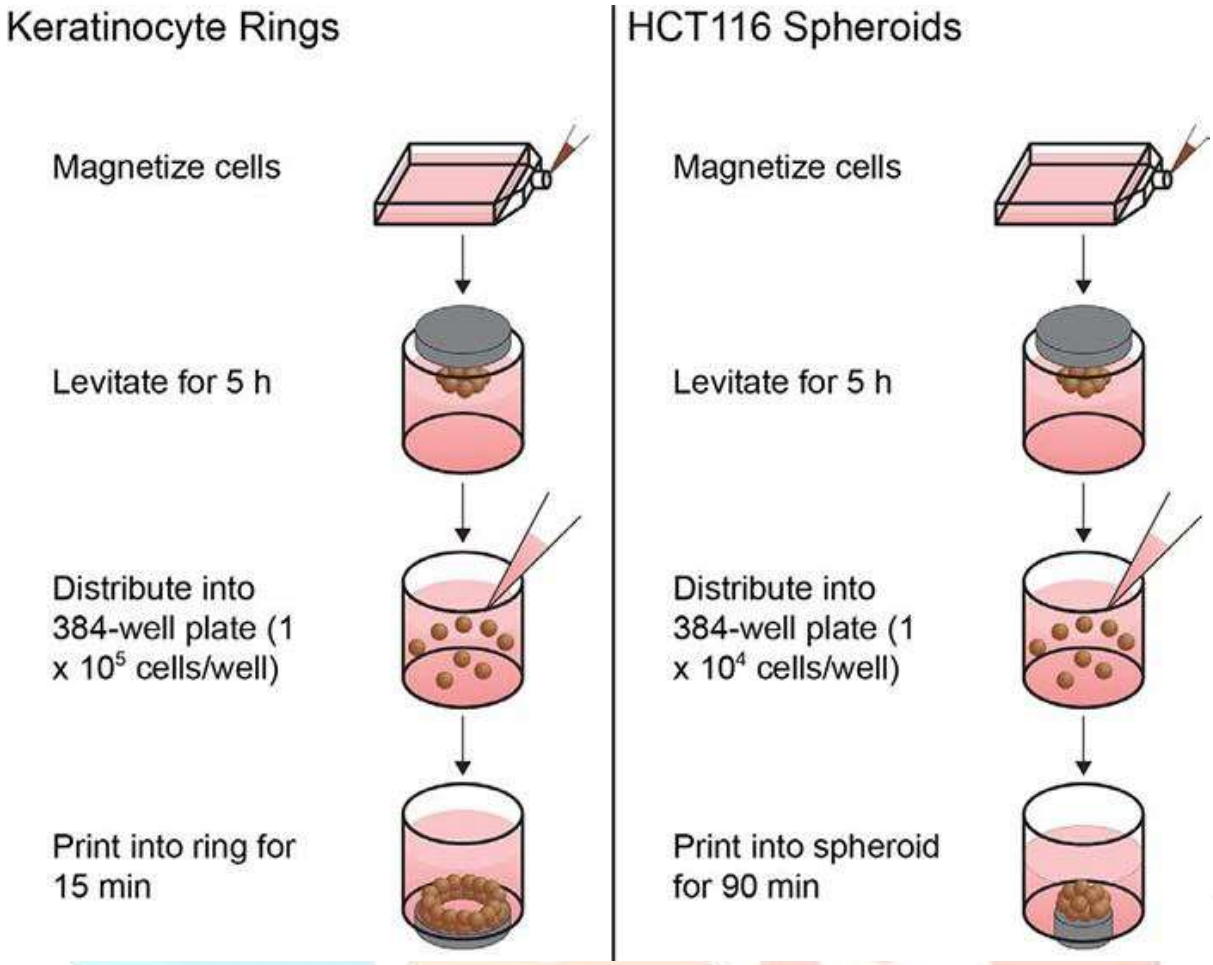
cell types and growth factors to construct biomimetic cell-laden scaffolds requires further exploration.

- **Stereolithography**



Stereolithography (SLA) bioprinting is an advanced method used to create 3D structured scaffolds at micro- and nano-scales . Traditional 3D scaffold fabrication methods often lack the ability to control properties such as porosity, resolution, and mechanical strength. SLA bioprinting involves using light to cure light-sensitive bioinks layer by layer, building up the material in a projection-printing system that cross-links bioinks plane-by-plane . Each layer's printing time is independent of its complexity and size, calculated based on the structure's thickness. SLA has achieved printing of 3D cell-encapsulated structures with resolutions as fine as 100 µm and exceptional cell viability (>90%) in under 30 minutes . Bioinks like polyethylene glycol diacrylate (PEG-DA) and gelatin methacrylate (GelMA), combined with NIH 3T3 fibroblast cells, have been successfully printed with a low-cost setup achieving 50 µm resolution and 85% cell viability . Recent research has integrated SLA bioprinting with electrospinning for neural tissue engineering, combining aligned electrospun fibers of polycaprolactone (PCL)/gelatin composites with SLA-printed microporous scaffolds of PEG-DA, showing promise in enhancing neural cell behavior and mechanical properties."

