

REVIEW

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# The preparation methods and types of cell sheets engineering

Danping Hu<sup>1,4†</sup>, Ce Gao<sup>1†</sup>, Jie Li<sup>1†</sup>, Pei Tong<sup>5</sup> and Yi Sun<sup>1,2,3,5\*</sup> 

## Abstract

Cell therapy has emerged as a viable approach for treating damaged organs or tissues, particularly with advancements in stem cell research and regenerative medicine. The innovative technique of cell sheet engineering offers the potential to create a cell-dense lamellar structure that preserves the extracellular matrix (ECM) secreted by cells, along with the cell-matrix and intercellular junctions formed during in vitro cultivation. In recent years, significant progress has been made in developing cell sheet engineering technology. A variety of novel materials and methods were utilized for enzyme-free cell detachment during the cell sheet formation process. The complexity of cell sheet structures increased to meet advanced usage demands. This review aims to provide an overview of the preparation methods and types of cell sheets, thereby enhancing the understanding of this rapidly evolving technology and offering a fresh perspective on the development and future application of cell sheet engineering.

**Keywords** Cell sheet engineering, Tissue engineering, Polymer materials, Three-dimensional structure, Stem cell and regenerative medicine

## Introduction

Cell therapy, a clinical approach with promising potential for treating previously untreatable diseases through the transplantation of living cells, has a long history spanning hundreds of years and has undergone extensive experimentation [1]. With advancements in stem cell research and regenerative medicine, cell therapy has emerged as a viable treatment option and has progressed to clinical trials [2]. Concurrently, researchers have been diligently exploring optimal cell delivery methods for the successful implementation of cell therapy. Cell suspension injection is a commonly utilized cellular transplantation method

due to its convenience in facilitating tissue regeneration and functional rehabilitation by injecting a single-cell suspension into the affected area [3]. However, this method may not be suitable for repairing osseous and cartilaginous tissues, as these tissues require the reconstruction of a three-dimensional structure with specific strength [4].

Intercellular interactions and the extracellular matrix (ECM) play crucial roles in the repair of tissue structure and restoration of function. Cell morphology, viability, and function may be compromised when cells are in suspension, leading to uncontrolled localization and non-homogeneous distribution, which can result in the risks of ectopic implantation and inefficacy [5]. These challenges have prompted the advancement of scaffold-free tissue engineering for cell delivery [6], with cell sheet engineering emerging as a promising technique to address these issues [7].

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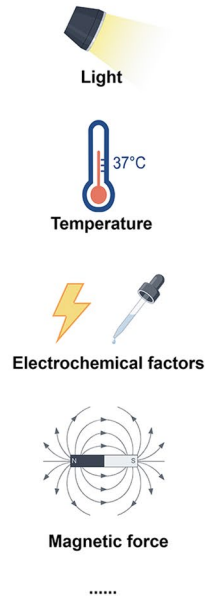


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### Graphical Abstract

## A. cell sheet fabrication

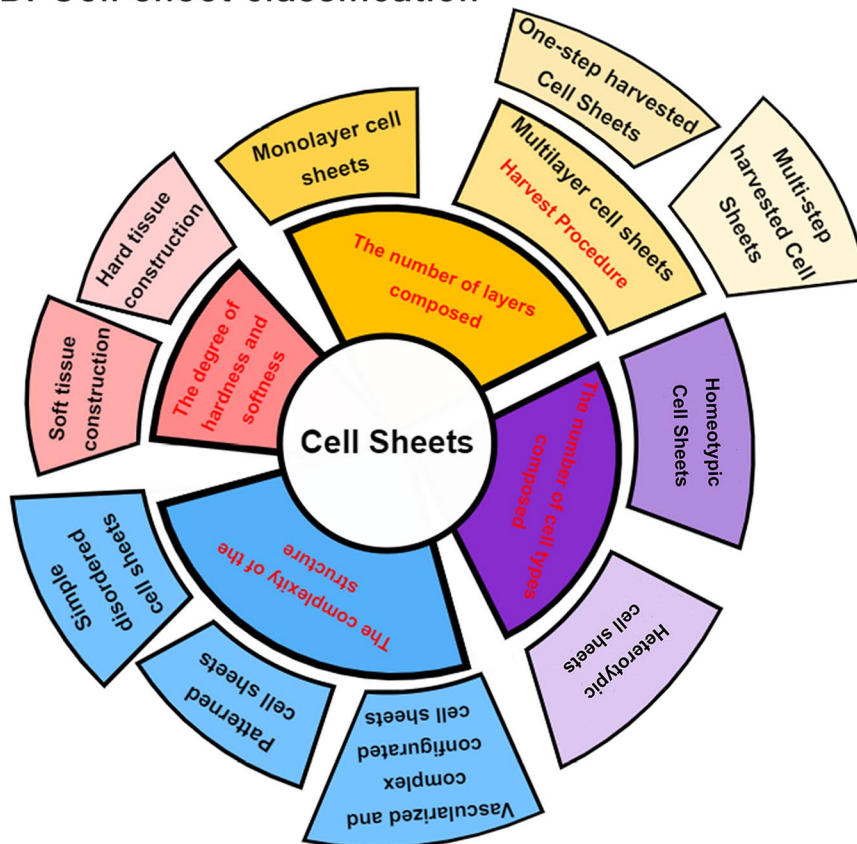
Fabrication methods



Cell sheet products



## B. Cell sheet classification



Cell sheet engineering is a scaffold-free tissue engineering technique that enables the formation of cell-dense tissues without the use of enzymes [8]. The assembly of cells into sheet-like structures facilitates the delivery of a large number of cells to targeted areas. Advancements in material science since the 1990s have spurred rapid development in this field. Temperature-responsive materials, photoresponsive materials, magnetic nanomaterials, and other innovative substances have been utilized to achieve enzyme-free cell detachment, resulting in various methods for cell sheet preparation. Additionally, to meet the needs of applications ranging from cell repopulation to functional recovery, different types of cell sheets have been developed, from simple monolayers to complex three-dimensional structures with multiple layers.

To address a variety of applications such as cell repopulation and functional recovery, various types of cell sheets have been developed, ranging from simple monolayers to complex three-dimensional structures with multiple layers. This study aims to consolidate the fabrication methods and classifications of cell sheets by conducting a comprehensive review of recent relevant literature. By doing so, it seeks to provide an overview of the preparation techniques and types of cell sheets, thereby enhancing understanding of this rapidly evolving technology and offering a novel perspective on its advancement and future applications (Table 1).

### The fabrication methods of cell sheet

The “sheet-like cell assembly” obtained through cell sheet engineering is an integrated structure that deposits ECM, allowing it to connect directly to the transplant site. Furthermore, essential substances for functional tissue regeneration, such as key membrane proteins (e.g., ion channels and growth factor receptors) and intercellular and ECM connections, are preserved and remain intact due to the use of dispase.

The preparation of cell sheets requires a delicate balance between cell attachment and detachment on the culture surface. With advancements in material science, materials with high biocompatibility and controllable cell adhesion properties have been synthesized and applied to cell sheet formation. Stimuli used for cell sheet release include temperature [9], electrochemical factors [10], mechanical force [11], magnetic force [12], and light [13], among others. Currently, the main methods for cell sheet preparation include: thermally-triggered fabrication using thermo-responsive polymeric surfaces; magnetic force-based fabrication employing magnetic nanoparticles (MNPs) and magnetic force; electrochemical polarization methods with electronic control coatings; pH-controlled fabrication; light-induced fabrication using light-responsive polymeric surfaces; ion concentration-related fabrication; and physical scraping

techniques. This summary highlights several strategies for cell sheet fabrication and provides an overview of the technical principles behind each method (Figs. 1, 2, 3, 4 and 5).

### Thermally-triggered cell-sheet fabrication

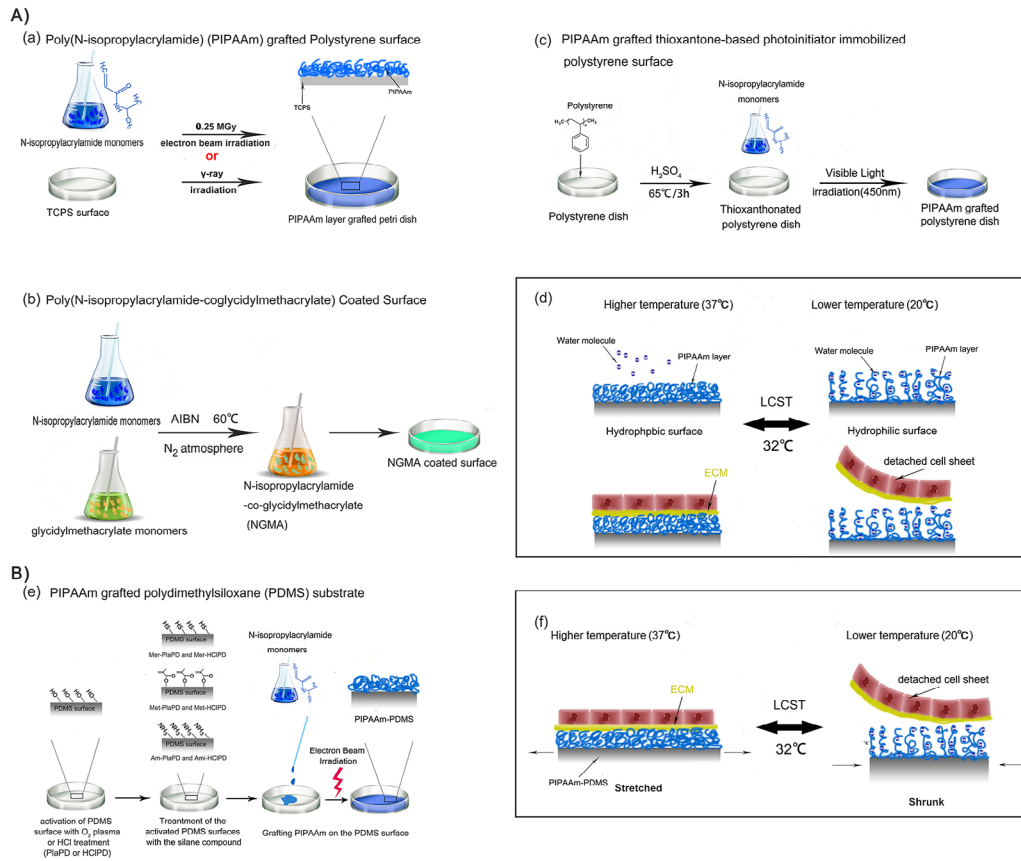
Okano et al. [14] reported their research on preparing a petri dish coated with poly(N-isopropylacrylamide) (PIPAAm) in 1990. They harvested bovine hepatocytes cultured on polystyrene dishes whose surfaces were grafted with PIPAAm by reducing the temperature. They demonstrated the possibility of switching between cell attachment and detachment using thermo-responsive polymer grafted surfaces by altering the culture temperature. In this technique, N-isopropylacrylamide monomers are polymerized simultaneously, and cross-linked polymer chains are uniformly grafted onto polystyrene or amino glass substrates under electron beam (EB) irradiation [15]. The nanometer-thick PIPAAm layer grafted on the petri dish is relatively hydrophobic and thus demonstrates a collapsed, compact conformation under suitable culture conditions at 37°C, facilitating cell attachment and proliferation. As the culture temperature drops to the lower critical solution temperature (LCST, 32°C) or lower, the PIPAAm surface becomes hydrophilic and transforms into a fully expanded chain conformation, resulting in spontaneous cell detachment and enabling the harvest of the “sheet-like cell assembly [16] (Fig. 1A).

