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Preservation Strategies that Support the Scale-up and Automation of Tissue Biomanufacturing

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Abstract

Purpose of Review Tissue engineering strategies to repair or replace tissues and organs that have been damaged by disease, trauma, or congenital issues usually require many weeks of production during which time patients are incapacitated or reliant on temporary devices. In order to fully meet the rising clinical demand of transplantable tissues/organs, various preservation technologies need to be implemented to create "off-the-shelf" availability of biological components and products. This review will focus on the preservation methods used for biological resources (cells, growth factors, and biological scaffolds) and also for the finished tissue-engineered constructs.

Recent Findings Recent studies have demonstrated that conventional cryopreservation and vitrification preservation methods can maintain functionality and properties of cells and cell-seeded scaffolds during long-term storage. Lyophilization can also be used as an alternative strategy for engineered tissues that are devoid of cells. Additionally, fabrication technologies combined with freezing/thawing processes will likely emerge as the preferred strategy to better control the physical and biological properties of engineered tissues while simultaneously providing a shelf life for the product.

Summary The development of preservation methodologies for tissue engineering would minimize the shortage of tissues/organs and offer an effective and commercialized strategy for improved automation of tissue biomanufacturing.

Keywords Cryopreservation · CPA · Dry preservation · Tissue engineering · Scaffolds · Biomanufacturing

Introduction

Tissue engineering (TE), a major component of regenerative and reparative medicine, can offer effective alternatives to conventional treatments for restoring functionality of organs and tissues impaired or damaged by disease and trauma. Tissue engineering comprises three main strategies [1, 2]: (1) cell-based, (2) growth factor-based, and (3) scaffold-based, as illustrated in Fig. 1. When injected into tissue defects, cells can be used for synthesizing the bulk of the tissue matrix, integrating with existing native tissues, maintaining tissue homeostasis in general, and providing various metabolic services to other tissues and organs. Cell-based strategies involve the direct in vivo implantation of isolated cells or cell substitutes and it is based on cells synthesizing their own matrix. Growth factors are soluble-secreted signaling polypeptides capable of instructing specific cellular responses in a biological environment and form the basis of growth factor-mediated tissue engineering. An example is the administration of bone morphogenetic protein (BMP) to a critical-sized skeletal defect to stimulate bone production and defect repair [3]. The third approach to tissue engineering is to use a scaffold-based construct that can serve as a carrier for other beneficial or reparative biological components. Scaffolds serve as substitute cellular microenvironments to support tissue formation, providing for cell attachment, proliferation, and differentiation, retention of cells and growth factors, and diffusion of cell nutrients and oxygen. They can also provide an appropriate mechanical and biological environment for regeneration of the tissue in an organized hierarchical manner.

Because cell- and growth factor-based approaches often fall short of delivering desired results, scaffold-based strategies that incorporate cells and/or growth factors have become increasingly prevalent. For example, a biodegradable scaffold

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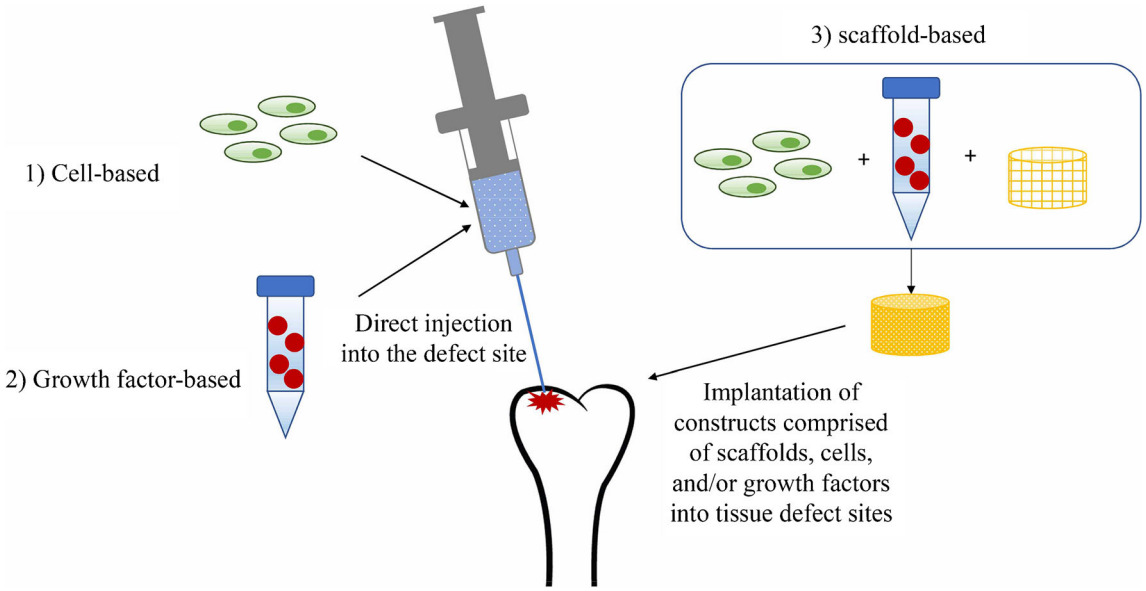

Fig. 1 Schematic representation of three tissue engineering strategies

system may be designed to carry cells into a defect while releasing growth factors into the surrounding tissue as it degrades. Whether produced using synthetic or biological materials, scaffold matrices enhance tissue growth and repair by facilitating delivery and localization of cells and growth factors to the injury site.