- **Magnetic bioprinting**



Magnetic bioprinting enables the assembly of 3D multitype co-cultures in laboratory settings through magnetic levitation principles . This technique offers precise spatial control, allows for the synthesis of extracellular matrix (ECM) without artificial protein substrates, and facilitates rapid printing of multiple tissue-like structures . It primarily operates as a contactless method using two strategies for manipulating and assembling cells into structured forms . In label-free diamagnetophoretic printing, cells mixed with a paramagnetic buffer are exposed to an external magnetic field to form aggregates . Alternatively, cells are incubated with a nanoparticle assembly containing poly-L-lysine, magnetic iron oxide (Fe3O4, magnetite), and gold nanoparticles (NanoShuttle-PL), forming a gel through electrostatic interactions. Upon uptake by cells, this gel renders them magnetic, allowing manipulation and levitation off the plate surface into the media to form aggregates . Magnetic forces from a pre-designed template guide these magnetized cell aggregates into 3D patterns, with the ability to alter spatial patterning by modifying the template shape . Various tissues such as adipose, lung, aortic valve, blood vessels, and tumors like glioblastoma and breast have been successfully fabricated using similar methods, demonstrating in vivo-like protein expression and ECM characteristics . Building on the foundation of M3DB, Tseng et al. validated spheroid contraction as a biologically relevant cytotoxic endpoint using 3T3 murine embryonic fibroblasts in response to five toxic compounds. This study highlights the utility of the developed assay in conjunction with M3DB. "Spheroids used to assess cytotoxicity in a 3D microenvironment could potentially address limitations in handling, speed, throughput, and imaging compared to other 3D cell culture platforms . Additionally, researchers proposed a 3D in vitro model to study uterine contractility physiology using human uterine myometrial cells. Patient-derived myometrium cells were magnetically arranged into hollow rings to analyze uterine contractility over time and in response to various clinically relevant agents. These printed uterine rings, sourced from diverse cell origins and patients, exhibited varied contractility patterns and responses to uterine contractility inhibitors such as nifedipine and indomethacin. This study aims

to fulfil the need for high-throughput evaluation of multiple agents and conditions in uterine contractility research .In another study, Hou et al. developed 3D pancreatic cancer organoids in standard flat-bottom well plates using M3DB and cell-repelling forces. They evaluated the inhibitory effects of approximately 3,300 clinically approved drugs on pancreatic cancer patient-derived cells, including cancer-associated fibroblasts (CAFs) . Furthermore, Baillargeon et al. utilized similar technology to upscale conventional 96- and 384-well microtiter plate densities to 1536-well plates for automated large-scale screening. This strategy proved effective for fully automated production of spheroids and organoids, supporting high-throughput screening."

- **Process parameters affecting 3D bioprinting**

"Extensive literature focuses on 3D bioprinting for applications in tissue engineering and bioengineering. However, the process parameters involved in extrusion-based bioprinting, which translate 2D or 3D designs of tissues or organs into synthetic structures under computer control, are often overlooked. Understanding these variables is crucial for creating user-defined 3D hierarchical structures that closely mimic native tissues. The diameter of the deposited or printed strand (strut) significantly impacts the overall porosity, mechanical strength, and layer height of the scaffold. Key factors affecting printing accuracy include solution viscosity, applied pressure, printing speed, and printing distance, all critical parameters for 3D bioprinters. Viscosity plays a critical role in producing 3D printed cell-laden constructs, particularly in inkjet and extrusion-based bioprinting . An ideal printable biomaterial must have adequate viscosity to facilitate smooth nozzle extrusion and rapid solidification post-printing, whether through gelation or shear thinning properties. Highly viscous inks can lead to nozzle clogging, while low viscosity materials may cause deformation or collapse of structures . Laser-assisted bioprinting, however, does not face viscosity limitations as there is no nozzle ejection . Therefore, bioink viscosity needs adjustment depending on the bioprinter type . Different bioprinters operate within varied viscosity ranges: common inkjet or droplet-based bioprinters typically use bioinks with viscosities around 10 mPa.s, while laser-assisted bioprinters (LAB) range from 1-300 mPa.s, and extrusion-based bioprinters span from 30 to 6 x 10^7 mPa.s .Moreover, increasing bioink concentration directly impacts cell viability, as higher concentrations can inhibit cell migration and proliferation due to polymer chain entanglement, thereby reducing cell viability . The composition of bioink significantly affects the printability, shape fidelity, structural resolution, and cell survival of 3D printed constructs, influencing cross-linking during pre- and post-gelation processes . In the following section, we provide a brief overview of various bioinks that influence scaffold properties."

- **Bioinks**

The solution or hydrogel form of biomaterials loaded with specific cell types is known as bioinks, essential for bioprinting to create functional tissue or organ constructs . The terms bioinks and biomaterial bioinks are often used interchangeably; however, the term bioink specifically refers to the cellular component constructed in 3D within or on hydrogels. In contrast, biomaterial bioink refers to hydrogel precursors or aqueous polymer formulations containing biological factors used for subsequent cell seeding or in vivo studies . Bioinks are categorized into four classes based on their functions: (1) Structural bioinks support cell adhesion, proliferation, differentiation, mimic the extracellular matrix (ECM) during cell growth, and maintain mechanical integrity. (2) Fugitive bioinks, or sacrificial bioinks, are temporary materials quickly removed to create internal voids or channels in 3D printed constructs. (3) Support bioinks are non-biological materials with strong mechanical strength to withstand loads and provide support for softer or complex structures during printing. (4) Functional bioinks provide mechanical, biochemical, and electrical signals to influence cellular behaviour post-printing . Sacrifical and support bioinks are technically biomaterial inks