Although the PIPAAm surface-based thermo-responsive culture system is the earliest developed and most widely used method for cell sheet fabrication in cell sheet engineering, it faces challenges such as the high cost of EB irradiation or gamma ( $\gamma$ ) ray irradiation equipment [17] and the technical difficulty of nanometer-thick layer surface plating. Moreover, the only commercialized product, ‘Thermo Scientific™ Nunc™ UpCell™ Dishes,’ is expensive. These limitations make it impractical for widespread use in average laboratories, leading to numerous studies focused on improving temperature-induced cell sheet fabrication. In 2014, an optimized, simple method for preparing temperature-responsive cell culture surfaces was reported [18]. Researchers applied PIPAAm monomer dissolved in water onto polystyrene (PS) dishes pretreated with thiosalicylic acid, followed by visible light irradiation at a wavelength of 405 nm using a high-pressure mercury lamp or commercially available light-emitting diode light. PIPAAm was successfully grafted onto the PS dishes with the thioxanethone-based photoinitiator. This method enabled the successful fabrication of cell sheets by lowering the temperature to 20 °C, demonstrating a solvent-free, cost-effective surface modification method with potential for cell sheet fabrication and tissue engineering applications (Fig. 1A). Four years later, Yoshikatsu et al. [19] introduced a method

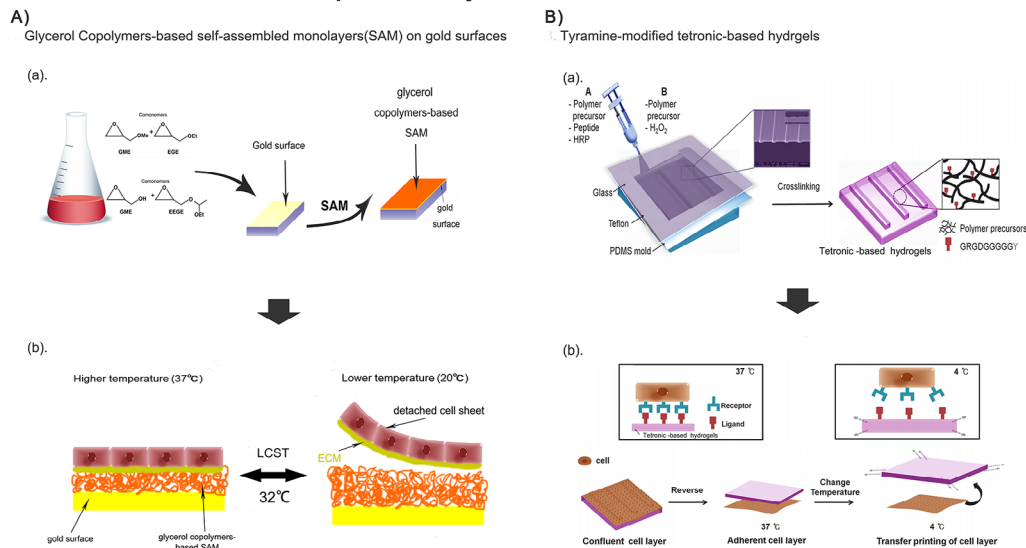
**Table 1** Features of different methods and techniques of cell sheet fabrication

Types	Fabrication techniques	Advantages	Disadvantages	Limitations
Thermally-triggered cell sheet [14–20]	<ol style="list-style-type: none"> <li>1. Coating petri dishes with poly(N-isopropylacrylamide) (PIPAAm).</li> <li>2. Cells attach at 37°C and detach when temperature is lowered to 32°C or below.</li> <li>3. Electron beam (EB) or gamma ray irradiation used for PIPAAm grafting.</li> </ol>	<ol style="list-style-type: none"> <li>1. Enables reversible cell attachment and detachment by changing temperature.</li> <li>2. Supports attachment and multiplication of cells.</li> <li>3. Easy cell sheet harvest.</li> </ol>	<ol style="list-style-type: none"> <li>1. High cost of EB or gamma ray irradiation equipment.</li> <li>2. Technical difficulty in creating nanometer-thick PIPAAm layers.</li> <li>3. Long time for cell sheet harvest (over 30 minutes).</li> </ol>	<ol style="list-style-type: none"> <li>1. High equipment cost limits average laboratory usage.</li> <li>2. Prolonged low temperature may deteriorate cellular vitality and function.</li> <li>3. Limited to specific cell types and conditions.</li> </ol>
Magnetic force-based cell sheet [31–39]	<ol style="list-style-type: none"> <li>1. using of iron oxide nanoparticles (MNPs) into target cells.</li> <li>2. Magnets placed on the reverse side of the culture plates to attract and assemble cells.</li> <li>3. Cell sheets harvested by applying and removing magnetic force.</li> </ol>	<ol style="list-style-type: none"> <li>1. Can construct multilayer cell sheets.</li> <li>2. Suitable for cell attachment in generally non-adhesive conditions.</li> <li>3. Proven safe and widely applicable in biological and medical contexts.</li> </ol>	<ol style="list-style-type: none"> <li>1. Nutrient transport and cell activity in deep tissue cannot be guaranteed.</li> <li>2. Long-term interaction of MNPs with cells may have adverse effects on cell viability, phenotype, and function.</li> </ol>	<ol style="list-style-type: none"> <li>1. Requires internalization of MNPs, potentially affecting cell viability and function.</li> <li>2. Complex magnet configuration affects harvesting efficiency.</li> <li>3. Limited in pre-vascularization and mimicking natural environment in thick tissue construction.</li> </ol>
Polyelectrolyte-based Electro-chemical controlling cell sheet [44–51]	<ol style="list-style-type: none"> <li>1. Using electronically addressable and responsive surfaces involving polyelectrolyte mono and multilayered films.</li> <li>2. Applying positive potential to induce cell sheet detachment.</li> <li>3. Using biocompatible polymers like RGD-modified PLL-g-PEG/PEG.</li> </ol>	<ol style="list-style-type: none"> <li>1. Enables precise control over cell attachment and detachment.</li> <li>2. Fast and efficient cell sheet formation.</li> <li>3. Potential for reusability of the substrate.</li> </ol>	<ol style="list-style-type: none"> <li>1. Requires specialized equipment and precise control of electrochemical conditions.</li> <li>2. Potential pH changes might affect cell viability.</li> <li>3. Limited to specific cell types and conditions.</li> </ol>	<ol style="list-style-type: none"> <li>1. Complex process involving multiple steps of dipping and coating.</li> <li>2. Stability and reproducibility of polyelectrolyte layers can be challenging.</li> <li>3. Cell viability may be affected by electrochemical conditions.</li> </ol>
Light-induced cell sheet [54–62]	<ol style="list-style-type: none"> <li>1. Use of Titanium dioxide (TiO<sub>2</sub>) nanodot film coated on a quartz substrate.</li> <li>2. UV illumination (below 380 nm) induces wettability transition to hydrophilic, reducing cell adhesion.</li> <li>3. Cells are released as a sheet within 5 minutes of UV exposure.</li> </ol>	<ol style="list-style-type: none"> <li>1. Rapid and efficient cell sheet detachment.</li> <li>2. Non-invasive and biocompatible method.</li> <li>3. Compatible with various cell types.</li> </ol>	<ol style="list-style-type: none"> <li>1. Requires precise control of UV light exposure.</li> <li>2. Potential UV damage to cells if not properly controlled.</li> <li>3. Equipment for specific light wavelengths needed.</li> </ol>	<ol style="list-style-type: none"> <li>1. Limited by the availability and cost of light source equipment.</li> <li>2. Potential long-term effects of light exposure on cells are not well-studied.</li> </ol>
Ions concentration-related cell sheet [65, 66]	<ol style="list-style-type: none"> <li>1. Poly(divinylbenzene-co-4-vinylpyridine) [p(DVB-co-4VP), or pDV], a copolymer, prepared using initiated chemical vapor deposition (ICVD).</li> <li>2. Adjusting the input flow ratio of DVB to 4VP for precise control of surface energy.</li> <li>3. Modifying cell-substrate interactions by soaking cell cultures in Ca<sup>2+</sup>-free and Mg<sup>2+</sup>-free D-PBS solutions.</li> </ol>	<ol style="list-style-type: none"> <li>1. Rapid cell sheet detachment process in approximately 100 seconds, expected to be non-cytotoxic.</li> <li>2. Stabilizes cell-cell adhesion proteins, aiding in cell sheet formation.</li> </ol>	<ol style="list-style-type: none"> <li>1. Requires precise control over chemical vapor deposition methods.</li> <li>2. Dependency on specific ion concentrations like Ca<sup>2+</sup> and Mg<sup>2+</sup> for cell sheet formation.</li> <li>3. Specific equipment and material requirements.</li> </ol>	<ol style="list-style-type: none"> <li>1. Limited by specific experimental conditions and equipment availability.</li> <li>2. Requires specific requirements for cell culture substrates and solutions.</li> </ol>
Mechanical methods for cell sheet [11, 67, 68]	<ol style="list-style-type: none"> <li>1. Utilizes tools such as cell scrapers, pipette tips, or bent syringe needles for rapid and efficient cell sheet formation.</li> </ol>	<ol style="list-style-type: none"> <li>1. Rapid and efficient formation of intact cell sheets.</li> <li>2. Applicable to various cell types.</li> <li>3. No special equipment or materials required.</li> </ol>	<ol style="list-style-type: none"> <li>1. Dependency on specific cell types and culture conditions.</li> <li>2. Requires a certain level of technical skill.</li> </ol>	<ol style="list-style-type: none"> <li>1. Some cell types may not be suitable for mechanical scraping methods.</li> <li>2. Ensuring cell integrity and health during preparation processes.</li> </ol>

## A. PIPAAm-based Thermally-triggered Cell-sheet Fabrication



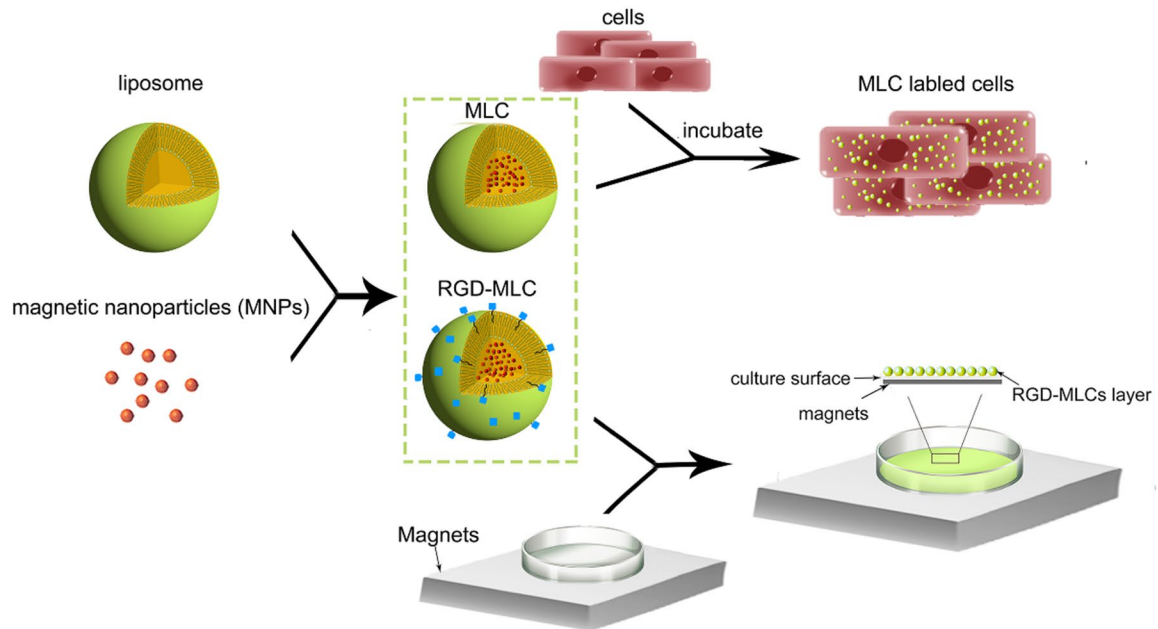
## B. Other Thermoresponsive Polymers-based Cell-sheet Fabrication