In order to fully implement biomanufactured tissues as a solution for the rising clinical need of "replacement parts," the challenges related to maintaining a steady supply of living tissue substitutes must be addressed. Prior to that level of commercial and clinical viability of engineered substitutes, there is also a need to consider the TE supply chain from a manufacturing point of view. A typical TE strategy starts from the isolation of autologous cells from recipients followed by in vitro expansion. These cells are then seeded and cultured in 3D porous scaffolds until a predetermined cell density and/or amount of extracellular matrix is generated. At that point, if the patient's health status is amenable, the tissue can be implanted. The whole process can require several weeks, during which time the patient is incapacitated or reliant on short-term substitutes. As shown in Fig. 2, each step in the process provides opportunities for creating "off-the-shelf" availability of biological components that can enable suspension of manufacturing at key points for just-in-time delivery at the clinic. Preservation also creates opportunities for quality control testing at key manufacturing junctures and can greatly reduce costs associated with storage and shipping of a clinical product. In 2014, the average cost for organ transplantation in the USA exceeded $1 million (e.g., heart, intestine, and double lung transplant), partly because of the need for rapid delivery of the organ by aircraft [4]. A research study published by Crystal Market Research projects that the organ preservation market will reach $285.63 billion by 2023 [5]. Whether donated or manufactured, the lack of preservation strategies for organs adds significant logistical and financial burden. Inadequate tissue preservation capabilities also place a significant constraint on basic and preclinical biomedical research aimed at the advancement of tissue engineering and regenerative medicine. Likewise, product development in the areas of biofabrication, tissue engineering, regenerative medicine, and tissue-based chip devices will all require capabilities emerging from organ and tissue preservation advances [6].

Preservation of Source Materials

Cells

Tissue engineering strategies that rely primarily on cell transplantation involve the direct implantation of cells into diseased/damaged tissues or the systemic circulation. Cells can be also employed in tissue engineering to form cell-seeded scaffolds that can be implanted in the body to support production of native tissue. Preservation of cells is thus one of the most important strategies for the "off-the-shelf" availability in tissue engineering. Stem cells isolated from autologous or allogenic tissues are generally used in conjunction with scaffolds to produce tissue for re-implantation. They include embryonic stem cells, bone marrow stem cells, umbilical cord stem cells, and adipose-derived stem cells [7, 8]. Cryopreservation of cells, including stem cells, is both a mature and an attractive technology, involving slow freezing (ex. 1 °C/min) of ~ 1 ml aliquots of cell suspension in the presence of 5–10% of a penetrating cryoprotectant, such as dimethyl sulfoxide (DMSO). Samples can then be stored in liquid nitrogen vapor for multiple years without loss of function [9, 10]. Woods and coworkers have

