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REVIEW

Advanced Biotechnology for Cell Cryopreservation

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Abstract
Cell cryopreservation has evolved as an important technology required for supporting various cell-based applications, such as stem cell therapy, tissue engineering, and assisted reproduction. Recent times have witnessed an increase in the clinical demand of these applications, requiring urgent improvements in cell cryopreservation. However, cryopreservation technology suffers from the issues of low cryopreservation efficiency and cryoprotectant (CPA) toxicity. Application of advanced biotechnology tools can significantly improve post-thaw cell survival and reduce or even eliminate the use of organic solvent CPAs, thus promoting the development of cryopreservation. Herein, based on the different cryopreservation mechanisms available, we provide an overview of the applications and achievements of various biotechnology tools used in cell cryopreservation, including trehalose delivery, hydrogel-based cell encapsulation technique, droplet-based cell printing, and nanowarming, and also discuss the associated challenges and perspectives for future development.

Keywords Cell cryopreservation · Biotechnology · Trehalose delivery · Hydrogel-based cell encapsulation · Droplet-based cell printing · Nanowarming

Introduction

Cell cryopreservation is a technology used to preserve living cells, while maintaining their cellular viability and functions even at cryogenic temperatures (usually at − 80 °C or − 196 °C). At such ultra-low temperatures, the chemical, biological, and physical processes normally occurring at cellular level can remain suspended for a long time. In recent times, cell cryopreservation has become an important supporting technology for various cell-based applications such as stem cell therapy, tissue engineering, assisted human reproduction, and transfusion medicine [1]. The importance of cryopreservation technology is correctly reflected by the burgeoning demand of stem cell therapy, owing to which approximately 400,000 units of umbilical cord blood have been stored worldwide for public use and 900,000 units for private use [2]. As per the report of Stem Cell Banking Market, the global stem cell banking market had a current value of ~ 18.2 billion dollars in 2017, and this will reach ~ 54.1 billion dollars by 2024. Red blood cell cryopreservation has also gained importance in the past few years. Cryopreservation of red blood cells (RBCs) can extend the storage time from 42 d (hypothermic preservation) to 10 years, which will ease the burden of short blood supply, especially in remote areas [3].

However, during freezing–thawing cycles, cells inevitably suffer from cryoinjuries, including solution injury and ice injury. The freeze concentration-induced excessive dehydration can damage cells resulting in solution injury. Besides this, ice formation and growth during cryopreservation can mechanically damage the biological structure of cells resulting in ice injury [4] (Fig. 1a). Cryoprotectants (CPAs) play a pivotal role in protecting cells against these cryoinjuries and allow their successful storage at cryogenic temperatures. CPAs can be broadly classified into two main categories on the basis of the permeability or non-permeability of CPAs into the cellular membrane [5]. Permeating CPAs mainly

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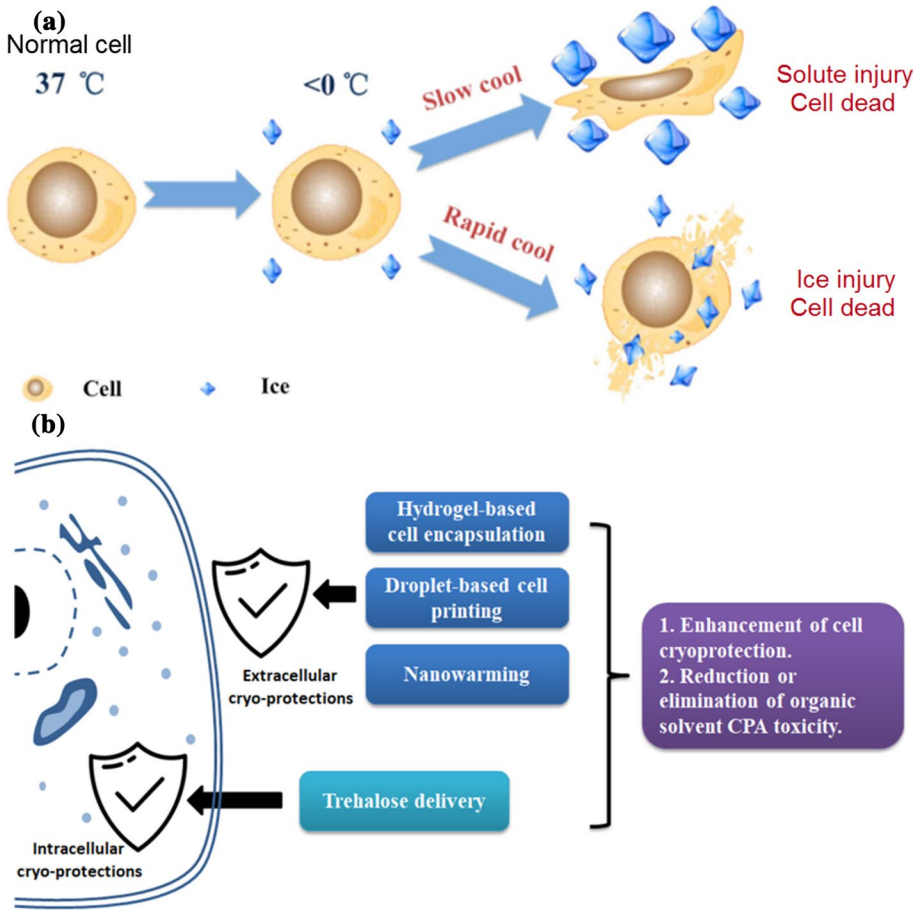

Fig. 1 **a** Schematic diagram for two types of cryoinjuries occurring during cryopreservation of cells; **b** schematic diagram for various biotechnology techniques used in cryopreservation and their method of protection

include organic solvents, such as glycerol and DMSO, which can permeate phosphate bilayers. This permeability is usually driven by a concentration gradient and confers intracellular protection to cells [6]. However, most of the organic solvent CPAs exhibit toxicity or poor biocompatibility that can cause serious side effects in patients like hemolysis, neurotoxicity, cardiovascular failure, respiratory arrest, and fatal arrhythmias [7, 8]. In comparison with this, non-permeating CPAs provide extracellular protection only. These include natural non-toxic carbohydrates (such as trehalose and sucrose) and biomacromolecules (such as proteins and polymers) [6]. Generally, non-permeating CPAs are combined with permeating CPAs to ensure both extracellular and intracellular protection, where the latter is required for the critical protection of cells from inside. This results in a compromise between high cryopreservation efficiency and CPA toxicity [9].