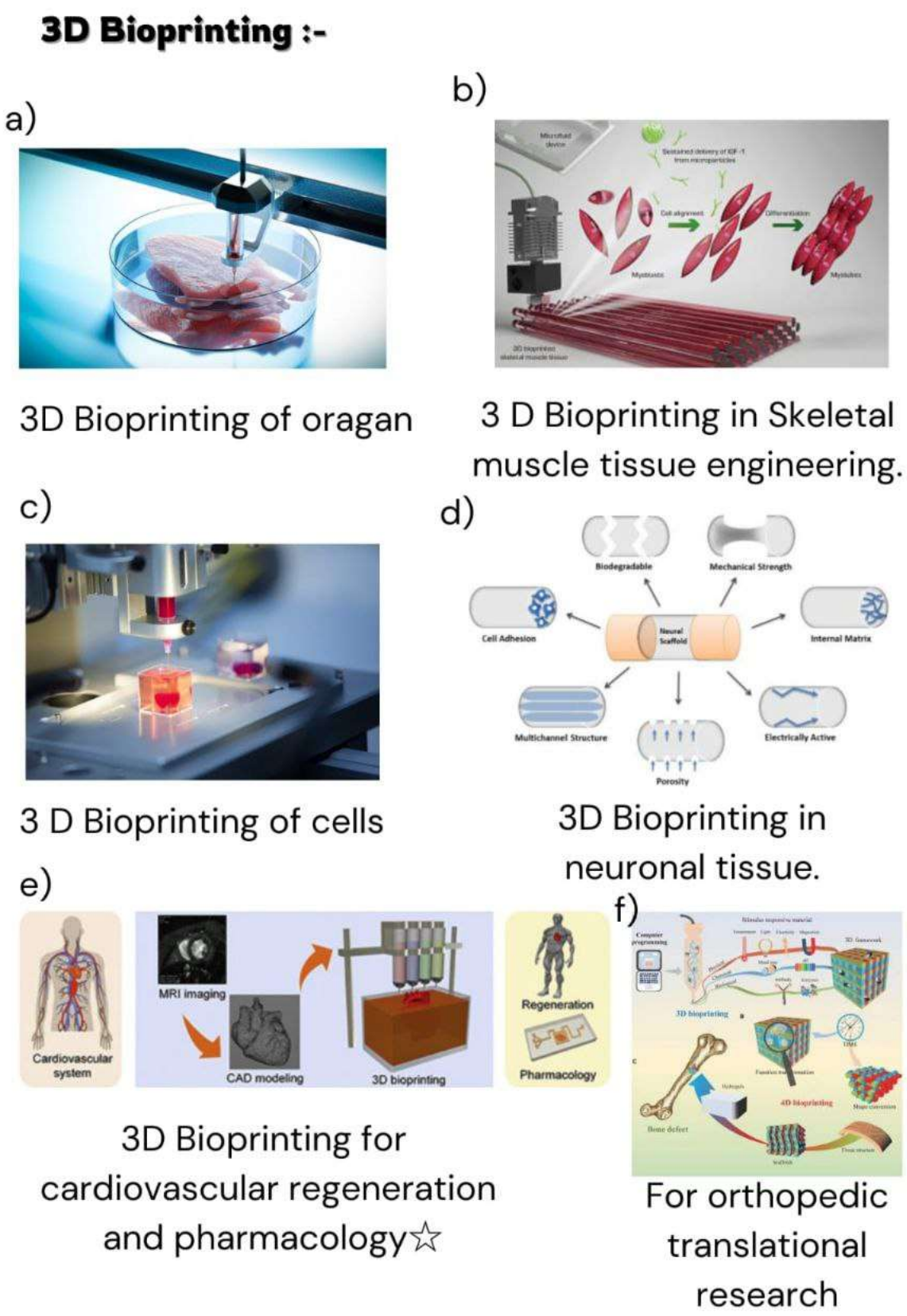
rather than bioinks. The functionality of final printed tissues and organs depends on the rheological, mechanical, and biological properties of the bioink .Polymers used in bioinks can be natural, synthetic, or a combination thereof, promoting favorable cellular interactions, increased proliferation, motility, and differentiation . Natural polymers commonly used as bioink bases include alginate , collagen , silk , dextran , gelatin , fibrin , agarose-chitosan , agarose , gellan gum , hyaluronic acid (HA) , decellularized matrix , matrigel , and hydroxyapatite (Hap) . Synthetic polymers include polyethylene glycol (PEG) , methacrylated hyaluronic acid (HAMA) - methacrylated poly[N-(2-hydroxypropyl)methacrylamide mono/dilactate (Phpma-lac)/PEG , polyvinylpyrrolidone (PVP) , poly(ε-caprolactone) (PCL) , pluronic , poly(glycidol)-HA (PG-HA) , and polyhydroxybutyrate (PHB) . Tables 1−3 summarize the use of various natural, synthetic, and composite materials as base biomaterials with specific cell types for formulating bioinks.

The prevailing approach in utilizing additive manufacturing technologies for tissue engineering involves seeding cells onto porous scaffolds after printing, using biomaterial inks rather than bioinks containing cells. However, this method often results in uneven distribution of cell density, with higher cell concentrations observed at the edges of the construct. This heterogeneous cell distribution can create unfavorable oxygen gradients from the periphery to the core of the structure, potentially compromising cell growth and proliferation .

In contrast, bioprinting or printing bioinks containing cells can overcome this limitation by ensuring homogeneous distribution of cells within the bioink and consequently throughout the printed construct. The properties of the biomaterials used as the base for bioinks significantly influence the cell encapsulation process and cell viability. One critical property is the modulus of the encapsulating hydrogel; hydrogels with low moduli (<1 kPa) generally exhibit better cell attachment, viability, expansion, and proliferation .

As emphasized, cells are the primary and crucial component of bioinks, and their selection should precede the choice of base biomaterials. The appropriate cell density and types or combinations of cells for bioinks should be tailored based on the specific tissue of interest. For instance, for bone tissue engineering, an optimal cell density of 5 to 10 million cells per milliliter of bioink may be recommended.