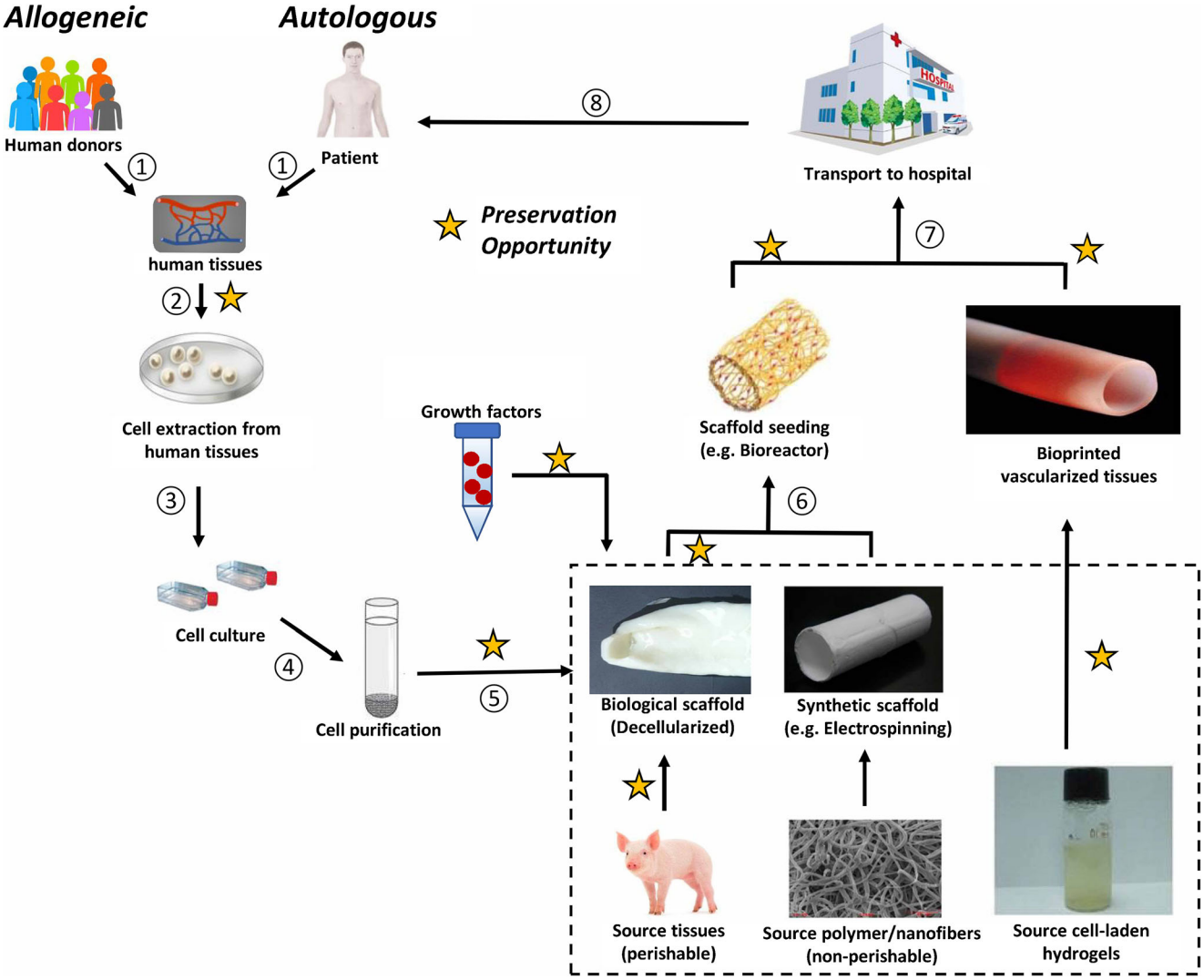

Fig. 2 Preservation opportunities that support biomanufacturing of engineered tissue constructs from autologous and allogeneic resources. Yellow stars indicate opportunities to use preservation methods to stockpile resources for just-in-time delivery of materials at critical junctures in the manufacturing and distribution pipeline, thus improving efficiency and reducing costs

recently reviewed the critical factors and considerations for successful cryopreservation and storage of human cells for clinical use in the "off-the-shelf" cellular therapeutics [11], and these methods are equally applicable to cells that have been sourced for tissue engineering purposes. Standard cryopreservation methods ensure good survival of source cells, but there is clinical interest in moving away from DMSO as a cryoprotectant due to patient aversion (strong odor) and its toxicity for stem and progenitor cells [12, 13]. Thus, it is highly desirable to develop cryopreservation protocols with non-toxic alternatives to DMSO. Devireddy's group has investigated cryopreservation and storage of human adipose tissue-derived adult stem cells (ASCs) using polyvinylpyrrolidone (PVP) as a cryoprotective agent (CPA) instead of DMSO. The results demonstrated that ASC viability and adipogenic and osteogenic differentiation abilities can be maintained when ASCs are frozen with 10% PVP in DMEM [14]. Furthermore, sugars, both monosaccharides and disaccharides, have been studied to minimize toxicity of CPAs. For example, Petrenko and his colleagues showed that the survival and metabolic activity of cells pretreated with sucrose, trehalose, and raffinose was ~50% after cryopreservation [15]. Matosevic's group recently demonstrated long-term cryopreservation of natural killer (NK)-92 cells in DMSO-free media [16]. As a critical part of the innate immune system, NK-92 cells are highly sensitive to freezing and thawing, while the use of DMSO during cryopreservation raises serious safety concerns. In their work, they demonstrated that a novel DMSO-free media containing combinations of dextran, ectoine, hydroxyectoine, trehalose, ethylene glycol, and polylysine maintained the cellular viability (70%), morphology, and cytotoxic activity following long-term cryopreservation for up to 2 months.

In cases where single cell suspension injection is not suitable for large tissue reconstruction, a unique, scaffold-free method of cell processing can be utilized to engineer "cell sheets." One or multiple layers of intact cell sheets can be grown and then subsequently detached from a thermosensitive surface. This approach is excellent for epithelium, endothelium, and cell-dense tissues. Cell sheets can also be slowly frozen in the presence of DMSO to − 80 °C, followed by storage in liquid nitrogen. For example, the Wei group cryopreserved periodontal ligament stem cell sheets (PDLSCs) in the presence of 90% FBS and 10% DMSO and stored them in liquid nitrogen for 3 months [17]. The PDLSCs derived from cryopreserved sheets and freshly controlled sheets showed no significant difference in their viability, proliferative capacities, multi-lineage differentiation potentials, and chromosomal stability, providing good evidence that cryopreservation does not alter the biological properties of PDLSC sheets.

Growth Factors

Growth factors are critical signaling molecules that direct specific cellular actions, such as cell survival and recruitment, and promote migration and differentiation during development in a biological environment. As such, they are an important component of the supply chain for tissue-engineered constructs. When growth factors (proteins) are used as a TE strategy, they are generally loaded onto scaffolds as a dilute solution to construct an in vivo delivery system for regeneration of damaged tissues. Because of instability problems of proteins in liquid formulation, they are typically lyophilized and preserved in the form of dehydrated powder to minimize degradation, which also offers advantages at the storage and shipping/distribution stages [18]. The stability of freeze-dried powders depends critically on how the freeze-drying process is carried out. Arakawa et al. have provided a comprehensive treatment of the factors affecting protein stability during freeze-drying. Their review makes a distinction between the freeze-thawing and freeze-drying processes in the context of protein stability and discusses the mechanisms by which the additives stabilize proteins against acute stress and the various factors to be considered for long-term storage of proteins in solution [19].