For most cell types, conventional cryopreservation protocol involves stepwise freezing of sample at slow cooling rates using 10–20% DMSO solution. In order to improve cell cryopreservation efficiency, previous studies have mainly focused on the optimization of CPA formulation, CPA introduction, and freezing–thawing protocol suitable for different cell types [10, 11]. Pollock et al. [12] reported the use of a differential evolution algorithm to optimize cryopreservation protocols for Jurkat cells (300 mmol/L trehalose, 10% glycerol, and 0.01% ectoine at 10 °C/min) and mesenchymal stem cells (300 mmol/L ethylene glycol, 1 mmol/L taurine, and 1% ectoine at 1 °C/min), which resulted in post-thawing cell viabilities of 95% and 96%, respectively. However, the optimization of cryopreservation protocol still suffers from two major challenges: (1) unfavorable post-thaw cell viability or functions and (2) safety concerns induced by CPA toxicity. For cryopreservation of hepatocytes, Mahler and co-workers [13] reported only 61–75% survival of isolated cells post-cryopreservation, and post-thawing cell attachment efficiency of 30–39%. Besides this, there are reports where the critical functions in some of the therapeutic cells such as mesenchymal stem cells (MSCs), natural killer cells, and dendritic cells (DCs) were compromised after use of conventional cryopreservation protocol [14–17]. Organic solvents glycerol and DMSO are widely used in intracellular protection; however, both lack biocompatibility. Glycerol can induce severe hemolysis [18], while the use of DMSO is found to be associated with many side effects in patients, like neurotoxicity, cardiovascular failure, respiratory arrest, fatal arrhythmias, and others [19]. Fetal bovine serum (FBS) is frequently combined with organic solvents to supplement

extracellular cryopreservation. However, FBS is derived from animals and has risk of inducing viral infections and immunogenicity inpatients [20, 21].

In recent years, the developments of biotechnology provided an opportunity to improve the final outcome of cell cryopreservation. Generally, biotechnology refers to use of scientific techniques to study and address the problems associated with living organisms. In this review, we summarize the applications and achievements of various advanced biotechnology tools used in cell cryopreservation. These include trehalose delivery, hydrogel-based cell encapsulation technique, droplet-based cell printing, and nanowarming. All these techniques broadly aim to enhance cell cryopreservation and reduce or eliminate CPA toxicity. As shown in Fig. 1b, trehalose delivery is proposed to deliver non-permeable but biocompatible trehalose into cells as an alternative to organic solvents, with the aim to provide intracellular cryopreservation. The other three techniques mainly enhance extracellular protection during freezing or warming process and thus aim to increase cryopreservation efficiency and significantly reduce the concentration of organic solvents. In addition to this, we discuss the challenges and provide future perspectives for the development of biotechnology tools used in cell cryopreservation.

Trehalose Delivery

Trehalose is a non-permeating disaccharide, which is used as a bio-inspired CPA to protect cells or organisms against cryoinjuries [22]. It facilitates the formation of a stable glassy matrix and promotes preferential hydration in cellular biomolecules, stabilizing their functional conformations [23–25]. However, trehalose generally provides extracellular protection only owing to its lower permeability. For intracellular cryopreservation, it is used in combination with organic solvent CPAs (glycerol or DMSO) [26–28]. In order to avoid the toxicity of organic solvents, several advanced biotechnology tools have been used to deliver trehalose into cells to provide both intracellular and extracellular cryopreservation. These techniques help to achieve organic solvent-free cell cryopreservation and high post-thaw cell survival efficiency [29]. These biotechnology tools, including both physical and chemical methods, can increase cellular membrane permeability and thus transport non-permeable trehalose into cells (Table 1).

Physical Delivery Method

The physical delivery methods, including freezing-induced membrane phase transition [30–32] and electroporation technology [33–35], have been used to promote the permeation of trehalose into cells. These methods help to achieve organic solvent-free cell cryopreservation. The loading of trehalose can be easily controlled by manipulating the concentration gradient. However, the increased membrane permeability achieved by these methods suffers from the issues of non-specificity, which results in uncontrolled influx and outflux of other molecules.

The use of freezing-induced membrane phase transition method for intracellular delivery of trehalose into cells was reported for the first time by Beattie et al. [36] in 1997, for cryopreservation of pancreatic islet cells. During the cooling process, changes occurring in the fluid-to-gel phase transition result in reorganization of membrane lipid components which increases the membrane permeability. A concentration gradient then drives the intracellular movement of trehalose to provide intracellular cryopreservation. Gläfke et al. [30] reported the use of high extracellular concentrations of trehalose for freezing platelets. This method resulted in 98% membrane intact platelets, 76% of which were in non-activated resting state. This platelet cryopreservation protocol avoided any use of DMSO.

For more than 40 years, electroporation technology has been widely used for intracellular delivery of xenomolecules such as saccharides, drugs, plasmids, DNA vaccine, siRNA, and proteins. It offers several advantages like controllability, reproducibility, and high efficiency. Application of an external electric pulse assists in the formation of hydrophilic pores on the membrane resulting in an increase in membrane permeability. This pore formation can be reversible or irreversible depending on the electric pulse conditions [37–41]. In two separate studies, Dovgan et al. [33] reported the use of electroporation for efficient loading of trehalose into human adipose-derived stem cells (hADSCs) and umbilical cord mesenchymal stem cells (UC-MSCs). For electroporation in hADSCs, the cells were incubated in 250 mmol/L trehalose and electroporated at the optimal conditions of 1.5 kV/cm^2, at 8 pulses, 100 µs, and 1 Hz, prior to programmable slow freezing. After thawing the cells, 83.8 ± 1.8% cell recovery rate was observed, which was similar to that of hADSCs (91.5 ± 1.6%) obtained using standard freezing protocol (10% DMSO in 90% FBS). In comparison with this, the electroporation of UC-MSCs under the optimal electroporation conditions (430 V, 8 pulses, 100 µs, and 1 Hz) resulted in 61% cell viability [34]. It has been previously shown that high voltage can result in great loss of cell viability, while insufficient voltage compromises the delivery of trehalose into cells. Therefore, to ensure efficient trehalose delivery and favorable post-thaw cell viability, it is important to optimize suitable voltage conditions for different cell types.

Table 1 Different trehalose delivery methods used for cell cryopreservation

Delivery methods		Cell type	Main results		Advantages	Disadvantages
Physical delivery	Freezing-induced lipid-phase transition	Platelets	Post-thawing, 98% platelets showed/were having intact membrane	Organic solvent-free cell cryopreservation based on trehalose	1. Trehalose loading into cells easily controlled by concentration gradient 2. Reversible based on external stimuli 3. Simple processing	1. Non-specific membrane permeability 2. Membrane injury concerns induced by thermal and electric shock
	Electroporation	hADSCs	1. 84% cell survival rate post-thawing 2. Maintenance of normal cell proliferation and differentiation potential			
		UC-MSCs	61% cell survival rate post-thawing			
Chemical delivery	PP50	Erythrocyte	1. 123 mmol/L of intracellular trehalose was achieved in erythrocytes 2. 83% post-thaw erythrocyte survival		Loading of high amounts of intracellular trehalose.	1. Non-specific membrane permeability 2. Long incubation time 3. Cytotoxicity concerns 4. Tedious washing step 5. Complex material preparation
		SAOS-2	The number of metabolically active cells at 24 h post-thaw was between 91% and 103%			
	α-Hemolysin	Fibroblasts and keratinocytes	Long-term post-thaw survival rate was 80% for fibroblasts and 70% for keratinocytes		1. Reversible based on external stimuli. 2. Trehalose loading into cells easily controlled by concentration gradient	
	Genipin-cross-linked Pluronic-F127 nanoparticles (GNPs)	hADSCs	Approximately 90% cell viability and normal differentiation potentials, and distinctive markers expression was maintained		1. Utilize natural endocytosis process 2. Specific transport of high amounts of trehalose	1. Long incubation time 2. Complex material preparation
	Apatite nanoparticles	RBCs	Increasing RBC cryosurvival up to 91%, which is comparable to FDA-approved cryopreservation protocol employing glycerol			