- **Overview**



- **Bioprinting for tissue regeneration**

  Different tissue constructs replicate native tissues and organs such as skin, cardiac tissue, bone, cartilage, liver, lung, neural tissue, and pancreas. These have been effectively fabricated using various 3D printing methods.

- **SKIN**

  Recent advancements in 3D bioprinting technology have revolutionized the fabrication of complex, multi-layered skin, the body's largest organ. This technology holds immense potential for diverse applications such as wound healing, skin grafts for burn patients, and the creation of human skin models for drug testing. Researchers have achieved significant milestones in bioprinting keratinocytes and fibroblasts in stratified arrangements, mimicking dermal and epidermal-like layers within 3D scaffolds. These efforts highlight the capability of bioprinted skin to support cell proliferation and maintain high viability across various surfaces.

  Studies using different bioprinting techniques, such as LIFT and extrusion-based methods, have successfully printed skin-derived cell lines and mesenchymal stem cells with high survival rates. These findings underscore the biocompatibility and minimal impact on cellular behaviors during and post-printing processes. Additionally, advancements in biomaterials like polyelectrolyte hydrogels have addressed challenges in vascularization and cell differentiation, crucial for enhancing the functionality of bioprinted skin constructs.

  Innovative approaches combining extrusion and inkjet bioprinting systems have enabled the creation of stable dermal and stratified epidermal layers, demonstrating cost-effectiveness and efficiency in skin model fabrication. Furthermore, the integration of growth factors and bioactive agents into bioprinted scaffolds has shown promising results in promoting enhanced tissue regeneration and wound closure in animal models.

  Overall, while laser-assisted bioprinting remains a staple for precision in skin bioprinting, advancements in nozzle-based bioprinters are expanding capabilities in multi-material printing. Skin's structural simplicity makes it an ideal candidate for bioprinting applications, particularly in situ bioprinting for accelerated wound healing and clinical use. Future directions aim to optimize printing techniques and biomaterial formulations to further improve the functional and morphological similarities of bioprinted skin to native tissue, paving the way for broader clinical adoption and personalized medicine applications.

- **Vascular Structures**:
  Creating vascular features in bioprinted tissues is often challenging, but innovative bioprinting methods offer potential solutions. For instance, a coaxial nozzle system was used to print carbon nanotube-reinforced alginate conduits over one meter long . These conduits supported human coronary artery smooth muscle cell growth and were perfusable, although they primarily achieved sub-millimeter diameters without approaching capillary sizes. Another approach involves incorporating magnetically controlled nanoparticles into bioinks to precisely position vessels using magnetic fields, though further research is needed on their efficiency and effects on cells and extracellular matrix (ECM). Sacrificial inks, like Pluronic F127, have been used successfully to print smaller vascular channels, down to 45 µm, which were endothelialized with HUVECs. This technique, combined with fibroblasts in gelatin methacrylate bioink, enabled the creation of multicellular bioprinted constructs. Different sacrificial materials have also been employed to create seedable channels in bioprinted tissues, allowing for efficient prepatterning and faster tissue printing speeds.

3D Bioprinting in Skeletal Muscle Tissue Engineering

3D Printing and Bioprinting in Skeletal Muscle Tissue Engineering3D bioprinting enables precise deposition of matrix and cells to create complex structures. For instance, Gao and Cui demonstrated that bioprinting can accurately place mouse myoblasts (C2C12) within a matrix on cantilevers, achieving a resolution of 85 µm, over 90% cell viability, and high reproducibility. Following differentiation, the myotubes on the cantilevers exhibited excitability (2 V, 40 ms, 5 Hz). Their findings suggest that bioprinting muscle cells using biological microelectromechanical systems (bio-MEMS) results in superior physiological responses due to precise cell positioning and alignment, in contrast to random seeding methods such as hand-based or syringe-based techniques. While cardiac cells exploit spontaneous beating to develop biological actuators, creating bioactuators with controlled movements requires skeletal muscle cells. Bashir and colleagues employed stereolithography to create biological devices (bio-bots) consisting of two rigid pillars of different lengths connected by a flexible beam. C2C12 cells, ECM proteins, and Matrigel solution were cast around and between the pillars to initiate gelation and form a cell strip (Figure 5a). Following differentiation, gel compaction and tension induced between the two pillars promoted myotube maturation, which, under electrical pulses at 1 Hz, contracted and facilitated an inchworm-like crawling motion of the structure at 117.8 µm s−1. Utilizing stereolithographic 3D printing enhanced the variety of materials and cell types applicable for developing biological machines. Recently, to address limitations such as spontaneous shrinkage of skeletal muscle tissue, which contracts on flexible substrates, researchers developed a biohybrid robot powered by opposing pairs of skeletal muscle tissues. This robot featured a 3D-printed resin skeleton equipped with electrodes for actively stimulating myoblast-laden hydrogel sheets mounted on both sides of the skeleton as antagonist muscles (Figure 5b). The study demonstrated that the biohybrid robot could perform significant movements (rotational angle close to 90°), suitable for basic actions like grasping and transporting small objects. Additionally, it sustained operation over an extended period (approximately 1 week). Despite these achievements, the field remains nascent, requiring further development to establish reliable hybrid robots for advanced applications. However, electrical stimulation for actuation presents challenges, including the coupling of the actuator with the environment and potential formation of bubbles due to electrolysis, which could harm skeletal muscle tissue and electrodes. To overcome these limitations, neural stimulation methods involve coculturing skeletal muscle cells with motor neurons, while optogenetic approaches genetically modify skeletal muscle cells to respond to light stimulation. Bashir and colleagues pioneered biological actuators driven by optogenetic skeletal muscle capable of generating up to 300 µN force in response to optical stimuli. Furthermore, they addressed tissue damage issues by developing a method to heal extensive muscle damage in bioactuators using new myoblasts, ECM proteins, physical exercise, and locally released insulin-like growth factor from a biological adhesive. Another emerging approach involves leveraging additive manufacturing, with or without other scaffold fabrication technologies, to assemble sophisticated constructs that mimic the organization and function of skeletal muscle tissue. Studies have focused on 3D fabrication of muscle fiber groups forming fascicles. For instance, Yeo and Kim produced bundles of aligned and random PCL microfibers using wet electrospinning. They achieved fiber alignment by stretching them at 45–50 °C. To mimic natural muscle further, they coated a second group of scaffolds with aligned microfibers with collagen. For homogeneous cell seeding, C2C12 cells were bioprinted onto scaffolds composed of 2% collagen-2% poly(ethylene oxide) (PEO).