**Fig. 1** Schematic diagram of PIPAAm-based thermally triggered cell sheet fabrication and Schematic diagram of other thermally triggered cell sheet fabrication methods. **A)** and **B)** in Panel A illustrate the PIPAAm-based fabrication process: (a), (b), (c), and (e) represent four methods for preparing PIPAAm-based thermosensitive petri dishes; (d) and (f) depict the processes of cell adhesion and cell sheet detachment. **A)** in panel B: (a) shows the method for preparing glycerol copolymer-based thermosensitive surfaces, and (b) illustrates the process of cell adhesion and cell sheet detachment. **B)** in Panel B: (a) shows the method for preparing tyramine-modified Tetronic thermosensitive hydrogels, and (b) depicts the process of cell adhesion and cell sheet detachment. **B)** in Panel B is reproduced from reference [21] with permission

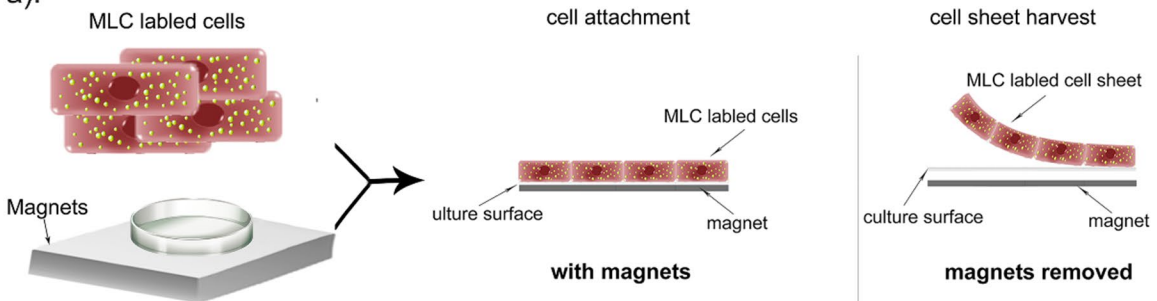
# Magnetic Force-based Cell-sheet Fabrication

## A. The preparation of magnetic force-based cell-sheet fabrication

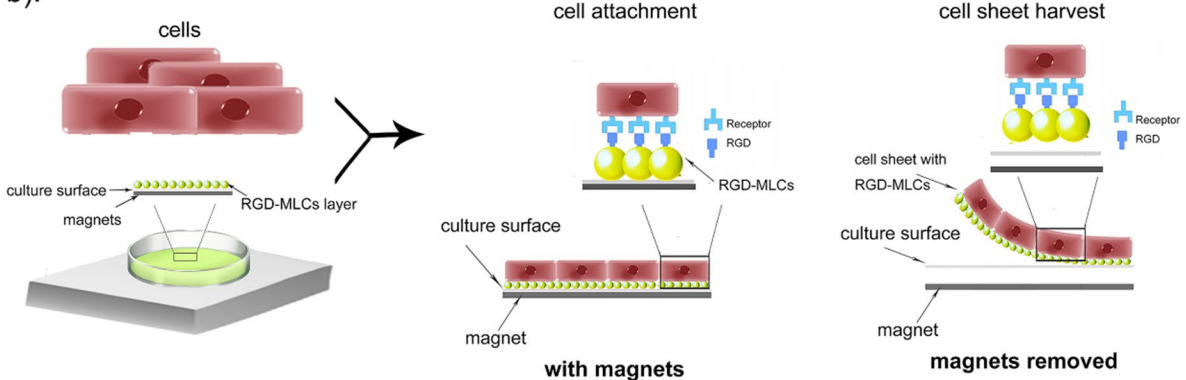


## B. The magnetic force-based cell attachment and harvest

a).



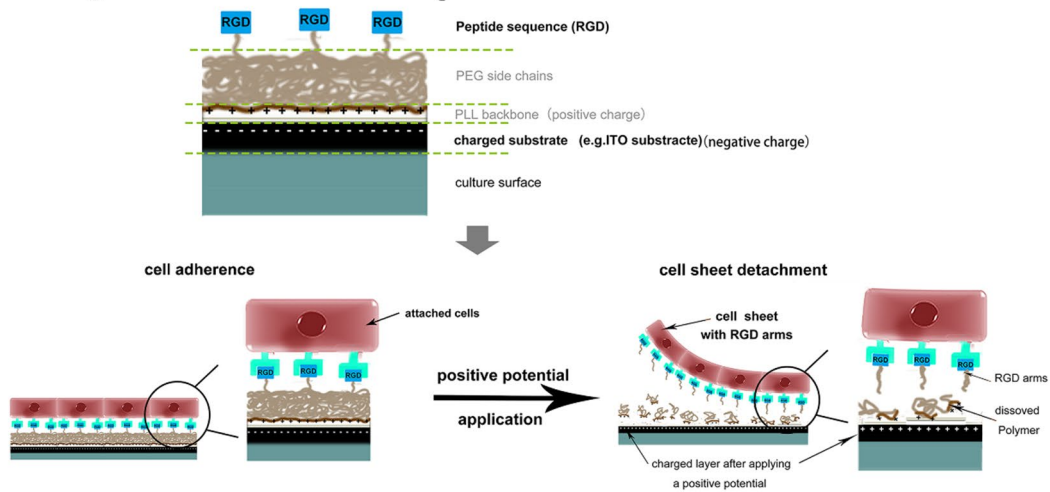
b).



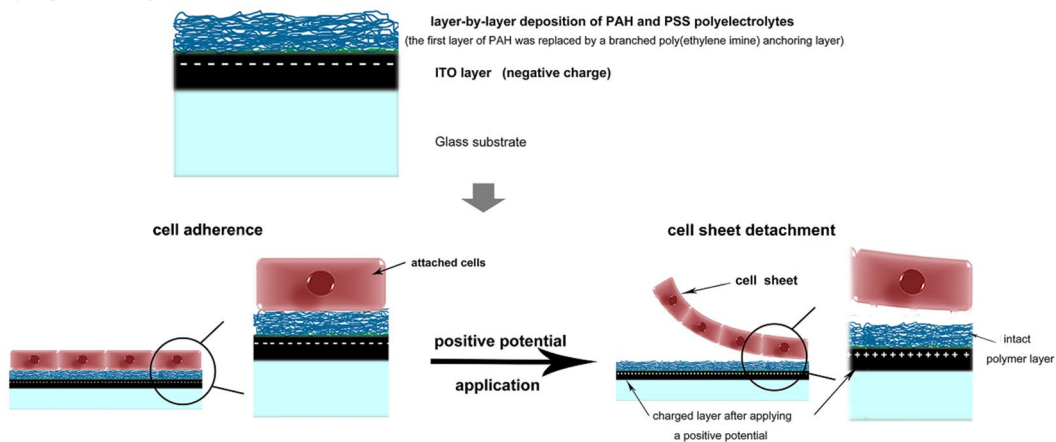
**Fig. 2** Schematic diagram of magnetic force-based cell sheet fabrication. Cells attach to a specialized culture surface with the aid of magnetic nanoparticles (MNPs) and magnets. Once the magnets are removed, the cells can be harvested as a sheet

# Electrochemical Controlling Cell-sheet Fabrication

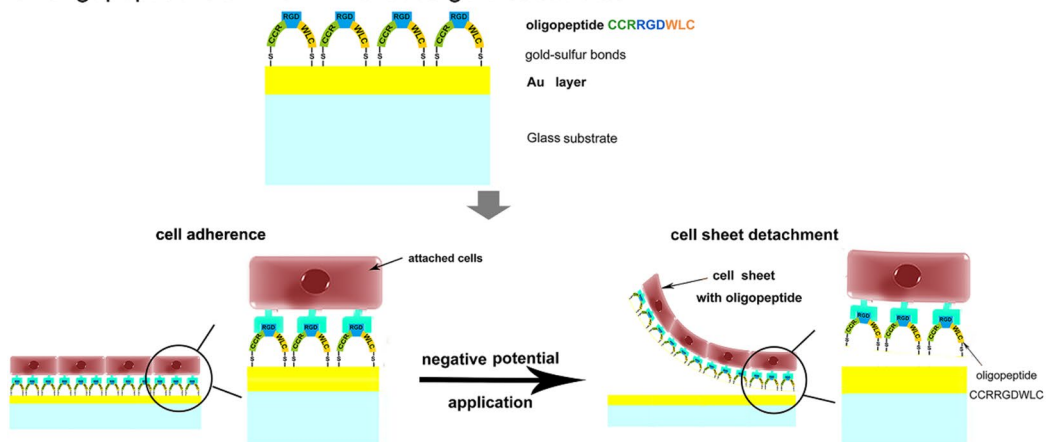
## A. PLL-g-PEG/PEGRGD coated charged substrate



## B. Cationic poly(allylamine hydrochloride)(PAH) and anionic poly(styrene sulfonate)(PSS) polyelectrolytes on ITO substrate

