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Overcoming ice: cutting-edge materials and advanced strategies for effective cryopreservation of biosample

Miaorong Huang[1,2], Minhua Hu[3], Gengyuan Cai[1,2], Hengxi Wei[1,2], Sixiu Huang[1,2], Enqin Zheng[2,1*] and Zhenfang Wu[1,2*]

Abstract

Cryopreservation techniques have been widely used, especially in biomedical applications and preservation of germplasm resources. Ideally, biological materials would maintain functional integrity as well as a normal structure and can be recovered when needed. However, this tool does not work all the time. Ice formation and growth are the key challenges. The other major reason is that the cryoprotective agents (CPAs) currently used do not meet these needs and are always accompanied by their cytotoxicity. A comprehensive and synergistic approach that focuses on the overall frozen biological system is crucial for the evolution of cryopreservation methods. In this review, we first summarize the fundamental damage mechanisms during cryopreservation, as well as common cryoprotectants and their limitations. Next, we discuss materials that interact with ice to improve cryopreservation outcomes. We evaluated natural and synthetic materials, including sugars and polymers, AFPs and mimics, ice nucleators, and hydrogels. In addition, biochemical regulation, which enhances the tolerance of biosamples to cryopreservation-induced stresses, was also mentioned. Nanotechnology, cell encapsulation, cryomesh, and isochoric freezing, such scalable approaches, are further discussed for cryopreservation. Finally, future research directions in this field for efficient cryopreservation are proposed. We emphasized the need for multidisciplinary progress to address these challenges. The combination of cryobiology mechanisms with technologies, such as synthetic biology, nanotechnology, microfluidics, and 3D bioprinting, is highlighted.