They observed improved sarcomeric organization and differentiation after 7 days of culture on collagen-coated aligned fibers compared to random fiber scaffolds. In another biomimetic approach to muscle bundle fabrication, Kim and colleagues used a melt-printing system to produce a microfibrous PCL bundle by printing a PVA/PCL solution (3:7 ratio) at 85 °C with a 350 µm nozzle at

a speed of 10 mm s−1 and pneumatic pressure of 250 kPa. After removing sacrificial PVA in water after 24 hours, they crosslinked the PCL structure with 0.5% collagen using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) for 30 minutes, followed by 12-hour freeze-drying. Due to PVA microfibrillation and leaching from the PVA/PC mixture, the scaffold exhibited a surface with an aligned microfibrous pattern and a section allowing cell penetration between the microfibers (Figure 6a). C2C12 myoblasts were seeded onto these scaffolds and cultured for two weeks. Cell analysis revealed longitudinal cell alignment, robust cell proliferation on the surface, extensive cell infiltration between the microfibers, and formation of a scaffold section mimicking a muscle bundle cross-section. Myosin heavy chain (MHC) development was also notable. Moreover, various bioinks have been developed to enhance cell viability, printability, and tissue formation. Engineering interface tissues requires materials with distinct mechanical and chemical properties tailored to specific cell types in different regions. To create a muscle-tendon unit, Atala and colleagues used a composite hydrogel bioink comprising gelatin, fibrinogen, hyaluronic acid, and calcium-free high-glucose DMEM to bioprint C2C12 onto a PU-aligned fiber scaffold mimicking muscle elasticity. Concurrently, NIH/3T3 cells were bioprinted onto a PCL-aligned fiber scaffold mimicking tendon stiffness, with the interface area overlapped by 10% of both fiber types. The constructs were crosslinked for 30 minutes in a thrombin solution (20 U mL−1) with 0.5 × 10−3 m CaCl2 and incubated in culture medium. After one day of culture, the medium was switched to differentiation medium for seven days, during which C2C12 expressed desmin and MHC, and fibroblasts secreted type I collagen. Notably, distinct secretion patterns were observed at the interfacial region between muscle and tendon regions. This study highlighted the versatility of integrated organ printing (IOP) in creating complex constructs with region-specific mechanical and biological characteristics. In another application of IOP, Atala and colleagues fabricated human-scale tissue constructs, including the mandible, calvarial bone, ear cartilage, and skeletal muscle (Figure 6c). They bioprinted C2C12 using a bioink comprising gelatin, fibrinogen, hyaluronic acid, and glycerol. They observed 97% cell viability post-printing, cell alignment at day three of culture, and myotube formation after seven days in differentiation medium. The constructs were subcutaneously implanted in nude rats with surgically inserted common peroneal nerves. After two weeks, nerve integration in the constructs was evident, with acetylcholine receptor (AChR) clusters observed on muscle fibers and nerve contacts. Vascularization was induced in the constructs, as indicated by endothelial cell marker expression. Electromyography confirmed the engineered muscles' response to electrical stimulation, although they remained immature. To enhance cell signaling, some researchers proposed using bioinks comprising decellularized matrix from skeletal muscle. For instance, Cho and colleagues developed a decellularized matrix bioink (mdECM) from porcine skeletal muscle, using it at a 1% concentration to print various patterns (parallel lines with a 500 µm width, diamonds, chains) of C2C12 at 18 °C (Figure 6b). After gelation at 37 °C and culturing the constructs for one, four, and seven days, they observed high cell viability and increased cell proliferation compared to collagen bioink constructs.

Following cell differentiation induction, higher myogenic gene expression was noted at day 14 of culture in C2C12 encapsulated in mdECM compared to collagen constructs. They also found that mdECM preserved major ECM components such as laminin, collagen, and glycosaminoglycans (GAGs), along with agrin, enabling acetylcholine receptor (AChR) pre-patterning. In another study, Lee and colleagues used an integrated tissue-organ printer (ITOP) system to engineer a skeletal muscle construct (10 × 7 × 3 mm3) by bioprinting human muscle progenitor cells (hMCs) isolated from biopsies in a fibrin bioink associated with gelatin and PCL deposition. They transplanted the construct into a rat tibialis anterior (TA) muscle defect model, noting enhanced tetanic force and TA muscle weight at four and eight weeks post-implantation. Furthermore, 82% of muscle force was restored eight weeks post-surgery compared to normal TA muscle. The construct integrated well with vascular and neural networks, as confirmed by immunostaining showing new blood vessels and mature neuromuscular junctions (NMJ). In another study, Kaplan and colleagues printed 40%