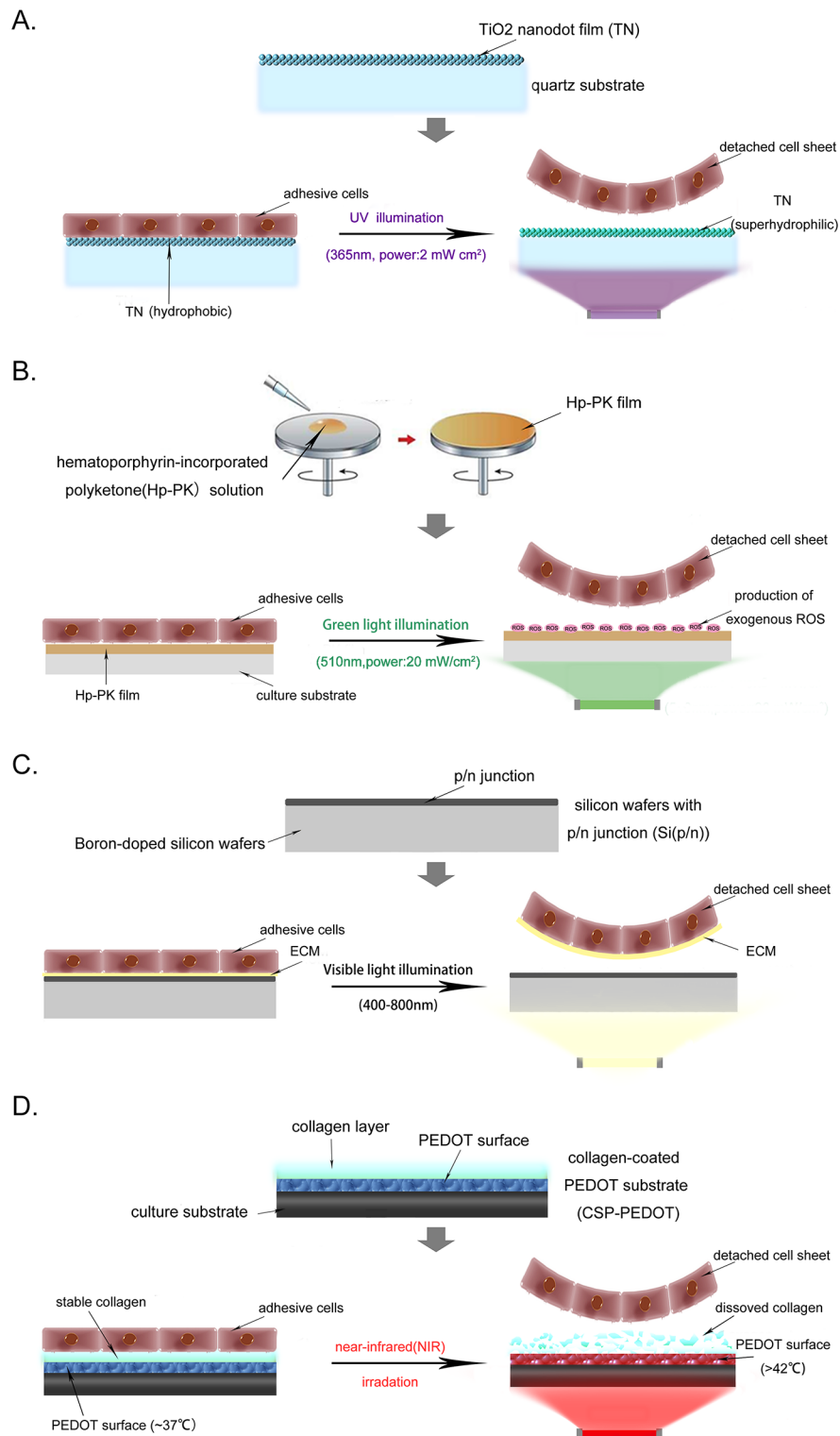


## C. Oligopeptide CCRRGDWLC-based gold substrates



**Fig. 3** Schematic diagram of electrochemical control in cell sheet fabrication. **A.** A biocompatible polymer, RGD-modified poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG/PEGRGD), coated charged substrate was used for cell sheet fabrication. **B.** Multilayered cationic poly(allylamine hydrochloride) (PAH) and anionic poly(styrene sulfonate) (PSS) polyelectrolytes coated charged substrate was used for cell sheet fabrication. **C.** Oligopeptide CCRRGDWLC was designed and used for cell sheet fabrication. Cells can be harvested as a sheet after applying a potential

## Light-induced Cell-sheet Fabrication

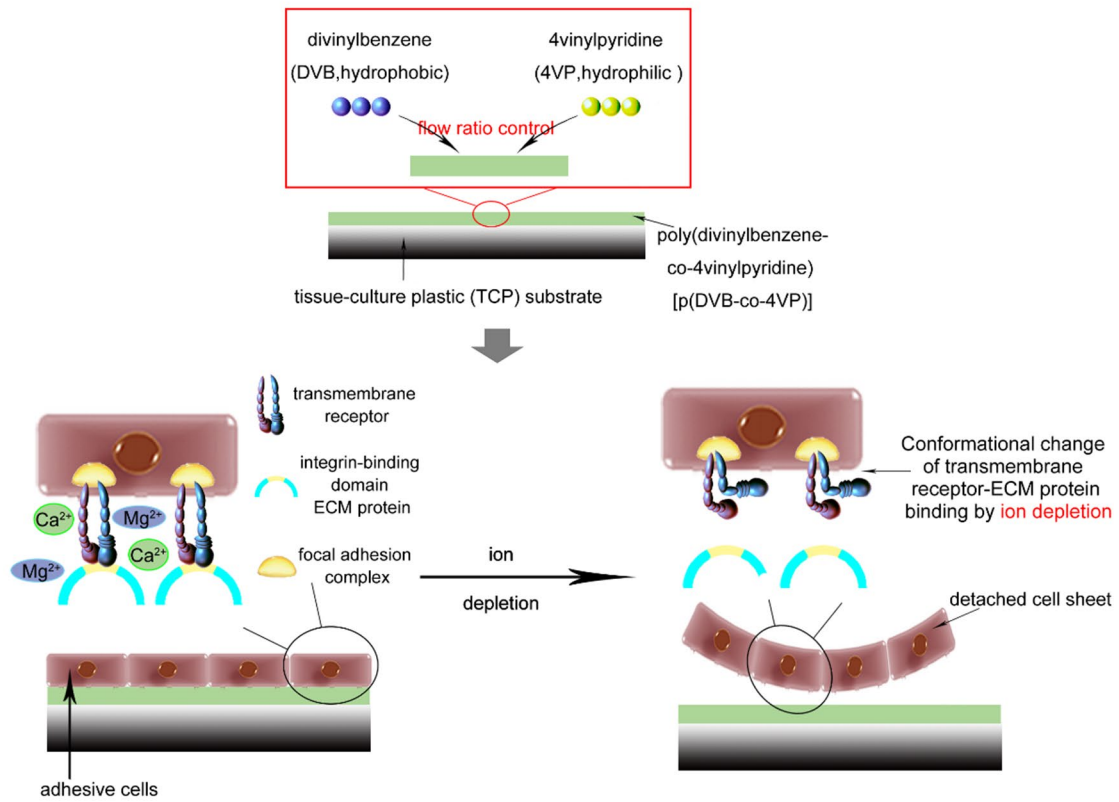


**Fig. 4** Schematic diagrams of light-induced cell sheet fabrication. **A.** UV illumination + TiO<sub>2</sub> nanodot film (TN); **B.** Green light (510 nm) illumination + hematoporphyrin-incorporated polyketone (Hp-PK) film; **C.** Visible light (400–800 nm) illumination + silicon wafers with a p/n junction [Si(p/n)]; **D.** NIR light (808 nm) irradiation + poly(3,4-ethylenedioxythiophene) (PEDOT) surface. Panel B is reproduced from reference [62] with permission

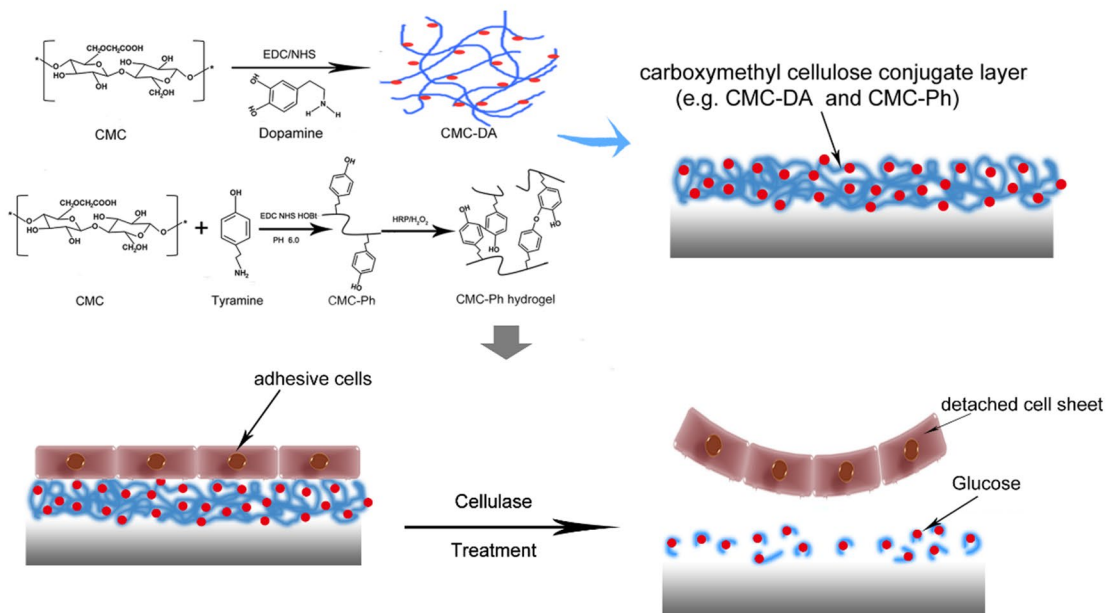


## Other Cell-sheet Fabrication Methods

### A. Ions concentration-related cell sheet fabrication



### B. Cellulose conjugate based cell sheet fabrication



**Fig. 5** Schematic diagrams of alternative methods for cell sheet fabrication. **A.** Cell sheet release through the depletion of ions crucial for cell-substrate adhesion. **B.** Cellulase treatment facilitates the detachment of cell sheets from cellulose conjugate-coated surfaces

to control cell adhesion and detachment through dual stimulation of temperature and mechanical stress. They developed stretchable temperature-responsive cell culture surfaces composed of poly(*N*-isopropylacrylamide) (PIPAAm) gel-grafted polydimethylsiloxane (PIPAAm-PDMS). This surface exhibited a reversible hydrophilic/hydrophobic change due to the phase transition of PIPAAm chains as the temperature dynamically changed by crossing the LCST. On the other hand, the thickness of the PIPAAm layer affects the adhesion and detachment properties of the cells [15]. In thicker PIPAAm layers, the outermost PIPAAm chains remain hydrated, preventing cell adhesion. Conversely, in thinner PIPAAm layers, the polymer chains near the base substrate can become well dehydrated, but they often fail to show significant temperature-induced transitions, leading to inadequate cell detachment [20]. When PIPAAm-PDMS was subjected to uniaxial stretching, the grafted nanoscale-thick surface became thinner, deformed, and modulated to a more hydrophobic phase, resulting in increased cell adhesion compared to unstretched PIPAAm-PDMS at 37 °C. Consequently, dual stimulation of lowering the temperature and shrinking the stretched PIPAAm-PDMS accelerated cell sheet detachment and harvesting more effectively than single stimulation. Additionally, these new culture surfaces can integrate various stimuli and adjust surface properties to accommodate different cell adhesiveness, meeting the requirements for fabricating various cell sheets (Fig. 1A).

So far, PIPAAm surface-based dishes have become a powerful technology in cell sheet-based tissue engineering. These dishes have been applied to various types of cells, including corneal epithelial cells, retinal pigment epithelial cells (RPE), and oral mucosal epithelial cells (OMECS), among others. However, harvesting and transferring cell sheets fabricated with this material often takes more than 30 min, and prolonged exposure to lower temperatures may potentially deteriorate cellular vitality and affect cellular function [22]. In addition to PIPAAm, several other thermoresponsive materials have been synthesized and developed for relatively nondestructive cell sheet fabrication. These include PNIPAAm, glycidyl methacrylate (GMA)-based thermoresponsive copolymers such as *N*-isopropylacrylamide-co-glycidyl methacrylate (NGMA) [23], and thermoresponsive nanofabricated substratum (TNFS) [24]. Weinhart et al. presented two glycerol copolymer-based linear thermoresponsive surfaces [25], which exhibited phase transitions after forming self-assembled monolayers (SAM) on gold surfaces and supported reversible cell adhesion and detachment within the physiological range [26]. These switchable, biocompatible copolymer SAM surfaces sustain protein adsorption, leading to cell adhesion and spreading under standard cell culture conditions at

37 °C. Conversely, decreasing the temperature causes the thermoresponsive polymers to become hydrated and repel proteins. Lowering the temperature to ambient allows adherent cells to detach from the functionalized surfaces within 20 min without the need for detachment agents like trypsin/EDTA. Some researchers have attempted to synthesize cell-interactive thermosensitive hydrogels from tyramine-modified Tetronic [27]. During the crosslinking reaction, bioactive molecules can be easily incorporated into the hydrogel, which forms rapidly using HRP/H<sub>2</sub>O<sub>2</sub> as catalysts [28]. The cell-interactive properties of these hydrogels can be readily tuned by adding peptides, such as cell adhesive peptide (RGD), or proteins, such as angiogenic growth factor (bFGF) [29]. Under standard culture conditions at 37 °C, cells can attach to Tetronic-based thermosensitive hydrogels, which are conjugated with bioactive molecules and cultured to form a confluent cell assembly. When the temperature is lowered to 4 °C, the hydrogels expand, disrupting the binding between integrin receptors on the cell membrane and the peptide ligands presented by the hydrogel. Consequently, the cell layer can be easily harvested and transferred to any substrate due to the intact cell-cell junctions [30] (Fig. 1B).