Chemical Delivery Method

Chemical delivery methods utilize cell membrane perforating agents like synthetic polymers [42, 43], α-hemolysin [44–46], and nanoparticles [47–49] to achieve intracellular delivery of trehalose. These chemical materials usually involve a complex preparation process. They can interact with cell membranes and increase membrane permeability temporarily. Trehalose has been used as sole CPA in combination with these chemical delivery methods to successfully cryopreserve RBCs, fibroblasts, keratinocytes, and human mesenchymal stem cells (hMSCs).

Mercado et al. [43] designed and synthesized a series of biomimetic derivatives of PLP polymer to facilitate intracellular delivery of trehalose. Particularly, co-incubation of PP50 (composed of PLP grafted with L-phenylalanine) with erythrocytes and trehalose suspension increased the intracellular concentration of trehalose to 123 ± 16 mmol/L. After cryopreservation of erythrocytes loaded with trehalose, the erythrocyte survival rate was $82.6 \pm 3.4\%$, which was $20.4 \pm 5.6\%$ higher as compared to the unloaded erythrocytes. PP50-mediated trehalose delivery method has been also used in organic solvent-free cryopreservation of nucleated human cell line SAOS-2. Although the post-thaw cell viability was only $60 \pm 2\%$, the number of metabolically active cells at 24 h post-thaw was in the range of 103 ± 4 to $91 \pm 5\%$. This was comparable to the results observed for cells frozen using DMSO [42]. Mechanically, PP50 adsorption onto the membrane contributed by its amphipathic characteristic induced the appearance of thinner phospholipid bilayer, which resulted in an increase in trehalose uptake [42]. However, longer incubation times in cryopreservation of erythrocytes might result in undesirable hemolysis of cells. Thus, PP50 must be removed to overcome the safety issues, which generally involves a tedious washing procedure.

α-Hemolysin is a genetically engineered endotoxin derived from *Staphylococcus aureus*. It has been shown to work via generation of pores in the lipid bilayers for both fibroblasts and keratinocytes, allowing an influx of trehalose [46, 50]. Buchanan et al. [46] used α-hemolysin to achieve intracellular trehalose concentrations of up to 0.5 mol/L. Trehalose at concentration of only 0.2 mol/L provided cryopreservation in fibroblasts and keratinocytes, with trehalose as sole CPA. After long-term cryopreservation, the post-thaw survival rates were 80% and 70% for fibroblasts and keratinocytes, respectively. Despite being so promising, α-hemolysin being a bacteria-derived pore protein may induce undesirable immune responses in patients. Therefore, it must be removed from cells prior to their use in clinical therapy. The safety concerns and necessary removal steps associated with the use of α-hemolysin limit its clinical application.

Nanomaterials are widely used in medical field especially as vehicles for the delivery of drugs, such as chemical molecules, DNA vaccine, and protein or peptide drugs, to therapeutic target [51–54]. In recent years, several studies have reported efficient delivery of trehalose into cells using nanomaterials. The nanoparticles generally utilize the natural process of endocytosis to specifically deliver trehalose into cells without any harmful effects [29]. The use of nanoparticles for trehalose delivery and cryopreservation of cells with trehalose as sole CPA has been reported to maintain cell viability and functions. Rao et al. [48] developed a pH-responsive genipin-cross-linked Pluronic F127–chitosan nanoparticle (GNP), which efficiently encapsulated trehalose for intracellular delivery (Fig. 2a). For cryopreservation of hADSCs, the cells were incubated with trehalose-loaded GNPs (nTre) for 24 h and cryopreserved in culture medium containing 200 mmol/L free trehalose. After rewarming, pre-incubation with nTre resulted in 90% cell viability which was comparable to the cell viability obtained post-cryopreservation with DMSO. Besides this, the differentiation potential and the expression of distinctive markers in hADSCs remained unchanged upon cryopreservation. Another nano-vehicle used for delivery of trehalose is a type of biomimetic (bone-like) apatite nanoparticle. These apatite nanoparticles have been shown to efficiently deliver drugs and nucleic acids into various types of cells. Stefanic et al. [49] utilized colloidal bio-inspired apatite nanoparticles to mediate intracellular delivery of trehalose into RBCs. The local interactions between apatite NPs and the bilayer enhanced the translocation of trehalose into the cells (Fig. 2b). Cryopreservation of trehalose-laden RBCs demonstrated that the use of this glycerol-free cryopreservation protocol tremendously increased survival of RBCs to 91%, which was 42% higher as compared to the control without NP treatment. These results were comparable to the FDA-approved cryopreservation protocol that utilized glycerol as CPA. Nanoparticles-mediated intracellular delivery of trehalose has shown great potential to achieve clinical cryopreservation of therapeutic cells without any use of organic solvents. A possible limitation of this method could be requirement of long incubation time to achieve sufficient intracellular trehalose concentration. The incubation time required for trehalose delivery was 24 h and 7 h for GNPs and apatite nanoparticles, respectively.

Hydrogel-Based Cell Encapsulation Technology

Hydrogel-based cell encapsulation technology refers to the encapsulation of living cells within semipermeable capsules prepared using hydrogel materials. This technology has been found to be highly promising for various