Scaffolds

Scaffolds used in engineered tissues ideally should mimic the extracellular matrix (ECM) of the target tissue in its native state, which mainly functions to: (1) provide structural support for resident cells, (2) contribute to the mechanical properties of tissues, (3) provide bioactive cues for cells to respond to their microenvironment, (4) act as a reservoir for growth factors, and (5) provide a flexible physical environment to allow remodeling in response to tissue dynamic processes such as wound healing. Currently, there are three major approaches to produce 3D scaffolds for tissue engineering that involve premade porous scaffolds, hydrogel matrix, and decellularized ECM from allogenic or xenogenic tissues.

In general, biomaterials used for making porous scaffolds for TE can be classified into two categories according to their source, namely natural and synthetic polymeric biomaterials. Natural polymers include proteins (silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, and myosin), polysaccharides (cellulose, amylose, dextran, chitin, and glycosaminoglycans), or polynucleotides (DNA, RNA) [20]. However, they have limited physical and mechanical stability and therefore often need to be co-developed or cross-linked with synthetic material. Synthetic polymers exhibit, in general, predictable and reproducible mechanical and physical properties. PLA, polyglycolic acid (PGA), and PLGA copolymers are among the most commonly used synthetic polymers in tissue engineering. Hydrogels are 3D networks composed of hydrophilic polymers cross-linked either through covalent bonds or held together via physical intramolecular and intermolecular attractions [21]. Cross-linked natural and synthetic polymers, as well as hydrogels, are generally stable and do not need specific preservation technologies before seeding with cells.

Decellularized scaffolds processed from allogenic or xenogenic tissues are obtained by removing allogenic or xenogenic cellular antigens from tissues with organ-specific ECM. Therefore, decellularized tissues are expected to be an effective scaffold with suitable components for the construction of tissues. The remaining ECM needs to be preserved to maintain the structure and properties before further use. Some studies have demonstrated that decellularized tissues stored using a hypothermic preservation approach do not meet the demands for a tissue engineering matrix and likely would not yield a suitable graft for lifelong implantation. For example, Baiguera and his colleagues directly stored human decellularized tracheas in PBS solution containing 1% antibiotic and antimycotic at 4 °C for 1 year [22]. Their results demonstrated that stored decellularized tracheas degraded steadily with time resulting in a loss of ECM architecture and decreased mechanical and angiogenic properties.

Cryopreservation has been widely studied for decellularized tissue preservation [23]. Urbani and his coworkers generated decellularized rabbit esophagi as a therapeutic alternative to treat congenital and acquired esophageal diseases. To biobank this product, they slow-cooled samples in media with 10% DMSO at 1 °C/min, then stored them in liquid nitrogen vapor and compared the outcome with cold storage in PBS solution at 4 °C. They quantified and detected no changes or increase in collagen/elastin/GAG composition during the cryopreservation storage period, from 2 weeks up to 6 months, whereas collagen fibers were preserved in 4 °C samples for only 4 weeks of storage.

Another study published by the Cook group [24] focused on the effect of freezing/thawing on decellularized organs

without using CPAs. Whole decellularized porcine kidneys were sequentially frozen at − 20 °C for at least 24 h, then at − 80 °C for 12 h, followed by slow warming (without phase transition) at − 20 °C for 24 h, and finally thawing at 4 °C for 12 h. Mechanical testing indicated that the elastic modulus of native kidneys was reduced by the decellularization, and the elastic modulus for decellularized ECM was essentially unchanged by the freezing/thawing process. Cellular damage and removal was found to be primarily responsible for reduced stiffness rather than fibril destruction. No adverse effect on the ability to recellularize after freezing/thawing was observed. Therefore, freezing/thawing without CPAs can be considered as a promising option for long-term preservation of decellularized porcine kidney.

Typically, fresh native tissues that are harvested for scaffold production are decellularized within 24 h to prevent degradation by bacterial growth. In order to provide the "off-the-shelf" availability of these resources, some studies have also investigated the use of cryopreservation to preserve native tissues/organs for this purpose [25–27]. In contrast to the decellularized scaffolds, the Cook group found that the elastic modulus of native kidneys was reduced by freezing/thawing and the increased porosity caused by ice crystal formation was also observed in the microstructure of frozen/thawed native renal tissues [24]. These adverse effects can lead to destructive effects that need to be mitigated or avoided using cryoprotectants in order to preserve the functionality of a whole organ. In addition, Tuan-Mu et al. observed that the efficiency of decellularization was influenced by freezing without any cryoprotectants [28]. In their study, native human umbilical arteries (HUAs) were frozen at cooling rate of 1 °C/min and then stored at − 20 °C (not cryogenic temperature) for 1 week prior to further decellularization or testing. Evidenced by DNA assay and histology every 12 up to 48 h, the efficiency of decellularization was observed to be significantly decreased in frozen HUAs compared to fresh ones, which may be attributed to condensation of ECM caused by ice crystal formation during freezing. Although little difference in the stiffness was observed in their studies, they suggested that avoiding freezing allowed more complete decellularization.