In summary, thermosensitive materials used for cell sheet fabrication must possess excellent biocompatibility and temperature response characteristics, support the formation of cellular assemblies, and allow phase transitions under biologically non-toxic conditions.

#### **Magnetic force-based cell-sheet fabrication**

Since the successful preparation of cell sheets on PIPAAm surface-based culture dishes, the field has rapidly developed, with several new thermosensitive materials synthesized to optimize cell sheet fabrication. However, this method often requires a long time to harvest cell sheets and struggles with the assembly and layering of heterotypic cells, limiting its effectiveness for organizing complex tissues such as tubular constructs. The detailed and precise construction of cellular structures is also challenging to achieve. In response to these limitations, magnetic force-based tissue fabrication methods have emerged. In this approach, MNPs, primarily iron oxide nanoparticles, are incorporated into target cells to enable their attraction by a magnet placed beneath the culture plates. Magnets positioned on the underside of the culture plates provide the magnetic force necessary for this process. Cells can then be assembled and harvested as a sheet through the application and removal of magnetic force (Fig. 2).

Ito et al. first presented their magnetic force-based cell sheet fabrication method in 2004 [31]. This technique utilized magnetite cationic liposomes (MCLs) containing magnetic nanoparticles (MNPs) that could label

biomolecules or living cells, demonstrating sufficient safety for widespread application in biological and medical contexts [36]. Specifically, they prepared MCLs using colloidal magnetite ( $\text{Fe}_3\text{O}_4$ ; average particle size, 10 nm) and a lipid mixture of N-( $\alpha$ -trimethylammonioacetyl)-didodecyl-D-glutamate chloride. They then magnetically labeled and co-cultured human aortic endothelial cells (HAECs), pre-cultured primary rat hepatocytes [31], and human keratinocytes in Petri dishes. With the magnetic force provided by a magnet placed on the underside of the culture plates, cells were able to adhere under conditions where attachment would generally be difficult [31]. Multilayered keratinocyte sheets could be detached from the bottom of the plates after removing the magnet and then harvested using the magnet [32]. Following this, they extended the technique to create a multilayered heterotypic cell sheet containing HepG2 and mouse fibroblasts NIH3T3 cells in 2007 [33].

Magnetic force-based cell sheet fabrication enables the construction of multilayered structures; however, it does not guarantee adequate nutrient transport and cellular activity in deeper tissues [34]. Ana et al. [35] addressed this limitation by proposing a stratified magnetic-responsive heterotypic cell sheet construction, which involved internalizing iron oxide nanoparticles within cells. They successfully constructed thick bone-like structures composed of heterotypic cell sheets, demonstrating effective osteogenesis due to the synergistic effects of human umbilical vein endothelial cells (HUVECs) and adipose-derived stromal cells (ASCs). The increased activity of Alkaline phosphatase (ALP), matrix mineralization, and elevated levels of osteopontin and osteocalcin further supported the success of the osteogenesis [35]. The inclusion of HUVECs facilitated the pre-vascularization of the entire cell assembly, leading to the formation of thicker tissue that more closely resembles the body's natural environment.

MNPs are critical in magnetic force-based cell sheet fabrication, involving the internalization of MNPs during cell magnetization labeling pretreatment. However, the direct and long-term interaction of MNPs with cells remains a significant concern due to potential adverse effects on cell viability, phenotype, and function [37]. The biocompatibility and potential cytotoxicity of MNPs are therefore crucial considerations [38]. To evaluate the biocompatibility of MNPs, Tomitaka et al. [39] characterized uncoated and oleic-acid-coated  $\text{Fe}_3\text{O}_4$  and  $\text{NiFe}_2\text{O}_4$  MNPs and assessed their cytotoxicity using human cervical carcinoma cells (HeLa). They found that  $\text{NiFe}_2\text{O}_4$  nanoparticles significantly reduced the number of HeLa cells in a dose-dependent manner, while  $\text{Fe}_3\text{O}_4$  MNPs did not exhibit a significant dose-dependent decrease in HeLa cell numbers. This result aligns with findings indicating low cytotoxicity for  $\text{Fe}_3\text{O}_4$  nanoparticles coated

with biocompatible substances [40, 41]. Although iron oxide nanoparticles have been widely used in tissue engineering due to their relative safety, the long-term effects of these exogenous particles on cell phenotype, viability, proliferation, and function remain uncertain. In response, magneto ferritin, a biological MNP, was introduced in 2014 [42]. This MNP demonstrated stronger magnetic capacity while being safer and causing no adverse cellular effects up to 7 days after incorporation into 3-D cellular spheroids. This provides confidence that magneto ferritin could be a viable alternative to iron oxide MNPs, potentially mitigating the adverse effects associated with metallic MNPs [42].

Dzamukova et al. [43] described a straightforward approach for cell surface engineering using cationic polyelectrolyte-stabilized MNPs. They reported the fabrication of viable layered planar cell sheets and 3-D multicellular spheroids. The MNPs used in this method rendered cells magnetically responsive by being deposited directly onto cellular membranes, creating mesoporous semi-permeable layers. Since the cationic polyelectrolyte-stabilized MNPs did not need to penetrate the cytoplasm, the deposition process was completed under cell-friendly conditions within a few minutes. Consequently, this method did not compromise cell membrane integrity, inhibit enzyme activity, disrupt adhesion or cytoskeleton formation, nor induce cell apoptosis [43].

#### **Polyelectrolyte-based electrochemical controlling cell sheet fabrication**

Electrochemical control was another effective method for constructing cell sheets (Fig. 3). Gentil et al. [47] proposed a cell sheet engineering platform using electronically addressable and responsive surfaces, involving polyelectrolyte mono- and multilayered films. Rat calvarial osteoblast (RCO) sheets, NIH3T3 sheets, and human periodontal ligament (PDL) cell sheets were formed on polyelectrolyte-coated electronic control surfaces after application of a positive potential. This method employed biocompatible polymer coatings, specifically RGD-modified poly(L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG/PEGRGD), to attract cells. The polycation promoted specific interactions between cells and the surface through the  $\alpha/\beta$ -integrin-binding sequence Arg-Gly-Asp (RGD) [44], while the unfunctionalized PEG arms prevented non-specific protein adsorption [45]. Monolayer polyelectrolyte coatings were obtained by simply dipping a charged substrate (e.g., ITO) into a dilute solution of polycationic polymers, which spontaneously adsorbed onto the conducting substrate with the opposite charge (indium tin oxide, ITO) via electrostatic interaction [46]. Multilayered polyelectrolyte coatings were applied layer by layer by alternating dips of the charged surface into

dilute solutions of a polycation and a polyanion [47]. Since polyelectrolyte monolayer and multilayer films can be desorbed from the conductive substrate through electrochemical polarization [48], cell sheet detachment was achieved by applying a positive potential to weaken the interactions between the polyelectrolyte and the conductive substrate. At the same time, the electrolysis of water during the electrochemical polarization process can alter the local pH, potentially leading to the dissolution of the polyelectrolyte surface and facilitating the separation of the cell sheet [47]. The PLL-g-PEG/PEG polymer, a weak polyelectrolyte, forms relatively soft and highly hydrated coatings that exhibit poor adhesion to certain types of cells, even after functionalization with bioligands such as fibronectin or RGD (Arg-Gly-Asp) [49] (Fig. 3). Further research by Gentil et al. [50] introduced a novel polyelectrolyte-based platform for the rapid formation of viable human placenta-derived mesenchymal stem cell (PD-MSC) sheets. Coatings achieved through layer-by-layer deposition of cationic poly(allylamine hydrochloride) (PAH) and anionic poly(styrene sulfonate) (PSS) polyelectrolytes on ITO substrates [51] allowed for cell sheet detachment by significantly reducing the stability of the adsorbed protein layers to which cells adhere, through changes in the protein microenvironment, such as decreased pH [52]. When the pH of the culture media was reduced to 4.0, continuous and intact cell sheets grown on PAH/PSS-coated ITO electrodes detached readily within 2 to 3 min [50]. Additionally, this polymer demonstrated better stability, maintaining its integrity after cell sheet detachment and thereby allowing for reusability [51] (Fig. 3).

Seto et al. [53] designed an oligopeptide, CCRRG-DWLC, featuring a cell adhesion region (RGD) at the center and cysteine residues (CCR and WLC) at both terminals to facilitate cell attachment and detachment. The thiol group of cysteine can adsorb onto a gold surface via gold-sulfur bonds, mediate cell adhesion, and enable reductive desorption of the oligopeptide and cell detachment when a potential is applied (Fig. 3). They prepared thin gold rods, chemically adsorbed with the oligopeptide CCRRGDWLC. HUVECs were seeded onto the rods, which were subsequently placed within a perfusion culture chamber. Following this, a collagen solution was introduced. Upon the formation of the collagen gel, the cells and rods were dissociated by applying an electric potential. The rods were then removed, creating tubular channels within the collagen gel matrix. The cells that had initially adhered to the rods subsequently attached to the surfaces of these collagen gel tubular channels and underwent spontaneous vascularization under continuous fluid perfusion stimulation. This method is applicable not only to the construction of planar structures

but also to various configurations, including cylindrical structures.

#### Light-induced cell-sheet fabrication

Hong et al. [54] introduced a less invasive method for cell sheet fabrication based on the transition in surface wettability of culture coatings through light illumination. This approach provided an effective and convenient means for cell sheet production. Titanium dioxide ( $\text{TiO}_2$ ) was chosen as the raw material for preparing  $\text{TiO}_2$  nanodot films (TN) due to its biocompatibility [55] and light-induced wettability transition under safe illumination below a wavelength of 380 nm [56]. This nanodot film was coated onto a quartz substrate to support cell attachment under normal culture conditions. Upon exposure to 380 nm ultraviolet (UV) light, the  $\text{TiO}_2$  nanodot film became hydrophilic, reducing cell adhesion [57]. Consequently, proliferating cells on this surface could be released as a sheet-like structure within 5 min. Additionally, cell sheets from mouse calvaria-derived pre-osteoblastic (MC3T3-E1) cells [54], NIH3T3 cells [58], bone marrow mesenchymal stem cells (BMSCs) [59], and pre-vascularized composite cell sheets consisting of human mesenchymal stem cells and human umbilical vein endothelial cells (MSCs-ECs) [60] have been engineered using UV (365–366 nm) and  $\text{TiO}_2$ -based surfaces.

As a novel approach with significant advantages, light-induced cell sheet technology has seen rapid advancements [13] (Fig. 4). In addition to UV illumination, visible light (400–800 nm) [61], green light (510 nm) [62], and near-infrared (NIR) light (808 nm) [63] have been utilized for the fabrication of MC3T3-E1 cell sheets, hMSCs sheets [64], and human fibroblast (hFCs) sheets, among others.