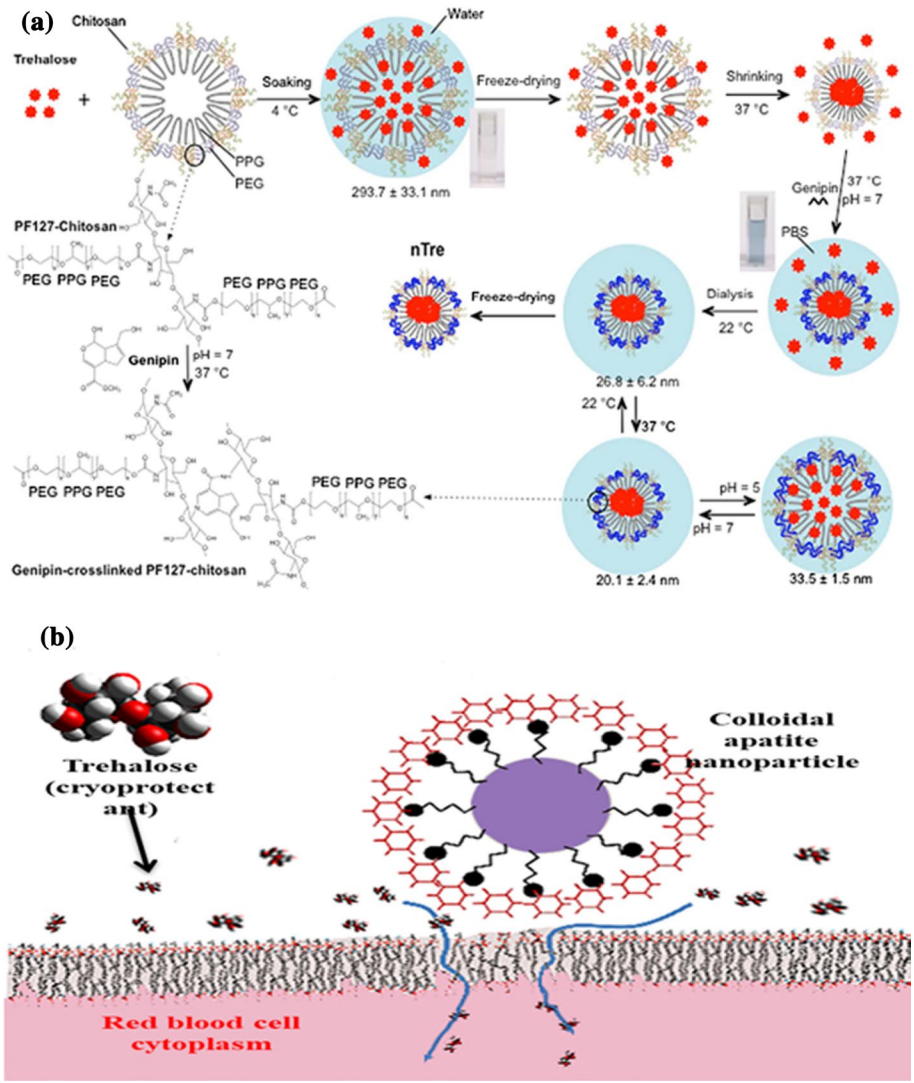

Fig. 2 Nanomaterials-mediated trehalose transportation into cells. **a** The encapsulation of trehalose in genipin-cross-linked Pluronic F127–chitosan nanoparticles (GNPs) to produce nanoparticle-encapsulated trehalose (nTre). **b** Interactions of biomimetic (bone-like) apatite nanoparticles with the lipid bilayer and enhanced delivery of trehalose into RBCs

cell-based studies and applications. This technology provides suitable 3D microenvironment similar to the extracellular matrix, blocks the immunogenicity of encapsulated cells, and directs the differentiation of stem cells [55–57]. In recent years, hydrogel-based cell encapsulation technology has been widely used in cell cryopreservation. The capsules not only protect the inner cells from mechanical and osmotic stress during the freezing and warming process, but also allow the bidirectional diffusion of nutrients, oxygen, and waste products. Numerous studies have established its positive effects on post-thaw cell viability and functions [58–60].

The method used for cell encapsulation greatly influences the final outcome of cell cryopreservation. These methods can be broadly divided into three categories, namely emulsion/thermal gelation, extrusion (electrostatic spray, air flow nozzle, and vibrating nozzle), and microfluidic method [58]. Choice of method depends on two main factors: capability to maintain high cell viability/function and ability to control the capsule phenotypic characteristic such as size, shape, strength, and permeability [61].

Emulsion Method

Emulsion is a generic method used to encapsulate cells within bulk hydrogels. In this method, pre-gel solutions are first prepared by suspending cells and gel materials. When the dispersion reaches an equilibrium state, gel formation is triggered by adding an initiator or changing the physical conditions, such as UV light. Although emulsion method is simple and easy to scale up, the process of gelation may result in cell death and loss of functions owing to the toxicity of initiator, chemical cross-linking, and unfavorable reaction conditions [58, 62].

In recent years, numerous novel cross-linking approaches have been used in the preparation of cell-loaded hydrogels to avoid the negative effects of conventional methods. PVA-based hydrogel including PVA–gelatin cryogels and

PVA–carrageenan (Car) scaffold was prepared via freeze-gelation technique. The encapsulation process only involved a freezing step without requirement of any external cross-linking agents. After cryopreservation, cell viability and functions were observed to be unaffected [63–66]. Zeng et al. [67] developed a supramolecular gel that also involved a cooling process to trigger the gel formation. For cryopreservation of encapsulated PC12 cells and RSC96 cells, a mixture of cell suspension, gelator, and DMSO was prepared and cooled in ice-water bath. Gelator self-assembled to form supramolecular gel at 8.2 °C and then programmed freezing of the system was carried out at − 80 °C (Fig. 3a). The post-thaw viability of PC12 and RSC96 cells increased significantly owing to the protection provided by the hydrogel during the freezing and thawing process. In addition to this, the thermo-reversible supramolecular gel could be removed easily by centrifugation. Jain et al. [68] reported a two-component molecular recognition gelation method that was