As an alternative to banking at cryogenic temperatures, storage of decellularized ECM in a stable dry state at room temperature is of high interest because it would render storage and transport easier and cheaper. Freeze-drying (also known as lyophilization) has already been used for preservation of the structural and mechanical properties of allografts (formerly called homografts) [29, 30]. Freeze-dried tissues are devoid of living donor cells and are capable of repopulation with autologous cells after transplantation [31]. For example, Wolkers' group freeze-dried porcine decellularized pulmonary heart valves using a 5% sucrose solution as a lyoprotectant to stabilize endogenous structural proteins and to form a protective glassy state at room temperature [32, 33]. A hypertonic sucrose solution with 80% concentration was used to diminish pore formation in freeze-dried ECMs by removing excess water from the decellularized ECMs prior to freeze-drying. Histological analysis indicated that matrix structures closely resembled those of control samples that were not freeze-dried. Heart valve matrices were shown to be in a glassy state after drying, suggesting that they can be stored at room temperature.

Growth Factor-Scaffold Delivery Systems

To facilitate the sustained release of bioactive molecules over an extended period of time, they are often incorporated into biomaterials during processing or otherwise are loaded into polymeric scaffolds after fabrication. The types, mechanisms of action, presentation strategies, and variables affecting growth factor delivery have been comprehensively reviewed by Lee and his colleagues [2]. Lyophilization has proven to be an effective growth factor integration approach while simultaneously enabling stabilization of the construct. In addition to providing storage stability, lyophilization has been a useful adaptive processing method for overcoming delivery problems such inaccurate dose, uncontrolled flow, and loss of loaded growth factors that can occur with other methods [34].

Despite success with lyophilization of a range of constructs, slow or decreased release characteristics have been somewhat problematic in bone tissue engineering applications [35, 36]. Lyoprotective agents, often used as stabilizers to protect proteins from freezing- and drying-derived stresses, can enhance the release of growth factors. Zhao and his coworkers used trehalose as a lyoprotectant to develop a porous calcium-deficient hydroxyapatite (CDHA) scaffold for delivery of bone morphogenetic protein-2 (BMP-2) [37], a protein used to stimulate MSC differentiation into osteoblasts as well as proliferation and function of both chondrocytes and osteoblasts [38]. Lyophilized BMP-2/CDHA constructs prepared with trehalose significantly promoted osteogenic differentiation of bone marrow stromal cells (bMSCs) and new bone formation and demonstrated retention of over 70% of protein bioactivity after 5 weeks storage at 25 °C.

Apart from polymer scaffolds, other natural matrices can be used for delivery of growth factors. For example, human dentin matrices obtained from premolar teeth have been shown to contain growth factors including dental matrix protein-1 (DMP-1), dentin sialophosphoprotein (DSPP), and growth factor (TGF)-β1 [39]. Dentin samples cryopreserved with a proprietary protein stabilizing cocktail were able to express DMP-1 and DSPP after storage in liquid nitrogen for up to 6 months. The released concentration of growth factors was found to be statistically equivalent to those of non-cryopreserved dentin matrix [40].

Cell-Seeded Scaffolds

Porous Scaffolds Seeded with Cells

Seeding of cells onto pre-made porous scaffolds made of degradable biomaterials is a commonly used methodology for creating tissue replacements. Cryopreservation has been identified as an efficient approach for long-term preservation of these tissue-engineered constructs. Permeation of these 3D constructs with protective CPAs is typically achieved using diffusion-based methods. Depending on the tissue geometry cells at different locations within a tissue construct can be subjected to varying local concentrations of the CPA. Since high local concentrations of CPA may damage cells due to toxic effects and insufficient local levels could lead to inadequate protection during cryopreservation, the structure of engineered tissues presents significant challenges but also designs opportunities for adaptation of conventional cryopreservation methods used for cells. In order to overcome the shortcomings of diffusion-based methods, a variety of perfusion strategies can be used for tissues with vasculature or other appropriate architecture for delivering CPAs under forced flow conditions (Fig. 3). For example, Petrenko and coworkers developed a perfusion-based bioreactor system to minimize exposure time of the cells to CPA. They found that using a perfusion bioreactor to control the addition and removal of CPA to/from cells within engineered tissues resulted in higher cell viability and higher recovery rates compared to conventional diffusion methods [41••].

Another critical challenge associated with the cryopreservation of engineered tissues is the choice of CPAs. DMSO has been used extensively as a CPA for the preservation of various cells and tissues [10, 42], but it may cause adverse effects upon infusion and has an epigenetic effect on cells [43, 44]. As a result, alternative methods that reduce the levels of or avoid DMSO in freezing solutions have recently been a focus of development efforts. Bissoyi and his coworkers evaluated nine different freezing solutions to cryopreserve cell-seeded engineered constructs [45]. The freezing medium consisting of trehalose (40 mM), ectoin (40 mM), catalase (100 μg), and DMSO (2.5%) was found to be the most effective to cryopreserve MSCs derived from umbilical cord seeded on electrospun nanofibrous silk fibroin scaffolds.