#### Ions concentration-related cell sheet fabrication

Scientists have suggested that the surface energy of the cell culture substrate can affect cell-substrate adhesion and mediate cell sheet fabrication. Baek et al. [65] developed a highly effective and mild surface-tailoring method to regulate the degree of adhesion between cells and their culture surface, achieving intact cell sheets under isothermal conditions for precise modulation of cell-substrate interactions. They prepared a homogeneous copolymer, poly(divinylbenzene-co-4-vinylpyridine) [p(DVB-co-4VP) or pDV], by combining the nonpolar, hydrophobic divinylbenzene (DVB) and the polar, hydrophilic 4-vinylpyridine (4VP) through initiated chemical vapor deposition (iCVD) [66]. The copolymer film deposited onto tissue culture plastic (TCP) allowed for precise control of surface energy and systematic modulation of cell attachment and detachment by adjusting the input flow ratio of DVB to 4VP [65]. Cells seeded onto the culture plate could be harvested after changing the ligand-binding

conformation of the integrin-binding domain ECM protein and cell adhesion molecules (CAMs) by soaking in D-PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Fig. 5A). The entire release process took approximately 100 s and was expected to induce no cytotoxicity. While divalent cations do not affect all transmembrane proteins related to cell-cell junctions, the stability of these proteins in the absence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contributes to the formation of the sheet-like structure.

### Mechanical methods for cell sheet fabrication

Mechanical methods, such as cell scrapers and specialized culture supplements, were used for cell sheet fabrication. These simplified methods can produce intact cell sheets with good flexibility in a short time using tools like cell scrapers, pipette tips, or syringe needles, whereas other methods may require special materials or equipment [11].

In 2009, a large scaffold-free bone was constructed from rabbit BMSCs-derived osteogenic cell sheets harvested using the scraping technique [67]. Researchers cultured rabbit BMSCs under osteogenic conditions and used a gentle roll-up force with a cell scraper to lift a thick cell sheet immediately after formation. Additionally, intact cell sheets of rabbit BMSCs, chondrocytes derived from rabbit BMSCs, human autologous OMECs, and Curcumin-mediated mouse BMSCs were fabricated using the same method [68].

Instead of single-cell suspensions after trypsinization. Jia et al. cocultured smooth muscle cells (SMCs) and endothelial progenitor cells (EPCs) and harvested intact cell sheets by pushing the edge of the confluent cell layer gently with a pipette tip. This finding suggested that cell sheets might be feasible through mechanical methods when cell-cell adherence surpasses cell-substrate adherence. They prolonged the coculture of SMCs and EPCs and obtained an intact, viable, and pre-vascularized SMC sheet by gently tapping the edge of the fused cell layer with a pipette tip [11].

A Vitamin C-mediated procedure assisted in mechanical cell sheet fabrication for certain types of cells. By adding appropriate dexamethasone and ascorbic acid phosphate (vitamin C, Vc) to the culture medium, cells could be scraped off as a sheet structure using a scraper once they were confluent. This approach enhanced the creation of rat BMSCs sheets and bone formation [69]. Later, Wei et al. explored the potential of vitamin C for cell sheet formation due to its properties of biosynthesizing ECM, mimicking the physiological environment, and regulating the proliferation and differentiation of MSCs. They formed cell sheets by adding vitamin C to the culture medium and harvested the sheets using a crooked syringe needle [70]. Maha and Liu et al. [71] also reported the preparation of periodontal ligament stem

cell (PLSCs) sheets, BMSCs sheets, and EPCs–BMSCs sheets using this method.

### Other approaches to the release of cell sheets

In addition to the methods mentioned above, alternative approaches are available for obtaining cell sheets suitable for specific types of cells (Fig. 5B).

Cellulose-based methods have been employed for cell sheet fabrication, utilizing enzymatic digestion of polysaccharides. Cellulose, a biocompatible and biodegradable polysaccharide with suitable mechanical strength, can be hydrolyzed into beta-glucose by biocompatible cellulase [72]. For cell adherence, cellulose modified to contain different types of ligands is pre-coated onto the culture surface. After the cells form a confluent layer, cellulase-containing culture medium is added, leading to the release of cell sheets within 10 min [73]. Following this principle, fibroblast cell sheets [74], endothelial cell sheets [75], human mesenchymal stem cells (hMSCs) sheets, and corneal limbal epithelial cell (hCLE) sheets [73] have been fabricated using carboxymethyl cellulose conjugates (e.g., carboxymethyl cellulose conjugated with dopamine (CMC-DA) [73] and carboxymethyl cellulose conjugated with tyramine (CMC-Ph) [76]). These cell sheets demonstrated excellent morphology and showed better proliferative activity compared to cells treated with trypsin.

### The categories of cell sheets

Cell sheet engineering has emerged as a creative and gentle method for cell harvest in the life sciences. With advancements in this technology, cell sheets have become diverse in shape and structure, meeting various experimental needs. It is now possible to fabricate cell sheets ranging from monolayered to multilayered three-dimensional structures, using single or complex cell types. In this review, we categorize the cell sheet structures developed to date based on their cell type compositions and structural characteristics.

### Categories classified by the standards of the number of cell types

Considering the classification standards based on the number of cell types present in cell sheets, they can be divided into two categories as outlined below.

#### Homeotypic cell sheets

The earliest example of cell sheet construction involved this category, which consists of a single cell type [14]. Due to the simplicity and convenience of this approach, homotypic cell sheets were the primary focus of research and development for some time. Various cells, including autologous oral mucosal cells, mouse BMSCs, NIH3T3 cells, HDFBs, mouse C2C12 cells, human SMCs, hMSCs,

and MC3T3-E1 cells [77], have been used to prepare these sheets.

The cell composition in this category is straightforward, and the variables to be controlled during preparation are relatively simple. This simplicity makes it the most basic and easiest category to achieve quality control and mass production, facilitating rapid clinical translation at the top speed [78]. Considering the physiological structure of human organs and tissues, cell sheets composed of a single cell type are particularly valuable for repairing damaged or pathological thin tissues, such as skin, mucosa, and cornea. Additionally, these cell sheets are advantageous for studying the therapeutic effects and mechanisms of specific cells in treating diseases [79]. Cell sheet transplantation allows for the retrieval of grafts from experimental animals [80], whereas cells injected as a suspension are difficult to recover [81].

#### **Heterotypic cell sheets**

The advancement of cell sheet engineering has led to the development of cell sheets composed of two or more cell types. Most reported heterotypic cell sheets contain two types of cells, with one type typically being endothelial lineage cells, which are crucial for vascularization and integration in vivo [82]. While the cell composition in these sheets is more complex than in homotypic sheets, this complexity enhances the capacity of the cell sheets to integrate with the host tissue and establish vascular connections [83]. Therefore, multi-cell type sheets are particularly valuable for repairing thicker tissues or those requiring vascularization and functional integration [84]. However, the distribution and arrangement of cells in heterotypic sheets remain relatively disordered, which limits their effectiveness in replacing damaged tissues and in tissue regeneration.

#### **Categories classified by the standards of the number of cell layers**

Cell sheets can also be classified based on the number of layers they possess: monolayer cell sheets and multilayer cell sheets. Multilayer cell sheets can be further divided into one-step harvested cell sheets and multi-step harvested cell sheets, depending on the harvesting procedure.

#### **Monolayer cell sheets**

Except for certain special cell types (e.g., keratinocytes and stem cells) that may naturally form multilayer sheets, cells seeded on a culture surface typically grow in a monolayer due to contact inhibition [85]. These cells can be harvested as a monolayer once they reach confluence. Monolayer cell sheets do not require complex techniques, only a suitable method and a basic harvesting protocol [86]. Due to their straightforward fabrication,

monolayer cell sheets were among the first types constructed and remain prevalent.

#### **Multilayer cell sheets**

As most organs and tissues are more than just thin layers but structures of significant thickness, the demand for a large number of cells to achieve therapeutic doses cannot be met by single-layer structures alone. Therefore, multilayer cell sheets were developed, and these multilayered tissues can be categorized based on the harvesting procedure.

#### **Multilayer cell sheets with one-step harvest procedure**

To construct multilayer cell sheets using a one-step harvest procedure, the multilayer structure must be established prior to harvesting. Yamato [87] achieved this by plating human keratinocytes on poly(N-isopropylacrylamide)-coated thermo-responsive culture dishes, resulting in intact multilayered human keratinocyte sheets. For other monolayer-adherent cells, there are three main approaches:

The first method involves seeding adherent cells at a much higher density initially or culturing adherent cells for an extended period to achieve a higher density on the culture surfaces, thereby promoting the spontaneous formation of a multilayer cell structure spontaneously [68]. Nishida [78] plated mitomycin C (MMC)-treated NIH3T3 cells onto the culture surfaces one day prior, then seeded human or rabbit limbal stem cells onto these surfaces. After 2 weeks of cultivation, three to five-layered corneal epithelial cell sheets, with flat basal and apical surfaces, were harvested.

Contact inhibition is a characteristic of normal adherent cells that results in a monolayer during in vitro cultivation [88]. To overcome contact inhibition and shape adherent cells into a multilayered sheet, magnetic force was employed as a facilitating device. This approach compels cells to adhere to culture surfaces, promoting intercellular connections between different layers [32]. Yamamoto et al. [89] successfully prepared skeletal muscle tissue constructs expressing muscle-specific markers and exhibiting physiological functions by accumulating MCLs-labeled mouse C2C12 myoblast cells onto an ultralow attachment surface using magnetic force.

Kito et al. [90] labeled induced pluripotent stem cell (iPSCs)-derived fetal liver kinase-1 positive cardiovascular cells (FLK-1<sup>+</sup> cells) with MCLs and fabricated an approximately 300  $\mu\text{m}$  thick cell sheet composed of 15 to 20 layers of cells using a magnetic force-based system [31]. Later, Ana et al. [35] constructed a triple-layer sheet with HUVECs sandwiched between two sheets of ASCs with the aid of magnetic force.

The final method relies on time lag. Cells are directly seeded onto a dish that already has an attached and

confluent cell layer [71]. The previously attached cell layer serves as a substrate, and the new cell layer adheres to the original layer through cell-cell connections and ECM effects [91]. Repeating this process enables the formation of multilayer structures [92].

**Multilayer cell sheets with multi-step harvest procedure** Constructing multilayer cell sheets with a one-step harvest procedure involves applying specific stimulation only once, which is gentler on the cells due to the limited impact of time consumption and changes in culture conditions. However, single cells seeded onto existing cell layers are more prone to migration and harder to control in terms of distribution within the structure. The multi-step harvest procedure addresses these limitations effectively.

This method primarily constructs multilayer cell sheets through the repeated superposition of prepared monolayer cell sheets, utilizing various advanced manipulators [93]. For example, Haraguchi et al. [94] developed a hydrogel-coated, plunger-like manipulator and fabricated triple-layer pulsating cardiac cell sheets by stacking detached monolayer cardiac cell sheets with the aid of this manipulator.

Multilayer cell sheets produced using the multi-step harvest procedure have a relatively defined cell composition and number of layers. These characteristics enhance their ability to mimic the original structure of damaged tissues and provide more effective therapeutic alternatives in tissue and organ regeneration.

#### **Categories classified by the standards of the complexity of the structure**

Considering the classification standards for the structural complexity of cell sheets that have been constructed, cell sheets can be classified into three categories ranging from simple to complex structures.

##### ***Simple disordered cell sheets***

Simple disordered cell sheets are the most basic type of cell sheets. Cells are seeded onto culture surfaces, where they may experience transitions between adherence and detachment under various stimuli. Although the fabrication methods for these cell sheets vary, they share a common characteristic: the arrangement of cells within the sheet is random and disordered. This category offers the advantage of easy operation but has limitations in replicating native structures and specific functions.