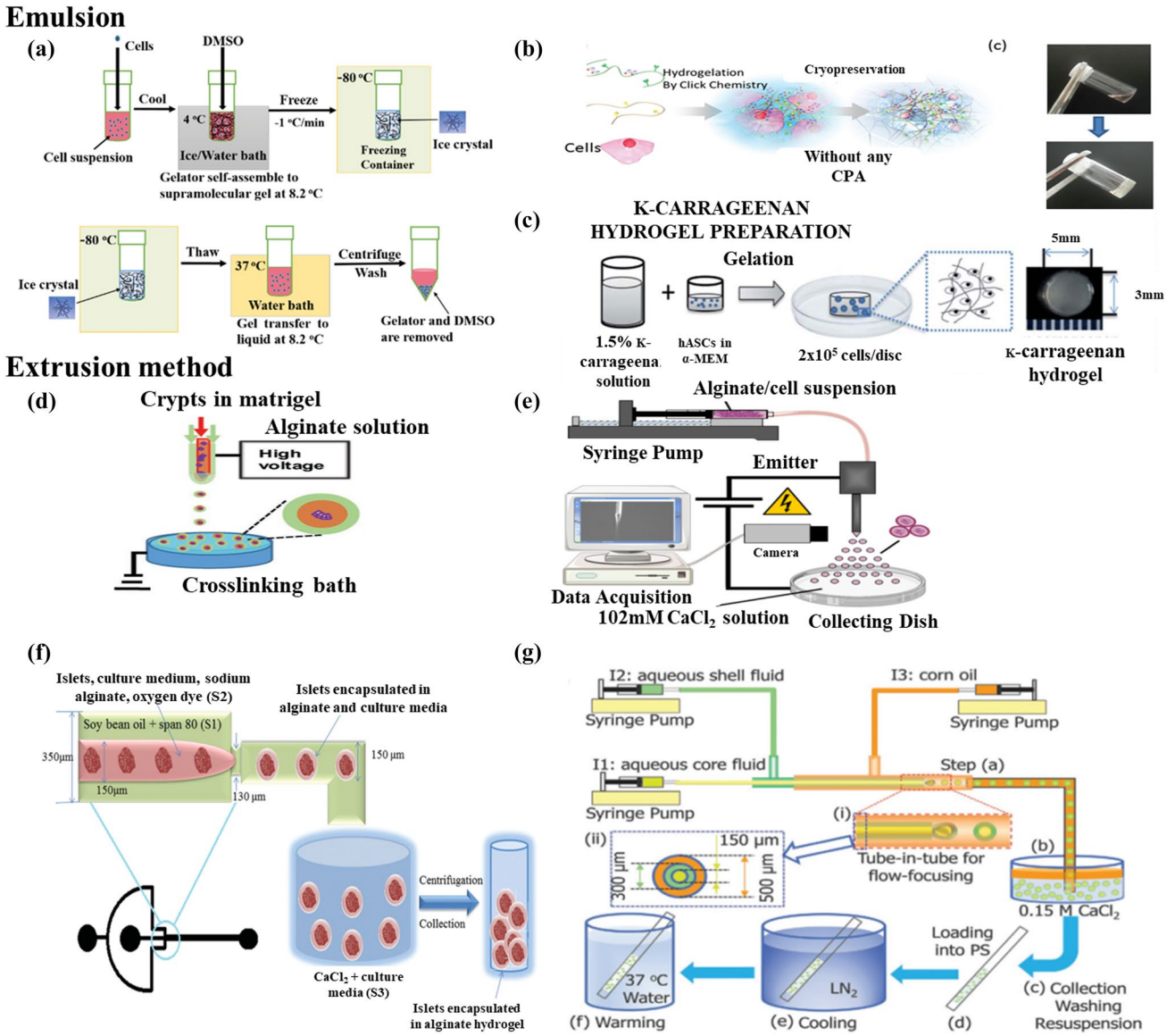

Fig. 3 Schematic diagram for cell encapsulation in hydrogel capsules by different methods. Emulsion method: **a** cooling and thawing process for cells encapsulated in supramolecular gel, **b** in situ hydrogelation via SPAAC click chemistry for cell encapsulation and cryopreservation, **c** preparation and cryopreservation process for hASCs–K-carrageenan hydrogel construct. Extrusion method: **d** a two-fluidic electrospraying method for encapsulation of cells in core–shell capsules. Microfluidic method: **e** electrohydrodynamic atomization (EHDA) method for fabrication of cell-laden microcapsules with uniform size, **f** encapsulation of individual rat islets into alginate hydrogel using a droplet microfluidic device at room temperature, and **g** preparation, vitrification and warming process for cell-laden alginate-based core–shell hydrogel produced using a double emulsion flow-focusing tube-in-tube capillary microfluidic device

adopted to develop a dextran-based polyampholyte hydrogel having cryoprotective properties. The gelation process was initiated by mixing of azide-Dex-PA and DBCO-Dex, and this gelation mainly depended on the reactant concentration (Fig. 3b). In the absence of any CPA, L929 cells encapsulated within these hydrogels showed a recovery rate of > 90% (in optimum gelation condition) after thawing. As a natural thermo-sensitive polymer, K-carrageenan could form stable hydrogel via ionic gelation process. Potassium chloride was used for the cross-linking of K-carrageenan, allowing further stabilization of the hydrogels (Fig. 3c). HMSCs encapsulated with K-carrageenan hydrogel showed improved proliferation and chondrogenic potential post-cryopreservation [69].

Extrusion Method

Extrusion methods majorly include two methods: electrostatic spray and air-jet encapsulation technology. These are commonly used for cell encapsulation owing to their high throughput and production of evenly sized beads [61].

Wolters et al. [70] developed an air-jet droplet generator and used it to produce small, uniform, and smooth alginate beads while maintaining high throughput. In 2010, Malpique et al. [71] investigated for the first time cryopreservation of brain cell neurospheres by encapsulating the cells within alginate hydrogel using the air-jet two-channel droplet generator. The results showed that the cell viability and metabolic activity were significantly higher in encapsulated neurospheres as compared to the non-encapsulated ones. It might be contributed by reduced fragmentation and better maintenance of spherical shape of aggregates upon encapsulation in alginate hydrogels. However, air-jet technology applied during microcapsule formation has several limitations such as use of harsh shearing forces and formation of air bubbles and "tails."

Cell encapsulation using electrostatic spray method involves generation of droplets containing cells and polymers from the nozzle, followed by spraying into a container with gelling bath to form hydrogel beads. The hydrogel beads formation is assisted by the electrostatic force between the gelling bath and the nozzle [71]. Zhang et al. [72] encapsulated mouse MSCs into small (~ 100 µm) Ca-alginate microcapsules generated by electrostatic spray method. The vitrification of cell-loaded microcapsules with low concentration of DMSO maintained high post-thaw cell viability in encapsulated cells. The Ca-alginate microcapsules provided great protection to the cells during cryopreservation. Two-fluidic electro-co-spraying technique was developed and adopted to continuously produce core–shell alginate capsules, which had better mass transfer and were used to encapsulate organoids (Fig. 3d). The core–shell structure of the capsules provided better cell recovery after cryopreservation of organoids, probably through prevention of intracellular ice formation [73–75]. Electrohydrodynamic atomization (EHDA) is an attractive approach that immobilizes living cells into biomaterials permitting localized and minimally invasive delivery. It minimizes cell leakage and maintains viability during the delivery process. Naqvi et al. [76] combined alginate and EHDA technique to fabricate bone marrow stomatal cells (BMSCs)-encapsulated microcapsules (Fig. 3e). The results of cryopreservation showed that micro-encapsulation of BMSCs within alginate maintained their cell viability and potential to synthesize sGAG and collagen. Electrostatic spray method offers several advantages including cytocompatibility, ease of operation, and high efficiency. Besides this, the manufacturing process of capsules could be performed in a sterile environment. Therefore, the electrostatic spray method is promising and suitable to encapsulate cell resources or CPTs for long-term storage purpose.

Microfluidic Method

Rapid development of micro- and nanotechnologies has allowed operation of cell encapsulation procedures on-chip [1]. Built on flow focusing, miniaturized devices are to encapsulate cells into capsules. Microfluidic methods permit a high degree of control over the morphological and dimensional properties. The experimental platforms are physically smaller than the macro-encapsulation systems. In addition to these, microfluidic method offers several advantages over macro-encapsulation systems, such as low cost, ease to scale up, disposability, specific designs, and rapid implementation [57, 76].