In addition to DMSO, fetal bovine serum is often included in the standard cryopreservation solutions for preservation of cells and tissues [27]. However, FBS carries the risk of transmitting viral and prion diseases and causing immunologic rejection [46, 47]. In the studies of Zhang and his colleagues, tissue-engineered vascular graft was constructed by seeding adipose-derived stem cells (ASCs) on the luminal surface of decellularized human saphenous vein, flow conditioned, and subsequently cryopreserved in 5% DMSO plus 95% autologous human plasma instead of fetal bovine serum for 10 days [48]. They found that replacement of fetal bovine serum with autologous human plasma does not significantly impair proliferation or endothelial differentiation of ASCs used as endothelial cell substitutes. In addition, it was also found that cryopreservation did not significantly alter ASC viability, proliferation, acquisition of endothelial characteristics, and retention after seeding onto a vascular graft.

A hydrogel that has cryoprotective properties could be a good alternative for the storage of tissue-engineered constructs or cell-based systems. Jain and his colleagues demonstrated that carboxylated poly-L-lysine (COOH-PLL), which is classified as a polyampholyte, yields excellent post-thaw survival efficiency of preserved cells [49•]. Cryoprotective properties are generally found in polyampholytes, and the balance of positive and negative charges is very important. Moreover, dextran has

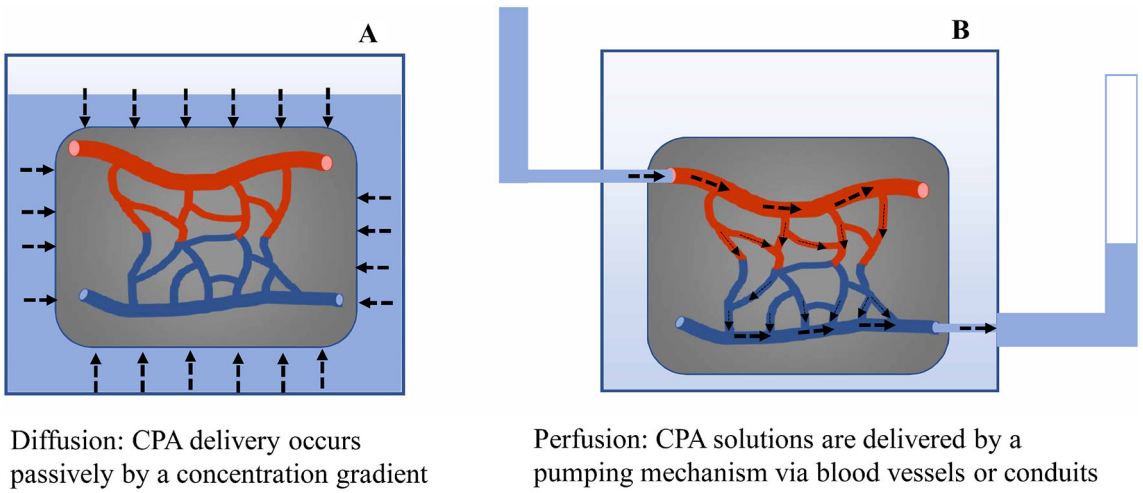

Fig. 3 Schematic of diffusion- and perfusion-based delivery of cryoprotectant agents (CPAs) for a conceptual vascularized tissue unit. CPA solutions (blue) can **a** diffuse into the tissue unit across a concentration gradient or **b** be delivered through the circulatory system into a tissue by a pumping mechanism, where the CPAs then diffuse from the blood vessels. Arrows indicated the direction of CPA delivery

reactive hydroxyl groups that can be modified to introduce positive and negative charges and also to introduce functional groups that enable the formation of an in situ hydrogel. This group attempted to make a dextran-based polyampholyte hydrogel that shows cryoprotective properties, which could be useful for cell encapsulation and tissue engineering applications involving hydrogel formation. In their studies, L929 cells encapsulated with such in situ hydrogels were cryopreserved well without the addition of any cryoprotectants. They succeeded in introducing 0.041 mg ml^{-1} of the cell attachment peptide RGDS into dextran and found that cells could attach to and proliferate on the hydrogel during culture over 7 days. Thus, these hydrogels can serve as scaffolds with cryoprotective properties that also provide structural integrity to tissue constructs.

Conventional cryopreservation usually leads to ice formation that causes extracellular matrix distortion and damage. As an alternative cryopreservation technology, vitrification has been widely used for the long-term storage of living cells and tissues [27]. To vitrify solutions, the viscosity is rapidly increased by decreasing the temperature using an extremely fast cooling rate until an amorphous "glassy" solid forms. This process avoids the crystallization of ice during cooling and rewarming and is a good preservation strategy for engineered tissues. Dahl and his coworkers used vitrification to preserve smooth muscle cells that were seeded onto a PGA mesh. They compared VS55 solution consisting of 3.10 M DMSO, 3.10 M formamide, and 2.21 M 1,2-propanediol in Euro-Collins solution, to a standard freezing method with a mixture containing 1 M DMSO, 2.5% chondroitin sulfate, and 10% fetal calf serum [50]. It was found that vitrified tissue had similar viability to fresh controls. The contractility results for vitrified samples were 82.7% of fresh controls, whereas the results for frozen samples were only 10.7% of fresh controls. Passive mechanical testing revealed excellent tissue strength after both freezing and vitrification.