(w/v) silk fibroin T-shaped cantilevers in a 12-well plate and used them as anchors to culture primary human myoblast-laden silk (1%)-collagen type I (3 mg mL−1)-Matrigel (8%) hydrogel. After three days of culture, the growth medium was replaced with differentiation medium. After 21 days, the formed myotubes were characterized. Concurrently, human-induced neural stem cells (hiNSCs) differentiated into motor neuron-like cells

- **3D bioprinting in neural tissues**
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3D Bioprinted Models of Brain Development

The brain, as the central nervous system (CNS), possesses a highly intricate cellular and structural framework. It is shielded by a triple-layered meninges structure and is extensively vascularized, ensuring adequate oxygen and nutrient supply to support its high metabolic needs. The brain consists of distinct functional and anatomical domains with unique developmental origins. Consequently, creating 3D models of these components poses significant challenges. Genetic factors and environmental toxins contribute to various CNS disorders, impacting brain function subtly or severely. Understanding the complex cytoarchitecture of the mammalian brain and modeling neurological diseases at the tissue level remains a substantial hurdle.

Incorporation of Neural Cells in 3D Bioprinted Scaffolds

Numerous studies in neural tissue bioprinting have focused on directly embedding neural cells into hydrogel bioinks to form 3D printed cellular scaffolds. These efforts have established optimal printing parameters, bioink considerations, and functionalization strategies that support cell differentiation, proliferation, and mimicry of in vivo characteristics. For instance, Gu et al. employed 3D bioprinting to create lattice-shaped minitissues using human neural stem cells encapsulated in hydrogels such as alginate, agarose, and carboxymethyl-chitosan. Post-printing, stem cells differentiated into GABAergic neurons and glial cells, demonstrating the platform's utility for modeling human neural cell development and neural network formation. Other studies have utilized fibrin-based bioinks to encapsulate human iPSC-derived neural aggregates, showing neurite extension and early neuronal marker expression over extended culture periods. Techniques like functionalizing bioinks with neurotransmitters have enhanced neural stem cell differentiation and network formation, underscoring the importance of mimicking native biochemical environments in bioprinted scaffolds.

Modeling the Cerebral Cortex

A defining feature of the mammalian brain is the laminated neuronal layers of the cerebral cortex, comprising six distinct layers each with specialized cell types crucial for information processing. Cortical development initiates with neurons migrating from the ventricular zone into the preplate structure, which subsequently divides into the marginal zone, cortical plate, and subplate. Layers II–VI of the cortex form through an "inside-out" process where deeper layers develop before superficial layers, driven by polarity cues, neural progenitor characteristics, and signals from surrounding cells and morphogens.

While traditional 2D cultures lack cortical lamination, recent advancements in 3D brain organoid cultures have shown partial success in replicating cortical layering using aggregated hiPSCs. However, these organoids typically lack controlled spatial organization of cortical layers. In contrast, 3D bioprinting enables precise layer formation and spatial control by prepositioning cells within printed constructs. For example, Lozano et al. demonstrated the layer-by-layer bioprinting of a six-layered cortical model using RGD-modified gellan gum bioink, allowing distinct layer visualization without compromising overall structure integrity. Other approaches, such as lipid-bilayer-supported droplet bioprinting, have facilitated the creation of 3D architectures with detailed

cellular organization, promoting high cell viability, differentiation into cortical cell types, and functional neural network formation. Incorporating astrocytes into these scaffolds revealed their role in axon bundling and their interactions with neurons, highlighting bioprinting's potential to replicate intricate brain architecture and study cell interactions crucial for cortical development.

3D Bioprinted Models of Spinal Cord

The spinal cord serves as a crucial relay between the peripheral nervous system (PNS) and brain, and its injury can have severe consequences. However, accurately modeling spinal cord biology using human cells is challenging due to its unique spatial architecture. One notable feature is the arrangement of gray matter (dorsal lateral and ventral "horns") surrounded by ascending and descending white matter axon tracts that carry sensory and motor signals. Sensory information is processed by neurons in the dorsal lateral horns, while motor neurons in the ventral horns send signals through ventral roots to initiate muscle movement. Both dorsal and ventral roots combine to form spinal nerves, part of the PNS. Understanding the intricate spatial organization of the spinal cord is crucial for insights into neuronal circuit formation and cellular cues that drive functional organization.

This section focuses on the application of 3D bioprinting to mimic spinal cord function and aid in repair. The spinal cord features distinct functional regions and diverse cell populations influenced by precise spatial and temporal morphogen gradients. These gradients induce specific gene expression patterns, regulate neural progenitor cell proliferation, and guide differentiation. Key morphogens involved include BMP, Wnt, Sonic Hedgehog (SHH), FGFs, and retinoic acid (RA). They establish molecular gradients that dictate the expression of transcription factors (TFs) along dorsal-ventral and anterior-posterior axes, thereby creating defined neural progenitor domains that differentiate into various neuronal subtypes (e.g., motor, sensory, interneurons).

Many research groups have integrated growth factors and signaling cues into bioprinted scaffolds to observe their effects on neural cell behavior. For instance, VEGF was bioprinted into fibrin scaffolds, enhancing murine neural stem cell migration compared to controls. Inkjet printing has been used to create macromolecular gradients within polyacrylamide-based bioinks, influencing neural stem cell differentiation. By incorporating factors like FGF2, CNTF, or fetal bovine serum (FBS), researchers observed varying effects on neural cell proliferation and differentiation. Specific combinations of small molecules have also been employed to induce differentiation of induced pluripotent stem cells (iPSCs) into motor neurons using microfluidic bioprinting techniques.