Many groups investigated the cryopreservation of cells encapsulated within hydrogel beads produced by microfluidic methods. In a droplet microfluidic platform, individual rat pancreatic islets were encapsulated with FOSD functionalized hydrogel microcapsules. This study aimed to establish single-islet-based quality control assay for assessing quality and functionality of individually cryopreserved islets (Fig. 3f). Hydrogel membrane surrounding the encapsulated islet effectively enhanced the insulin secretion after thawing. The unique microstructure of the hydrogel was characterized by the presence of a compact 3D porous network and considerable amount of non-freezable bound water, which may alleviate the cryoinjury to the cells, playing a role similar to the CPAs [77]. Large-volume low-CPA cell vitrification was achieved by microfluidic-based alginate hydrogel micro-encapsulation system (Fig. 3g) [78, 79]. HASCs in low concentration of CPA medium (2 mol/L) were encapsulated into core–shell microcapsules using an elaborate microfluidic system and then loaded into 0.25 mL conventional plastic straw (PS). These were directly plunged into liquid nitrogen to realize/induce vitrification. After thawing, hASCs liberated from the microcapsules showed no changes

in viability and differential capacity. In terms of mechanism, the microcapsule effectively inhibited the ice formation and further propagation during cooling and warming process. The IRI activity of microcapsules especially protected the cells against severe mechanical injuries.

Droplet-Based Cell Printing

Droplet-based techniques find wide applications in various fields, such as inkjet printing, emulsion polymerization, and DNA arraying, owing to their high efficiency and low cost [80–84]. Introduction of this advanced technology into cell cryopreservation created a series of novel vitrification protocols that are characterized by lower CPA concentration and higher cooling and warming rates. This is mainly applied to smaller volumes of cryo-system [85]. Droplet-based cell vitrification process not only solved the problem of high CPAs concentration required for conventional vitrification procedure, but also conferred significant protection to cells by reducing the time required for ice crystal formation and alleviating the osmotic shock. Besides this, the whole process is easy and quick, allowing a possibility of large-scale automation [86–88]. Therefore, this innovative approach may contribute significantly in the development of cell cryopreservation.

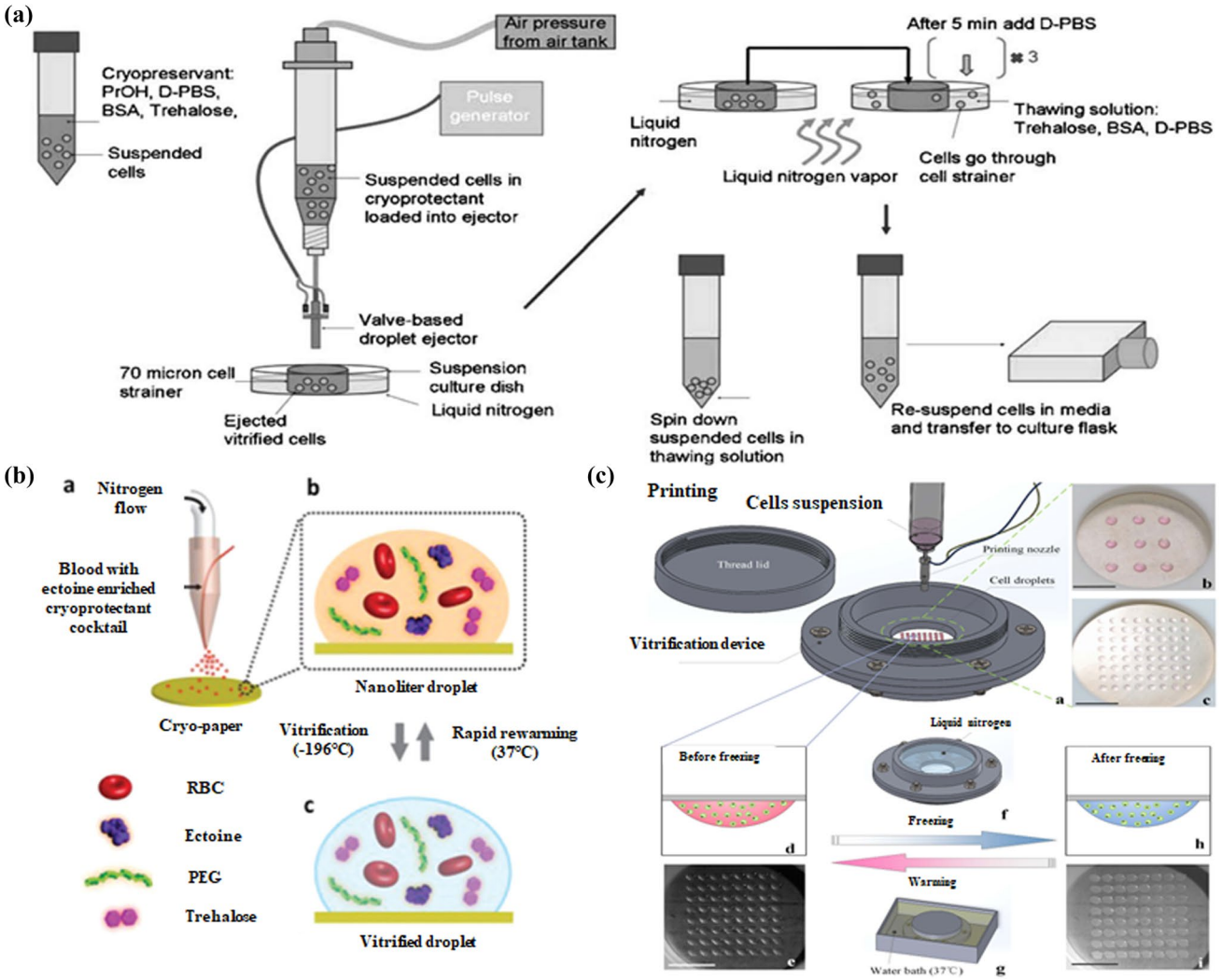

Fig. 4 Schematic diagram for preparation of cell-laden droplets using different devices. **a** Cell-CPA solution was loaded into valve-based droplet injector, and the resulting droplets were directly injected into liquid nitrogen. The ejected cells were collected in a cell strainer and were rapidly transferred (in nitrogen vapor) to the thawing media, followed by a step-by-step thawing process. **b** RBC-CPA droplets were printed onto a cryo-paper as nitrogen gas flowed through a droplet ejector, which transformed the bulk of the RBC-CPA mixture into nanoliter droplets. Vitrification was achieved by submerging the cryo-paper into liquid nitrogen. Warming process was performed by thawing the cells on a cryo-paper in phosphate-buffered saline at 37 °C. **c** Cell-laden droplets were rapidly ejected onto a freezing film using a cell printer with high throughput and precise spatial controllability. Vitrification/thawing process was achieved by pouring the liquid nitrogen/warm water onto the other side via boiling heat transfer that helped to maintain high cooling/warming rate

Demirci et al. [84] reported successful vitrification of many cell types by means of optimal droplet-based procedure. This technique involved generation of droplets containing cell and CPAs solution using a modified jet device, which were further received/transferred in a container filled with liquid nitrogen and warmed in 37 °C water bath (Fig. 4a). Before vitrification, droplet generation process did not affect the call viability and cell survival rate was maintained to approximately 90%. Propanediol and trehalose were used as CPAs instead of toxic DMSO for cell vitrification. Even after thawing, the viability of cells was maintained well.