Dry preservation of engineered tissues has also been receiving increasing attention due to the convenience of storage and transport compared to cryopreservation. Sun and his coworkers reported for the first time the freeze-dried preservation of 3D tissue-engineered skin grafts with trehalose and DMSO and their effect on the repair of mouse skin defects [51]. DMSO prevented ice crystal formation and protected fibroblasts during freezing, whereas trehalose acted to inhibit protein denaturation and to maintain the plasma membrane structure during desiccation, thus maintaining the viability and function of the engineered skin substitutes. The combination of trehalose and DMSO maintained cell viability at 37.55% of normal fibroblasts after treatment, which was significantly higher than that of the trehalose or DMSO group. The activity of cellular enzymes could be partially preserved by trehalose. The in vivo experiments demonstrated that freeze-dried TES were effective at stimulating skin defect healing after 4 weeks of preservation.

Cell Encapsulation in Self-assembled Hydrogel Matrix

Encapsulation is a process whereby living cells are entrapped within the confines of a semi-permeable membrane or within a homogenous solid mass. The biomaterials used for encapsulation are usually hydrogels, which are formed by covalent or ionic cross-linking of water-soluble polymers. Naturally occurring polysaccharides (ex. algal sodium alginate) are commonly used materials while other natural materials such as agarose and chitosan and synthetic materials such as poly (ethylene glycol) (PEG) and polyvinyl alcohol are also used. Artificial hydrogels can enable maintenance of cell-cell interactions via immobilization during preservation processing and, when combined with CPAs to protect against intracellular ice crystal formation, can support a complete preservation solution.

Popa and coworkers examined the effects of cryopreservation on the chondrogenic differentiation characteristics of human adipose-derived stem cells (hASCs) encapsulated in κ-carrageenan hydrogels, a very versatile, thermosensitive hydrogel used to develop ionotropic matrices for in situ immobilization of cells. The results at a macroscopic level suggested that the procedure did not significantly affect hydrogel structural integrity, as the morphology and stability of hASCs-kCR hydrogels were not altered radically by the cryopreservation process. DMA data indicated that the hASCs-hydrogels exhibit typical viscoelastic behavior and mechanical properties after a freeze-thaw process. Thus, cell encapsulation systems based on natural-based hydrogels seem to be an interesting approach for the preservation of cartilage tissue-engineered products [52]. However, their studies demonstrated that ice formation/nucleation within the hASCs-hydrogel resulted in a decrease in cell viability and proliferation, suggesting that further optimization is necessary.

Vitrification is a promising alternative to conventional cryopreservation of hydrogel-encapsulated cells because it avoids ice formation. Wu and his coworkers studied a vitrification strategy for human mesenchymal stem cells (hMSCs) seeded onto the surface of microcarriers made from alginate coated with chitosan and collagen [53]. The vitrification strategy included stepwise exposure to a vitrification solution (40% *v*/*v* EG and 0.6 M sucrose) and immersion into liquid nitrogen. They found that by using a vitrification strategy together with advances in their hMSC-expansion platform, they were able to completely preserve the ability of stem cells to proliferate and subsequently differentiate. In a different study, alginate-encapsulated mesenchymal stromal cells (AE-MSCs) were vitrified in a solution containing 10% DMSO, 20% ethylene glycol, 20% 1,2-polyethylene glycol, and 0.5 M sucrose (total 8.5 M) and stored in liquid nitrogen for 1–3 days [54]. After vitrification, AE-MSCs were able to differentiate into adipogenic and osteogenic lineages, confirming the preservation of the unique functional properties of MSCs.

Using Preservation Technologies as Part of the Manufacturing of Scaffolds

The generation of 3D scaffolds for tissue engineering can be achieved with various advanced fabrication techniques, including both conventional methods and additive biomanufacturing approaches [1]. Conventional techniques, including solvent casting/salt leaching, phase separation, forming, and textile meshes, usually do not enable proper control of pore size, pore geometry, and spatial distribution of pores and are not amenable to the construction of internal channels within the scaffold. Therefore, biomanufacturing additive fabrication processes are generally preferred for the fabrication of scaffolds for tissue engineering. The main advantages of these techniques are the capacity to rapidly produce very complex 3D models in a layer-by-layer fashion and the ability to use a range of raw materials. Scaffolds can be produced with customized external shapes and defined internal morphologies, allowing good control of pore size and pore distribution. These techniques include stereolithographic processes, laser sintering, extrusion, and 3D printing. Because of the tendency for biological components to rapidly degrade, it is not surprising that preservation is now becoming embedded as part of the biomanufacturing process.

The conventional 3D printing process typically employs plastics, and the printing process usually occurs in open air and at room temperature. The phase transition temperature of the molten plastic is higher than the room temperature. Although scaffolds made by conventional 3D printing have fully controlled shape, tailored interconnectivity, and sufficient mechanical strength, the hierarchical porous structure and biological properties of scaffolds must also be controlled to enable appropriate cell behavior. The biggest shortcoming of conventional 3D printing technology is that the melting of polymer wires to form fluidic inks generally requires high temperature (between ~180 °C for commercial polylactic acid and ~220 °C for acrylonitrile butadiene styrene), a process that would damage any incorporated biomolecules. 3D printing thus has been adapted to produce scaffolds that protect biological function via the use of "bio-inks" that support biological function [55].