Spatial organization of cells is crucial for tissue function, particularly in the spinal cord, which houses multiple cell types with distinct architectural arrangements. Techniques such as extrusion-based bioprinting have enabled the precise positioning of spinal neuronal progenitor cells within neurocompatible scaffolds. This method allows for the creation of functional networks where spinal neural progenitor cells differentiate and extend axons along bioprinted channels. Co-printing with oligodendrocyte progenitor cells facilitates close physical associations between different cell types, laying the groundwork for further studies on myelination within bioprinted neural tissues.

This research underscores the potential of 3D bioprinting to advance our understanding of spinal cord biology, offering new avenues for studying neural differentiation and modeling the complex cellular diversity within this critical tissue.

Nervous System Repair

Injuries to the nervous system disrupt neural networks and impair information transmission throughout the body, potentially leading to loss of motor and sensory functions. Current therapeutic options face significant challenges, including the lack of survival and regenerative signals, difficulties in replicating the mechanical and chemical environment at the injury site, and inefficiencies in delivering stem cells to damaged tissues. 3D bioprinting offers promising solutions to these challenges in several ways: 1) printed scaffolds can be infused with small molecules or proteins to enhance regeneration, 2) bioink properties can be tailored to mimic specific extracellular microenvironments, and 3) scaffolds can encapsulate and deliver stem cells directly to the injury site in a targeted and sustained manner. Here, we explore recent advancements in applying 3D bioprinting to facilitate regeneration following injuries to peripheral nerves and the spinal cord.

4.1. Peripheral Nerve Regeneration

The peripheral nervous system (PNS), lacking the protective bony structure of the CNS, is susceptible to mechanical injuries, toxins, and pathogens. While peripheral nerve fibers can regenerate after damage, severe injuries often require surgical intervention beyond simple suturing. Current treatments often involve autologous grafts from patients, which present challenges such as tissue rejection and mismatched geometries. Bioprinted scaffolds offer a personalized approach to creating nerve guide conduits that mimic the natural environment and promote regeneration. Advances in bioprinting technology have revolutionized synthetic nerve conduits for both in vivo regeneration and in vitro studies.

Recent studies have demonstrated the application of bioprinting techniques to enhance nerve regeneration. For instance, researchers have developed composite hydrogel scaffolds encapsulating Schwann cells to support axonal growth and survival. These scaffolds, composed of alginate, hyaluronic acid, fibrin, and/or RGD peptides, guide neurite outgrowth from dorsal root ganglion neurons cultured on their surfaces. Other approaches involve bioprinting Schwann cells in alginate-gelatin bioinks, which enhance nerve growth factor (NGF) release and support neuronal organization. Additionally, microfluidic-assisted bioprinting techniques have been used to create multiscale composite scaffolds that promote neurite elongation and improve neuronal organization.

In vivo studies have shown promising results with bioprinted scaffolds for peripheral nerve repair. Gelatin-sodium alginate scaffolds containing Schwann cells and neurotrophic factors were implanted into mouse models of nerve injury, demonstrating minimal inflammatory response and sustained cell viability. Other studies have utilized digital light processing bioprinting to create drug-loaded nerve conduits that support axonal elongation and neurotrophic factor upregulation, essential for nerve regeneration. These efforts highlight bioprinting's potential to replicate the peripheral nerve microenvironment and facilitate regeneration, although further validation through animal studies is needed.

4.2. Spinal Cord Repair

Traumatic injuries to the spinal cord often result in the breakdown of neuronal networks responsible for motor and sensory functions. Due to limited regenerative capabilities, various approaches have explored implantable scaffolds containing cells and biomolecules to induce spinal cord regeneration. Traditional methods have shown some success but struggle to replicate the

complex architectural and cellular organization found in vivo. 3D bioprinting offers precise spatial control to design scaffolds that match the mechanical and chemical properties of native spinal tissue.

Researchers have employed microscale continuous projection printing to create scaffolds for spinal cord injury repair, encapsulating neural progenitor cells. These scaffolds, implanted into spinal cord transection models, facilitated host axon infiltration and myelination by Schwann cells, resulting in improved functional recovery and neuronal connectivity. Other studies have utilized modified extrusion bioprinting techniques to create collagen-based scaffolds containing growth factors like basic FGF, demonstrating enhanced mechanical strength comparable to native spinal cord tissue. These scaffolds have shown promise in improving locomotive function and electrophysiological properties post-implantation.

Further advancements include the use of neural stem cells within collagen-silk fibroin scaffolds to reduce glial scarring and promote axonal regeneration. These bioprinted constructs have exhibited superior mechanical properties and functional outcomes compared to traditional scaffolding methods. By mimicking the native tissue architecture and incorporating biologically active components, 3D bioprinting holds potential to significantly advance spinal cord repair therapies.

In conclusion, 3D bioprinting offers a versatile platform to address challenges in nervous system repair by providing customizable scaffolds that mimic natural environments and promote tissue regeneration. While ongoing research and animal studies are essential to validate these approaches, bioprinting stands poised as a promising tool to enhance the treatment and rehabilitation of nerve and spinal cord injuries.

3D Printing of Anatomical Tissue Scaffolds and Nerve Conduits for CNS and PNS Regeneration

In the realm of spinal cord and nerve repair, there are various FDA-approved nerve guide conduits available on the market. These conduits are typically collagen-based, composed of synthetic biomaterials, or derived from allogenic tissue. While beneficial, these conduits often struggle to bridge larger gaps and lack customized mechanical properties or geometries. To overcome these challenges, many research groups utilize 3D printing (distinct from bioprinting) to develop constructs for regeneration and repair. Here, 3D printing refers to additive manufacturing technology without the incorporation of cells, biomolecules, or biomaterials in specific spatial arrangements. It shares similarities with 3D bioprinting modalities such as stereolithography, extrusion-based printing, and inkjet printing. Design considerations remain consistent across both processes, emphasizing biocompatibility, biodegradability, and mechanical support to facilitate tissue alignment.