Assal et al. [89] developed a novel cryo-printer that could transform a bulk volume of human blood into nanoliter cryo-inks on a cryo-paper, which was immersed into liquid nitrogen for rapid vitrification (Fig. 4b). After rewarming, the recovered human RBCs showed normal characteristic features. In addition to this, there was no effect on the essential functions of recovered RBCs, including phosphorylation of band 3 protein, expression of complement receptor 1, and maintenance of intracellular nitric oxide and reactive oxygen species levels. Besides these intrinsic advantages offered by droplet-based vitrification, the cryo-ink containing CPAs medium such as ectoine, trehalose, and PEG also reduced the injuries suffered by RBCs during the cooling and warming processes.

High-throughput non-contact vitrification of cell-laden droplets was reported by Shi et al. [90] in 2015. Cell printing generated droplets containing cell CPAs onto an ultra-thin freezing film. Vitrification/thawing process was operated by pouring liquid nitrogen/warm water onto the other side via boiling heat transfer. This ensured maintenance of high cooling/warming rate and avoided direct contact between cells and liquid nitrogen/water, preventing chances of potential contamination (Fig. 4c). The use of this novel method provided successful vitrification in NIH 3T3 cells and hASCs. After thawing, both cell viability and differentiation potential remained unaffected.

Recently, a CPA-free cryopreservation method-based inkjet cell printing technology was developed by Akiyama et al. [91]. It was successfully used for the vitrification of several mammalian cell types such as 3T3 cells, C2C12 cells, and rat MSCs at ultra-rapid cooling rates. The droplets containing cells and culture medium were printed onto a glass substrate cooled with liquid nitrogen to realize/induce solid-surface vitrification. Immediately after thawing, the viability of 3T3 cells for 40-pL droplets on thick substrates (thickness: 150 µm) was comparable to the cell viability obtained using conventional freezing method. The ultra-rapid cooling and warming rates significantly inhibited ice formation and ice recrystallization and also protected the cells against cryoinjuries during the freezing and warming processes.

Nanowarming

Conventional warming method (37 °C water bath) fails to provide sufficient warming rates and uniform warming effect with the increasing scale of bio-specimen. During rewarming process, once the warming rates go below the critical warming rates, ice recrystallization/devitrification occurs, which is one of the major causes of cell suffered injuries. However, advances in bio-specimen cooling for cryopreservation have not been matched well by similar developments in rewarming procedure [92]. Nanomaterials-mediated nanowarming technology has a potential to be established as a new approach to allow ultra-rapid and uniform rewarming [93]. Nanowarming generally involves use of some nanomaterials such as Fe_3O_4 nanoparticles (Fe_3O_4 NPs) or gold nanorods (GNRs) that can rapidly convert electromagnetic or light energy into heat energy. Thus, rapid and uniform rewarming of bio-specimens can be realized/achieved by incorporating these nanomaterials into CPA solution and heating with external electromagnetic, radiofrequency (RF) or laser fields [94–96]. Numerous studies have affirmed that nanowarming technology is significantly effective and can be used for improving cryopreservation of cells, tissues, and organs.

RF Inductive Warming Process

Magnetic iron oxide nanoparticles are capable of transforming external electromagnetic energy into heat energy rapidly. Modified iron oxide nanoparticles (msIONPs) characterized by compatibility, colloidal stability, and capability to remain in solution at higher concentration were synthesized by Manuchehrabadi et al. [93]. Human dermal fibroblasts (HDFs) were vitrified using VS55 cryo-solution loaded with msIONPs and warmed by different methods. In the 1 mL system, the viability of nanowarmed HDFs was statistically similar to the fresh control sample and higher than the viability of slow-warmed sample.

Liu et al. [97] successfully achieved low-CPA vitrification of stem cell-alginate hydrogel constructs by combining nanowarming and micro-encapsulation technology (Fig. 5a). Fe_3O_4 NPs were mixed with low-CPA solution for RF inductive warming process. After nanowarming, porcine adipose-derived stem cells (pADSCs) showed viability > 80%, while the attachment efficiency improved by three times as compared to the pADSCs treated with slow warming process. Besides these, the expression of surface markers and multi-lineage potentials of pADSCs after nanowarming remained unaffected. Mechanically, in addition to the cryopreservation provided by cryo-solution and alginate hydrogel, Fe_3O_4 NPs uniformly present outside of hydrogel further suppressed devitrification and recrystallization during nanowarming