##### ***Patterned cell sheets***

Patterned cell sheets are designed to meet more specific requirements in cell sheet engineering. The fabrication of patterned cell sheets primarily relies on the preparation of patterned culture surfaces. Tsuda et al. [96] reported preparing a patterned surface using EB irradiation and

fabricated a patterned cell sheet consisting of rat primary hepatocytes and bovine carotid endothelial cells (HCS-ECs) in 2005. In this method, n-butyl methacrylate (BMA) was grafted onto a PIPAAm-grafted surface using a graphic metal mask. This dual thermoresponsive polymer-grafted surface was patterned as the mask obscured part of the EB [96]. Another advanced patterned thermoresponsive surface was reported, where BMA was replaced by site-selective biofunctionalized carboxyl-functional thermoresponsive polymers [97]. The 'patterning' procedures mentioned are closely related to the areas (graphics) modified to achieve specific cell adhesion patterns [98]. The critical process in these methods is the adherence of seeded cells, meaning that patterned cell sheets are already shaped before harvest.

Efforts have also been made to obtain patterned cell sheets during the harvesting procedure [13]. Kim et al. [63] fabricated hFCs sheets with various patterns by diffracting NIR light into a light pattern using a micropatterned optical lens. They generated photothermal patterns on PEDOT, which converts NIR absorption into localized heat, thereby mediating the dissolution of the collagen layer on the PEDOT layer and enabling the rapid harvesting of patterned cell sheets [99].

Additionally, magnetic force-based cell sheet engineering is well-suited for producing patterned cell sheets. The only requirement for 'patterning' is the strategic placement of magnets. Pretreated cells seeded on petri dishes will gather and adhere to areas under magnetic control [100].

In contrast to simple disordered cell sheets, patterned cell sheets have a designed and well-aligned cell arrangement or macroscopic shape. This design allows for relatively precise control over the proportion of co-cultured cells, further enabling spatiotemporal regulation of cellular functions and better mimicking of original tissues.

##### ***Vascularized and complex configured cell sheets***

Superposing functional cell sheets has been a method for constructing scaffold-free 3D tissues, facilitating the regeneration and repair of tissue defects [87]. In 2006, thick and functionalized myocardial soft tissues [101] were created by stacking monolayered cultured cell sheets confluent with each other. However, fabricating 3D tissue from monolayers is time-consuming. Additionally, simply superimposing monolayer cell sheets may result in a lack of capillaries, which supply oxygen and nutrients. This can lead to cell necrosis in the center of the constructed thick sheets, limiting the number of superimposed cell layers [102]. Sakaguchi et al. used cell sheet engineering and a perfusion bioreactor with collagen-based microchannels to prepare a multi-layer construction of vascularized cell sheets and reported cell viability within them [103]. Sekine et al. also constructed

functional vascularized 3D tissues in vitro by stacking cell sheet units [104]. Specifically, every three layers of neonatal rat cardiac cell sheets were considered one unit and cultured on a vascular bed, which consisted of resected tissues with a connectable artery and vein. Endothelial cells within each unit connected with the capillaries on the vascular bed to form lumens. Once lumens were formed, the unit served as the vascular bed for the subsequent unit. Compared to structures with cell sheets superimposed in one step, the pre-vascularized cell sheet units' superposition is thicker and better mimics heart tissue.

Additionally, tissues usually possess complex 3D geometries, and their special configurations must be accommodated during repair and reconstruction. Recently, Williams et al. [105] reported the formation of a skeletal myotube using a flexible thermoresponsive nanofabricated substratum (TNFS) and a gel casting method [106]. Monolayers of SMCs, skeletal muscle cells, and cardiomyocytes were prepared using TNFS [24], harvested, and neatly stacked on a new TNFS, eventually folding into a cylinder.

With ongoing advancements in cell sheet engineering technology, the construction of more complex and thicker vascularized tissues, curved surfaces, and hollow cavity structures in vitro has become possible. This progress enhances the preservation of cell viability in complex structures or larger-scale tissues and better simulates the functions of natural tissues and organs.

#### **Categories classified by the standards of the degree of hardness and softness**

Based on the classification standards for the hardness and softness of in vivo tissues that the fabricated structures correspond to, cell sheets can be classified into soft tissues and hard tissues. The classification primarily depends on the cells used in the cell sheets. So far, soft tissue construction has been more commonly reported. Hepatocytes [107], ASCs [108], human skeletal muscle cells (HSMCs) [109], MSCs, RPE [110], and thyrocytes [111], among others, have been used to construct cell tissues that mimic soft tissues in vivo.

Cell sheet engineering technology also has broad applications in forming hard tissues, such as bone and cartilage structures [112]. Ma et al. [67] prepared BMSCs-derived osteogenic cell sheets using a cell scraping method and constructed large bone tissues by rolling the harvested cell sheets. Additionally, vascularized neotrachea, cartilage sheets, and various dental-derived stem cell sheets [113] have been constructed. These engineered scaffold-free structures offer new strategies for the construction and regeneration of bone and cartilage repair.

#### **Brief summary**

The categories of cell sheet types under different classification conditions summarize and evaluate fabricated cell sheets from various dimensions and are not mutually exclusive. For instance, a cell sheet might be classified as a patterned soft tissue cell sheet with multiple cell types and multi-layer structures. Furthermore, different categories of cell sheets may present varying levels of difficulty in construction. For example, monolayer cell sheets composed of single cells are relatively easy to prepare, while cell sheets with pre-vascularized complex structures are more challenging to create. Ultimately, the most suitable type of cell sheet can be selected from the various categories available to meet specific needs. Figures 6 and 7 illustrate some reported cases.

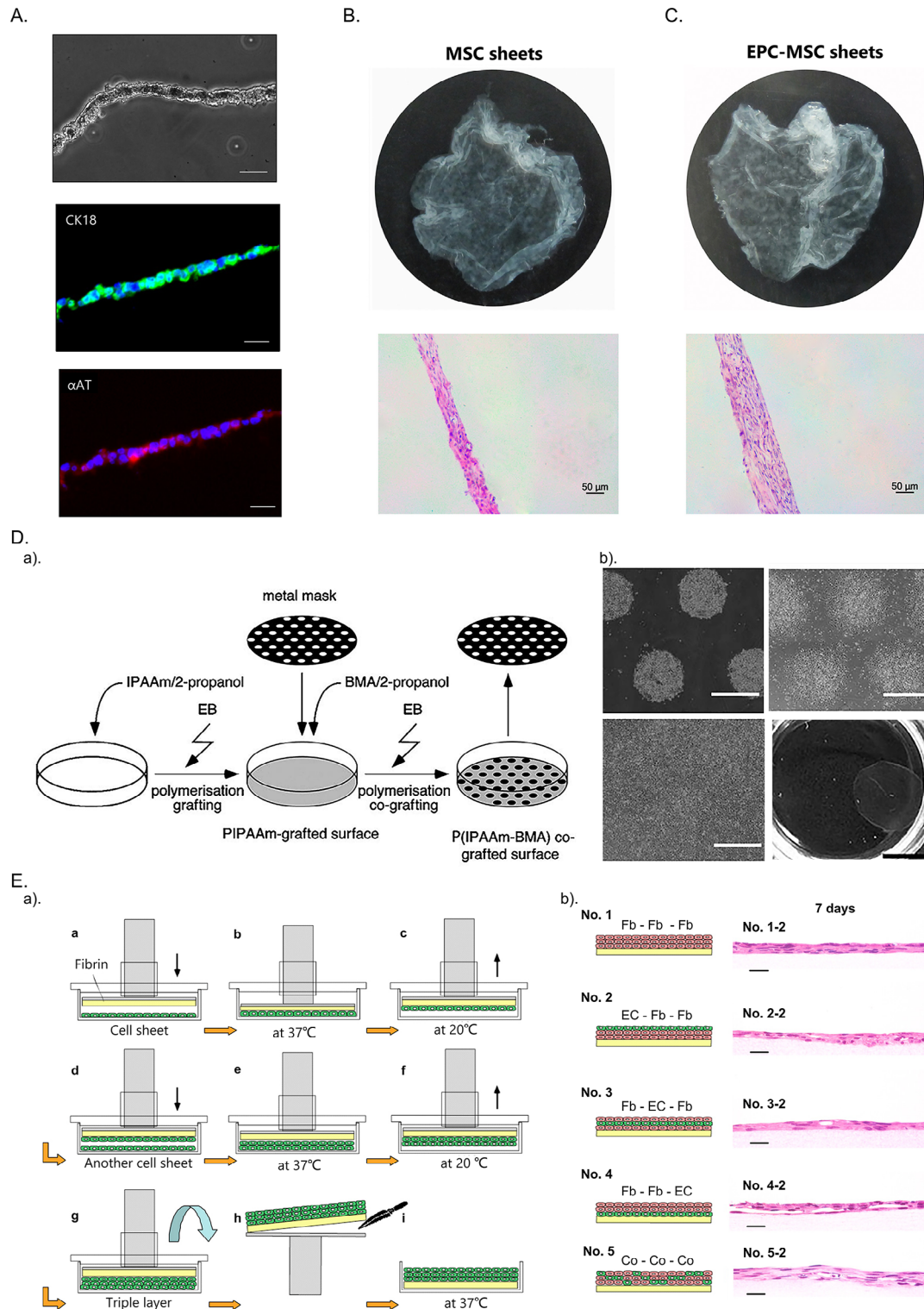
#### **Conclusions and future outlook**

Cell sheet engineering has found broad applications in basic research on regenerative medicine and has even advanced to clinical trials due to its unique characteristics and advantages. Various stimuli, including temperature variations, magnetic force, controllable electrochemical changes, and light, have been utilized to control cell adhesion and detachment. Different types of cell sheets have been fabricated using these stimuli to create diverse 3D tissue constructs without the need for 3D scaffolds.