3D printing has successfully produced single- and multilumen PEG nerve conduits and high-content graphene scaffolds. Leveraging scans from native tissue, 3D printing achieves nerve scaffolds that faithfully replicate in vivo geometries. Additionally, macroarchitecture scaffolds from materials like poly(caprolactone) have been tailored to influence spinal cord regeneration. The advantages of 3D printing include higher printing resolution, a wider range of available hydrogel materials, and reduced concerns regarding handling, storage, and shelf-life due to their acellular nature. However, compared to 3D bioprinting, the absence of cells or growth factors within 3D printed scaffolds may limit their regenerative potential.

To advance CNS and PNS repair, it is crucial for the field to harness the high printing resolution and diverse bioink materials available in 3D printing, while also integrating the biomimetic capabilities offered by 3D bioprinting. This hybrid approach holds promise for developing scaffolds that effectively support and facilitate nerve and spinal cord repair processes.

3. 3D Bioprinting of the Cardiovascular System

3D bioprinting encompasses advanced techniques in rapid prototyping and additive manufacturing, crucial for creating functional living constructs. It enables the precise fabrication of complex 3D architectures with high precision, throughput, reproducibility, and repeatability. This technology offers precise control over the placement of cells and bioactive factors to mimic native physiological environments accurately. Applications in cardiovascular tissue engineering require considerations for complex anisotropic structures, perfusion, mechanical adaptability, and electrical signal propagation.

Elements and Strategies of 3D Bioprinting the Cardiovascular System

The successful regeneration of cardiovascular tissues demands the accurate recapitulation of cellular structures and functions. Understanding tissue components, structures, and microenvironments is crucial for designing constructs that integrate various cell types, extracellular matrix compositions, gradients of biological molecules, and native biophysical cues. Proper bioink selection, structural characteristics, mechanical properties, and electrical stimuli are essential for fabricating engineered cardiovascular constructs.

Cell Sources

In cardiovascular bioprinting, selecting appropriate cell sources is critical for effective regenerative therapies. Cell-based strategies such as direct injection, cell sheet engineering, 3D organoids, injectable hydrogels, cardiac patches, and engineered scaffolds have been explored. Essential criteria for cell sources include accessibility, proliferative capacity, differentiation potential, preservation of phenotype and function, pathogen safety, and non-antigenicity. Stem cells, including mesenchymal stem cells (MSCs), cardiac stem cells (CSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), have shown promise for cardiovascular regeneration due to their ability to differentiate into various cardiac cell types.

Mesenchymal Stem Cells (MSCs)

MSCs, sourced from bone marrow, umbilical cord tissue, adipose tissue, and other sources, offer clinical potential due to their availability, accessibility, and immunosuppressive properties. Although they have limited cardiac differentiation capacity, MSCs contribute to therapeutic effects primarily through paracrine mechanisms rather than direct trans-differentiation into cardiomyocytes.

Cardiac Stem Cells (CSCs)

Resident cardiac stem cells within the heart present endogenous regenerative potential, capable of differentiating into myocytes, smooth muscle cells, and endothelial vascular cells. Isolation and amplification of CSCs offer a minimally invasive, autologous approach for myocardial repair.

Embryonic Stem Cells (ESCs)

ESCs, derived from early mammalian embryos, possess pluripotency and can differentiate into all cell types, including functional cardiomyocytes. Despite their regenerative potential, challenges such as tumorigenicity and ethical concerns limit their clinical application.

Induced Pluripotent Stem Cells (iPSCs)

Similar to ESCs, iPSCs offer the advantage of pluripotency and can be derived from adult cells, avoiding ethical issues associated with ESCs. iPSCs can differentiate into cardiomyocytes and other cardiac cell types, although challenges such as low efficiency and tumorigenicity remain hurdles for clinical translation.

Endothelial Cells (ECs), Hematopoietic Stem Cells (HSCs), and Endothelial Progenitor Cells (EPCs)

ECs play critical roles in cardiovascular function, contributing to barrier functions, angiogenesis, and vascular homeostasis. EPCs, derived from bone marrow, support endothelial regeneration and angiogenesis, aiding in cardiovascular repair.

Cardiac Fibroblasts (FBs)

Cardiac FBs are pivotal in cardiac development and physiology, regulating extracellular matrix synthesis and remodeling. They interact with cardiomyocytes through mechanical and electrical coupling, influencing cardiac function and structure.

Coculture
Combining multiple cell types such as MSCs, ECs, FBs, and potentially neuronal cells in coculture systems enhances cardiac tissue formation and functionality. These systems replicate physiological interactions necessary for cardiovascular regeneration and drug screening.

Extracellular Matrix (ECM) Environment

The ECM provides structural support and biochemical cues for cell growth and tissue remodeling. Engineered cardiac constructs must exhibit biocompatibility, biodegradability, appropriate mechanical properties, and biomimetic architecture to facilitate tissue integration and function.

Bioprinting Cardiovascular Tissues for Regeneration

Current research focuses on bioprinting myocardial patches, heart valves, and vascular structures using synthetic or biological materials. Techniques such as Laser-Induced-Forward-Transfer (LIFT) and extrusion printing have been utilized to create functional cardiac constructs capable of improving cardiac function and promoting vascularization in preclinical models.

This overview highlights the potential and challenges of 3D bioprinting in cardiovascular tissue engineering, emphasizing the importance of cell source selection, biomaterials, and tissue architecture in achieving successful tissue regeneration.

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