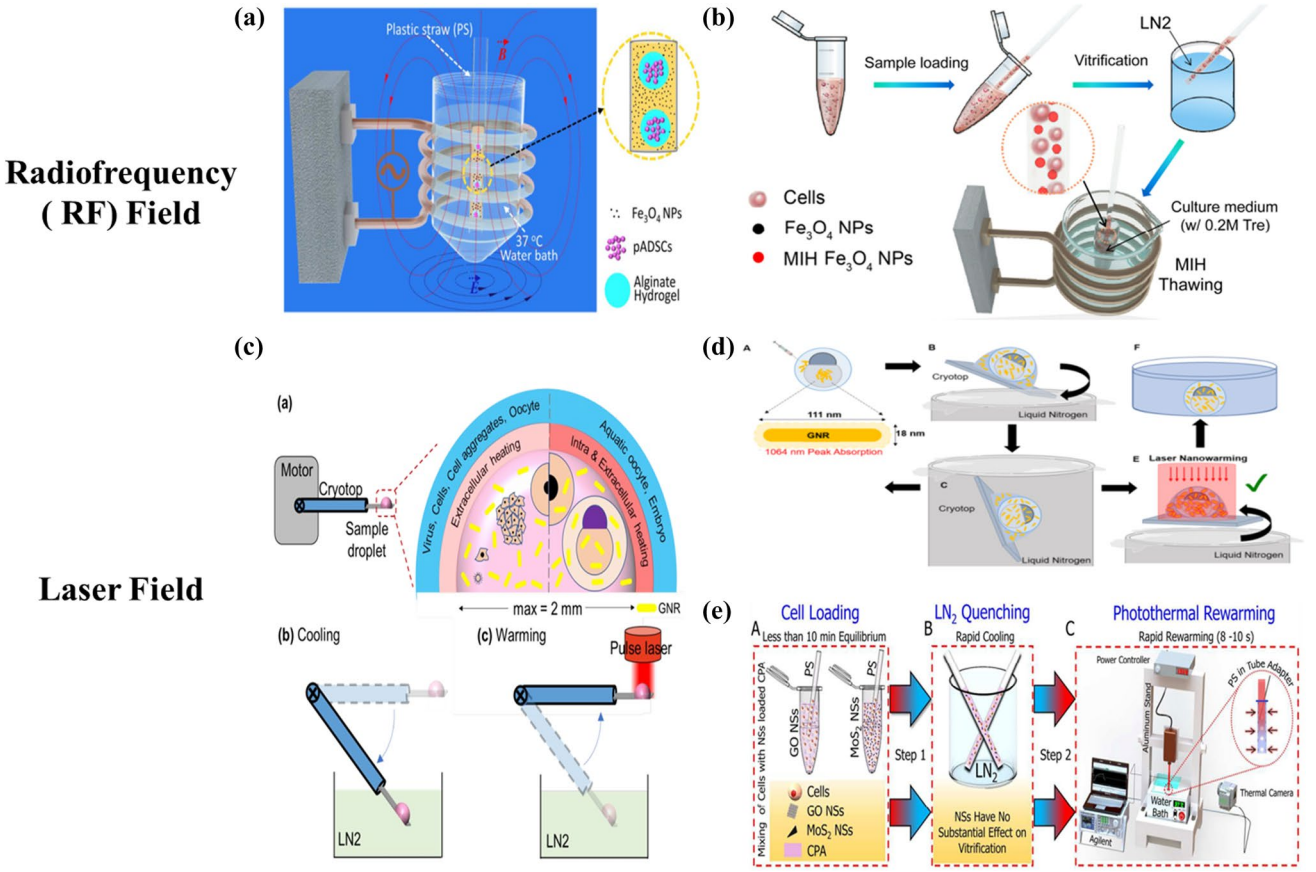

Fig. 5 Schematic diagram for different cell vitrification techniques and nanoparticles-mediated nanowarming process. RF field-mediated warming process: **a** Nanowarming procedure for vitrified PS loaded with cell-alginate hydrogel constructs and NPs in CPA solution. **b** Vitrification and nanowarming of hUCM-MSC-laden PS with magnetic induction heating. Laser field-mediated warming process: **c** Sample droplet consisting of biomaterial(s), CPA, and gold nanorods (GNRs) with a maximum volume of 1.8 µL was loaded onto a customized cryotop. For rapid cooling, cryotop was directly immersed into liquid nitrogen. Laser warming was achieved by pulsed laser irradiation yielding ultra-rapid rewarming at rates up to 2×10^7 °C/min. **d** Overview of zebrafish embryo cryopreservation and laser GNRs rewarming. Micro-injection consisting of 1064 nm resonant GNRs and PG was injected into the space of the embryo, between yolk and chorionic. Rapid cooling was achieved by immersing the modified cryotop into liquid nitrogen. Laser irradiation of the embryo taken from liquid nitrogen was used to achieve nanowarming. **e** Multistep addition of vitrification solution and loading in PS, followed by rapid cooling and photothermal rewarming with GO and MoS_2/NSs at 37 °C water bath

process. Nanowarming was the primary reason for promoted attachment of thawed pADSCs [97]. This technology was also found to be efficient in human UC-MSCs and resulted in an improved vitrification outcome [98] (Fig. 5b).

Laser Radioactive Warming Process

Gold nanoparticle-based laser warming has the potential to provide a platform for both extra- and intracellular heating of vitrified biomaterials, ranging in size from nm to mm in µL-sized droplets. Khosla et al. [99] mixed GNRs with cryo-solution for rapid cooling and nanowarming of HDFs ($D_{cell} = 10$ µm) in droplet volume. The method of nanowarming used was laser warming technology irradiated with a 1064 nm laser pulse for 1 ms (Fig. 5c). A cell viability of > 90% was maintained in HDF cells post-laser warming. Similar warming method was used to rewarm vitrified zebrafish embryos. Before vitrification, biocompatible PEGylated GNRs were microinjected directly into zebrafish embryos with 2.3 mol/L PG, thereby helping to distribute the laser energy throughout the embryo during warming (Fig. 5d). As compared to the conventionally warmed control group, the GNRs-mediated laser warming of embryos resulted in 31% viable embryos with consistent structure at 1 h, 17% viable embryos continuing development at 3 h, and 10% viable embryos showing movement at 24 h post-warming [100].

Two-dimensional (2D) graphene oxide (GO) and molybdenum disulfide (MoS_2) nanosheets (NSs) were used to improve warming process of bio-samples owing to their photothermal effects. Human umbilical vein endothelial cells

(HUVECs) were chosen to study this novel NSs-mediated spatial heating approach. For rewarming, cryopreserved HUVECs were placed into warming solution (37 °C) under a near-infrared laser field and photothermal effect was achieved at 5000 mW/cm^2 for 8–10 s (Fig. 5e). This warming technology significantly improved the cell viability as compared to the conventional rewarming method and also maintained normal cell function and subcellular ultrastructure. Further investigation showed that near-infrared laser irradiation effectively decreased ice formation and restricted recrystallization growth via micro- and macro-effects during rewarming [101].

Conclusions and Future Perspectives

With the advancement of cell-based applications, conventional cell cryopreservation clearly failed to keep pace with current and emerging needs. This review summarized the recent advances in biotechnology tools, including trehalose delivery, hydrogel-based cell encapsulation, droplet-based cell printing, and nanowarming technology, used in cell cryopreservation. Trehalose delivery technology helped to overcome the major limitation of ultra-low permeability of trehalose, and its application resulted in favorable post-thaw cell survival rates without need of any organic solvent. Trehalose delivery method provided both extracellular and intracellular cryopreservation with trehalose as sole CPA [29]. Hydrogel-based cell encapsulation technology created a new platform for efficient cell transportation and preservation. It has promoted long-term storage of cell resources and banking "off-the-shelf" cell-based therapy products at large scale. Since hydrogel capsules supplement excellent cryopreservation, satisfactory outcomes of cell cryopreservation can be also achieved at reduced DMSO concentrations [59, 102, 103]. Droplet-based cell printing can improve cell vitrification and can be helpful to achieve high efficiency at low-CPA concentration and reduce CPA toxicity and osmotic stress during CPA loading and unloading process [86]. Nanowarming technique has revolutionized the progress in warming method. Its use can achieve ultra-fast and uniform rewarming, while avoiding the adverse effects of devitrification on cells [93].

Remarkable progress has been made in the application of advanced biotechnology tools to improve cell cryopreservation. However, a serious challenge associated with cell cryopreservation has been identified that needs to addressed. Cryopreservation-induced delayed onset cell death has been reported to result in significant loss (> 50%) in the total cell population and compromises cellular functionality [104–106]. In order to further explore the true potential of biotechnology for cell cryopreservation, future work should focus not only on the advancement of the existing applications, but also discover new "binding domain" to introduce other innovative biotechnology techniques.

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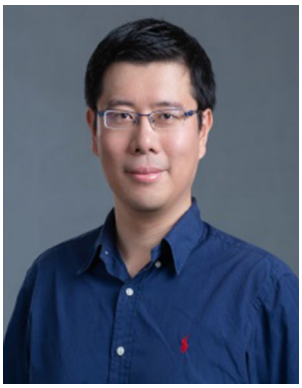

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