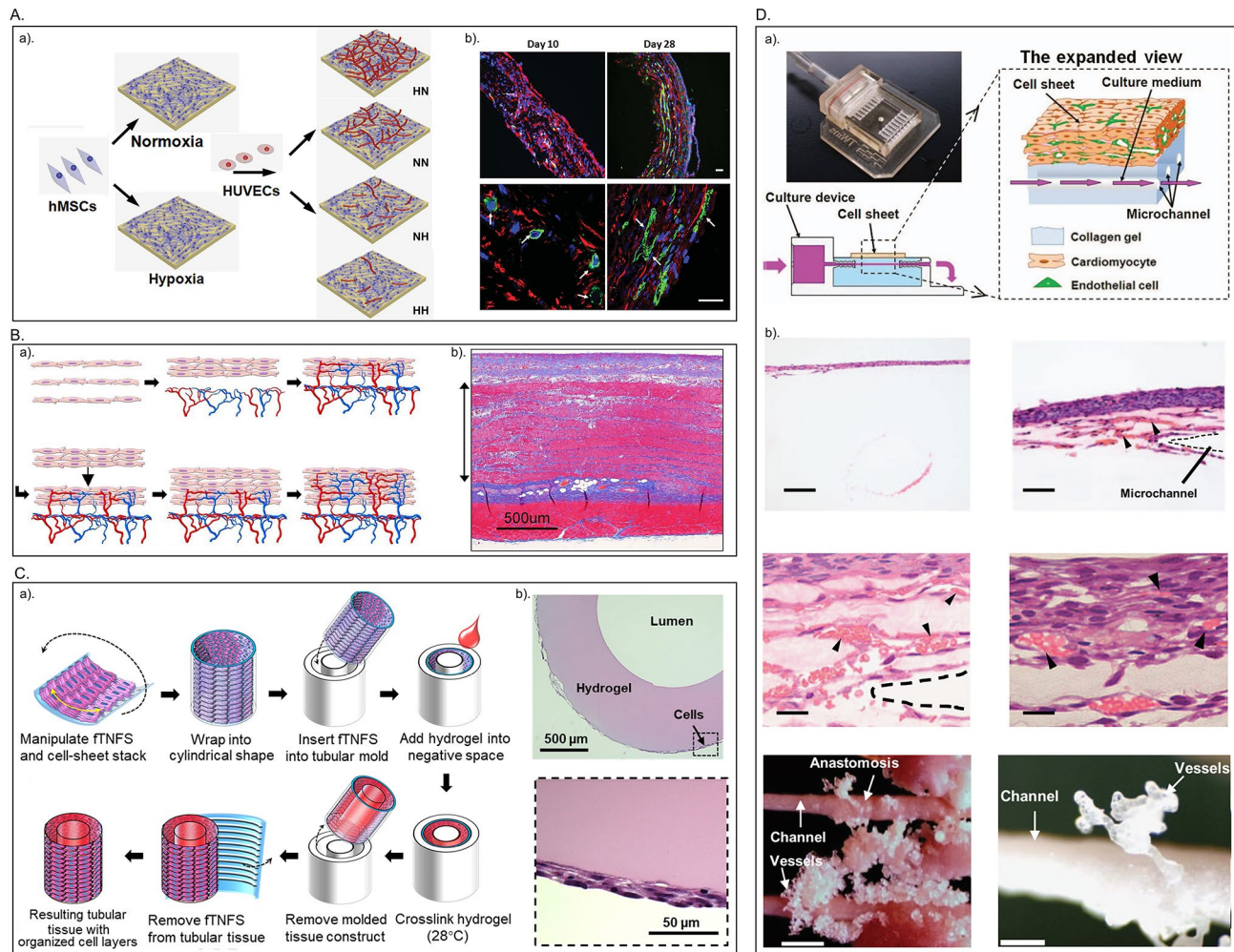
Thermally triggered cell sheet fabrication involves lowering the culture temperature and has been applied to various cell types. This method is relatively simple and widely used. However, it has a long production time, results in single-layer structures, and imposes stringent requirements on heat-sensitive materials. Magnetic force-based cell sheet fabrication involves impregnating MNPs into target cells, allowing them to be attracted by magnets under culture plates. By adjusting the magnetic force, cells can be assembled into sheets, including multilayer cell sheets. However, long-term exposure to MNPs may adversely affect cell viability. Magneto ferritin has been reported as a safer alternative and is expected to replace metallic MNPs in the future. Polyelectrolyte-based electrochemical cell sheet fabrication and light-induced cell sheet fabrication involve creating petri dishes with special coatings. By altering the culture medium environment (such as pH) and the wavelength of light, the adhesion properties of cells can be changed, leading to their detachment.

In terms of the number of layers, cell sheets can be categorized into monolayer and multilayer cell sheets. Monolayer cell sheets exhibit a single layer of growth due to contact inhibition and are relatively easy to manufacture. In contrast, multilayer cell sheets better mimic the original structure of damaged tissue and can demonstrate synergistic effects when different types are combined, thus enhancing the repair of affected sites.





**Fig. 6** Application examples of basic and relatively simple cell sheets. **A.** Monolayer human iPSCs-derived hepatocyte sheets [86]. **B.** Multilayer rat MSCs sheets [95]. **C.** Multilayer rat EPCs-MSCs sheets [95]. **D.** a): Schematic diagram of preparing thermo-responsive culture dishes for fabricating patterned cell sheets; b): Co-cultured, patterned monolayer bovine carotid artery endothelial cells (ECs) - rat primary hepatocytes (HCs) sheet [96]. **E.** a): Schematic diagram of multi-step fabrication of cell sheets using a cell sheet layering manipulator; b): Multi-step stacked homeotypic or heterotypic cell sheets. Sample No. 1: Triple-layered neonatal normal human dermal fibroblasts (NHDFs) sheet. Sample No. 2: Triple-layered NHDF sheet-NHDF sheet-human umbilical vein endothelial cells (HUVECs) sheet. Sample No. 3: NHDF sheet-HUVECs sheet-NHDF sheet. Sample No. 4: Triple-layered HUVECs sheet-NHDF sheet-NHDF sheet. Sample No. 5: Disordered triple-layered HUVECs-NHDFs co-culture sheet [82]. All panels are reproduced with permission



**Fig. 7** Application examples of advanced prevascularized and complex cell sheets. **A. a)** Process of preparing prevascularized 3D tissue constructions. “HN” indicates hypoxic hMSCs sheets, normoxic co-cultures, while “NN” indicates normoxic hMSCs sheets, normoxic co-cultures; **b)** 3D tissues constructed from prevascularized HN hMSCs sheets [83]. **B. a)** Schematic of constructing vascularized cell-dense tissues by using previously vascularized grafts as the vascular bed for subsequent grafts; **b)** Thick cell-dense tissues fabricated after 10 surgeries [101]. **C.** Schematic diagram and example of cell sheet engineering-based tubular tissue fabrication [105]. **D. a)** Schematic diagram of the culture device and collagen-microchannel-based perfusion culture system for in vitro vascularized tissue engineering; **b)** Vascularized multi-layer structures consisting of cardiac-endothelial cell sheets fabricated using this system [103]. All panels are reproduced with permission

In vitro vascularization using a vascular bed has been successfully implemented, leading to increased thickness and volume of densely packed cellular tissue. These advancements improve the benefits and effectiveness of this technology. However, simple stacking of cell sheets can result in oxygen and nutrient deficiencies, leading to cell necrosis in the center. Genetically modified cell sheets have been shown to stimulate angiogenesis. Recently, researchers have been exploring advanced vascular engineering techniques to maintain blood supply post-transplantation and improve the survival rate of transplanted cells. The process of cell sheet preparation is flexible, allowing for the selection of appropriate methods based on specific functional requirements. With the development of emerging technologies such as 3D

printing and microfluidics, increasingly delicate and personalized cell sheets are being created.

Future advancements will rely on continued development of materials and techniques, a deeper understanding of cellular behavior, clinical translation, customization, and interdisciplinary collaboration. These factors will enable cell sheets to better adapt to complex tissue and organ environments, simplify preparation methods, and effectively replicate the original structure at the transplant site.

Firstly, developing more efficient, cost-effective, and scalable methods is imperative. This involves exploring novel biomaterials with enhanced biocompatibility, mechanical properties, and functionalization capabilities, as well as incorporating advanced manufacturing

techniques like 3D printing and microfabrication to create precise and complex tissue structures. Understanding the molecular and cellular mechanisms underlying these processes is also crucial. Detailed investigations into how different fabrication methods impact cellular behavior—including differentiation, proliferation, and function—can help optimize cell sheet engineering for specific applications. This may include gene expression studies and long-term assessments of cell sheet stability and functionality in vivo. Clinical translation is a critical step, requiring rigorous preclinical and clinical trials to evaluate the safety, efficacy, and therapeutic potential of cell sheets in various medical conditions. Additionally, the trend towards personalized medicine necessitates the development of patient-specific cell sheets, which can minimize the risk of immune rejection and enhance therapeutic outcomes. Tailoring cell sheet properties to meet individual patient needs based on unique physiological conditions will further this goal. Finally, the advancement of cell sheet engineering will greatly benefit from interdisciplinary collaboration. By uniting experts from materials science, bioengineering, molecular biology, and clinical medicine, innovative solutions can be fostered, and progress can be accelerated. Continued investment in research and development, along with collaborative efforts across disciplines, will be essential to fully realize the potential of this transformative technology.

#### Abbreviations

ECM	Extracellular matrix
MNPs	Magnetite nanoparticles
PIPAAm	Poly(Nisopropylacrylamide)
PNIPAAm	Poly(N-isopropylacrylamide)
EB	Electron beam
LCST	Lower critical solution temperature
PS	Polystyrene
PDMS	Polydimethylsiloxane
PIPAAm-PDMS	Poly(Nisopropylacrylamide gel-grafted polydimethylsiloxane
pH	Potential of hydrogen potential of hydrogen
RPE	Retinal pigment epithelial cells
OMECS	Oral mucosal epithelial cells
GMA	Glycidylmethacrylate
NGMA	N-isopropylacrylamide-co-glycidylmethacrylate
TNFS	Thermoresponsive nanofabricated substratum
SAM	Self-assembled monolayers
RGD	Cell adhesive peptide
bFGF	Angiogenic growth factor
MCL	Magnetite cationic liposomes
HAECs	Human aortic endothelial cells
HUVECs	Human umbilical vein endothelial cells
ASCs	Adipose-derived stromal cells
RCO	Rat calvarial osteoblasts
PDL	Periodontal ligament cells
PLL-g-PEG/PEGRGD	RGD-modified poly(L-lysine)-graft-poly(ethylene glycol)
ALP	Alkaline phosphatase
HeLa	Human cervical carcinoma cells
ITO	Indium tin oxide
PD-MSCs	Placenta-derived mesenchymal stem cells
PAH	Poly(allylamine hydrochloride)
PSS	Poly(styrene sulfonate)
TN	TiO <sub>2</sub> nanodot film

TiO <sub>2</sub>	Titanium dioxide
UV	Ultraviolet
MC3T3-E1	Mouse calvaria-derived pre-osteoblastic
BMSCs	Bone marrow mesenchymal stem cell
MSCs-ECs	Human mesenchymal stem cells and human umbilical vein endothelial cells
hFCs	Human fibroblasts
Hp-PK	Hematoporphyrin-incorporated polyketone
PEDOT	Poly(3,4-ethylenedi-oxythiophene)
DVB	Divinylbenzene
4VP	4vinylpyridine
iCVD	Initiated chemical vapor deposition
TCP	Tissueculture plastic
CAM	Cell adhesion molecules
Vc	Ascorbic acid phosphate (vitamin C)
SMCs	Smooth muscle cells
EPCs	Endothelial progenitor cells
hMSCs	Human mesenchymal stem cell
hCLEs	Corneal limbal epithelial cells
CMC-DA	Carboxymethyl cellulose conjugation with dopamine
CMC-Ph	Carboxymethyl cellulose conjugation with tyramine
MMC	Mitomycin C
iPSCs	Induced pluripotent stem cells
FLK-1 <sup>+</sup>	Fetal liver kinase-1 positive cardiovascular
HCS -ECs	Hepatocytes and bovine carotid endothelial cells
BMA	N-butyl methacrylate
HSMCs	Human skeletal muscle cells
NHDFs	Normal human dermal fibroblasts

#### Supplementary Information

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Supplementary Material 1

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#### Author contributions

Danping Hu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. Ce Gao: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. Jie Li: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. Pei Tong: Investigation, Software, Visualization, Writing – review & editing. Yi Sun: Conceptualization, Data curation, Funding acquisition, Formal analysis, Methodology, Resources, Project administration, Validation, Visualization, Writing – review & editing. All authors read and approved the final manuscript.

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#### Data availability

Not applicable.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

All authors have read the journal's policy on disclosure of potential conflicts of interest. All authors declare that they have no known competing interests. DH, after graduating from central south university, was employed by HANG-ZHOU CHEXMED TECHNOLOGY CO., LTD which provided the opportunity to continue cell-sheet and organoids-related research. The experience in related fields contributes to this review.

### Artificial Intelligence

The authors declare that they have not used Artificial Intelligence in this study.

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