Adamkiewicz and Rubinsky recently described a prototype that facilitates 3D printing with freezing in order to support tissue engineering [56••]. They found that the outcome of a freezing process can be controlled by immersing the 3D printed object in a liquid with a lower temperature than the phase transition temperature of the printed material and that previously printed frozen layers remain at a constant temperature, as illustrated in Fig. 4.

Wang and his colleagues investigated a novel technique combining extrusion-based 3D printing and emulsion freezing/cryodrying for producing hierarchically porous structured bone tissue engineering scaffolds with tunable osteoconductivity and osteoinducivity [57]. In their study, water-in-oil emulsions and recombinant human bone morphogenetic protein-2 were used as printing inks and extruded onto a cryogenic platform (− 30 °C) through the nozzle of a modified commercial 3D printer in a programmed way. After cryogenic 3D printing, the frozen scaffolds were cryo-dried in a fume hood for 1 h to remove organic solvents. Using this

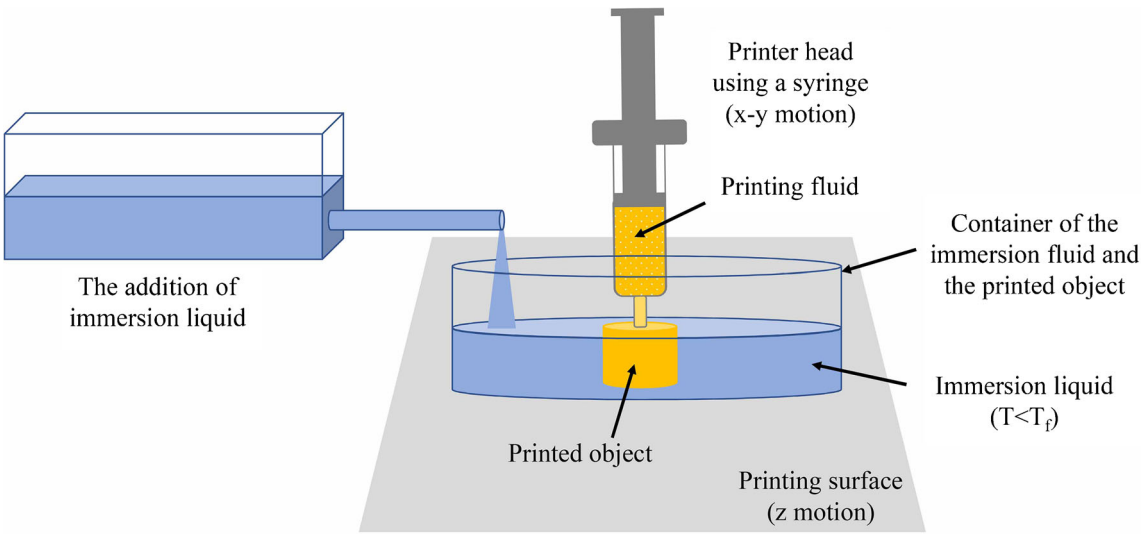

Fig. 4 Schematic of cryogenic 3D printer. The printing head can dispense the printing fluid at a controlled rate, through a syringe, and can move in the x-y plane, while the printer surface has a computer controlled z-direction motion. A container for the immersion fluid and the printed object rests on the printing surface. The immersion fluid temperature should be controlled and lower than the freezing temperature. The immersion liquid height is increased simultaneously with the printer head

method, the scaffolds displayed hierarchical porous structure similar to human cancellous bone. The presence of micropores on the strut surface may be attributed to the formation of ice particles during the cryogenic 3D printing and the subsequent removal of solvents, while the larger pores on the strut surface of the poly(L-lactic acid)-based scaffolds may be attributed to the higher water volume-induced water droplet fusion, which subsequently formed larger ice particles. Because the cryogenic 3D printing did not require the use of any cross-linking agent and all organic solvents could be completely removed through subsequent cryodrying, the 3D-printed scaffolds have shown high cytocompatibility, which would facilitate the growth of hBMSCs throughout the 21-day culture period. This new technology has great potential for manufacturing scaffolds.

The development of biomanufacturing processes that incorporate preservation science can enable the faster scale-up and deployment of engineered tissues that are amenable to storage via conventional means and thus supported by existing infrastructure. Success on this front will require more engagement between communities that tend to work along parallel, but not necessarily integrated, pathways.

Conclusions

In this review, we summarized preservation technologies that can be used to preserve the key supply chain components involved in tissue engineering strategies (cells, growth factors, and biological scaffolds), as well as the mature constructs. To support "off-the-shelf" availability of engineered tissues, cryopreservation technologies can be an effective strategy. In the case of acellular scaffolds and growth factor-based scaffolds, lyophilization yields a dried product that is more convenient for commercialized storage and shipment. A strategy for fabricating cell-seeded scaffolds at cryogenic temperatures in order to cryopreserve engineered tissues as part of the manufacturing process was also presented. While still an early stage concept, this approach could provide a synergistic manufacturing and preservation approach that would be transformative for 3D bioprinting applications.

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Compliance with Ethical Standards

Conflict of Interest Shangping Wang and Gloria D. Elliott declare that they have no conflict of interest.

Human and Animal Rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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