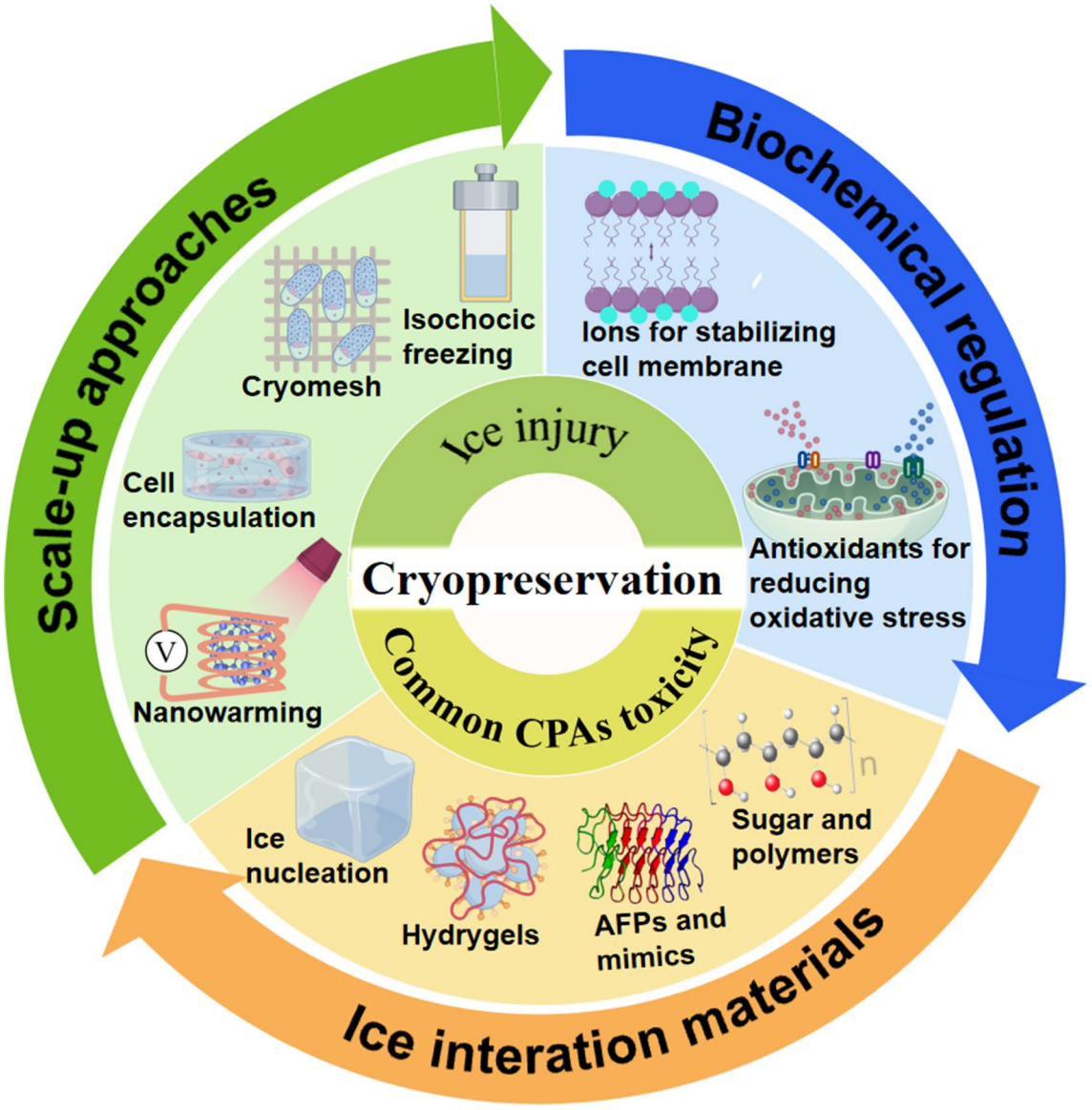
Introduction

The long-term storage of organs, tissues, cells, and other biosamples can be achieved through cryopreservation at very low temperatures (– 80 °C– – 196 °C). By significantly reducing or even completely stopping all chemical and biological reactions during frozen storage, biological materials maintain functional integrity as well as normal structure after thawing and can be used in scientific research and future clinical applications. This is the fundamental mechanism of cryopreservation. This technique has been widely used, especially in biomedical applications and germplasm resource preservation [1, 2]. Using cryopreservation, for example, will increase the availability of organs, eliminate waiting lists, reduce costs, and improve transplant outcomes in biomedicine. For reproductive therapies, the storage of gametes (sperm and oocytes), whole reproductive organs or parts of tissues (ovarian tissue and testicular tissue) and embryos has revolutionized in vitro fertility treatment,

*Correspondence:
Enqin Zheng
eqzheng@scau.edu.cn
Zhenfang Wu
wzfemail@163.com

Full list of author information is available at the end of the article

Graphical Abstract



Keywords Cryopreservation, Hydrogels, Antioxidant, Nanotechnology, Microfluidics, 3D bioprinting

allowing individuals to preserve their fertility for many years, even in emergency situations that might otherwise end in sterility [3]. In germplasm banks, the preservation of genetic material from animals has advanced significantly over the past few decades [4]. This work is essential for biodiversity conservation and endangered species protection. Moreover, cryopreservation overcomes the spatiotemporal limitations of animal reproduction, therefore accelerating the genetic progress of animals.

The cryopreservation of biomaterials is essential for various applications. However, in the process of cryopreservation, ice formation and growth are major challenge [5] leading to mechanical damages and oxidative stress. These effects can harm intra- and intercellular structures and functions, such as microtubule disruption, DNA fragmentation, RNA degradation, and nucleoprotein complex structure alteration, ultimately leading to cell death [6–8]. To moderate cryopreservation-induced damage, cryoprotective agents (CPAs), the most common of which are dimethyl sulfoxide (DMSO) and glycerol, are employed [9]. However, in comparison with somatic cells, tissues and organs are large in volume [10], sperm are very sensitive to temperature changes [6], oocytes have a low surface area-to-volume ratio and high

susceptibility to intracellular ice formation [11], and cryoinduced trauma to the embryos is subsequently manifested downstream [12]. Therefore, the cryopreservation and recovery of a number of biological materials are still highly challenging.

In this review, we first summarize the fundamental damage that occurs during the cryopreservation of biomaterials. Next, we introduce the advanced strategies applied in the freezing–thawing process. We will evaluate natural and synthetic materials that interact with ice, biochemical mechanisms for freezing tolerance, and some tool systems for high-throughput application research. Finally, we discuss future research directions and some emerging methods that may add to this issue.

Fundamental challenges during cryopreservation

To achieve systematic optimization, it is crucial to understand how cryoinjuries occur during freezing and thawing. The potential challenges were showed in Fig. 1.

Mechanical damage by ice

A remarkable aspect of the freezing and thawing process is the formation and growth of ice crystals. This is the primary cause of cell viability loss. When the temperature falls below the freezing point, water molecules tend to arrange in an orderly manner. This occurs because of the competition between the disordered thermal motion of water molecules and their ordered arrangement [13]. The phase change of intracellular and extracellular water leads to cryoinjury. Generally, extracellular aqueous solutions below the equilibrium freezing point form ice crystals, reducing the concentration of water in the solution and increasing the concentration of the extracellular matrix. Consequently, the osmotic pressure outside the cells increases, leading to severe dehydration and shrinkage, which is common in a slow freezing process. A certain degree of cellular dehydration is beneficial for cryopreservation since it reduces the possibility of excessive accumulation of intracellular ice. However, excessive dehydration may be irreversible and is a primary factor harmful to biological functions.

Another challenge is sample supercooling, where the actual crystallization temperature (often between − 15 °C and − 60 °C) is lower than the theoretical freezing point. This means that when the temperature falls below zero, biosamples do not immediately undergo a phase change from liquid to solid. A supercooled solution is in a metastable state [14] where the phase transition can easily be triggered by minimal thermal disturbance. As soon as ice nucleates, it rapidly propagates throughout the entire biosample, accompanied by the release of latent heat and warming of the solution, resulting in more severe mechanical damage to the samples [15]. This cryoinjury is more severe when a fast freezing process with little cryoprotectant is used. As the cooling rate accelerates, intracellular water cannot flow out as quickly, thus forming intracellular ice and leading to fatal cryoinjury to cells.

It is also a challenge when biological materials are rewarmed to physiological temperatures. Similar to the freezing process, the major obstacle in the thawing process is bypassing the water–ice transition phase. When the temperature is between − 15 °C and − 60 °C, the cells become increasingly supercooled, which promotes the transformation of free water into ice, and then, the ice crystals grow larger as the temperature increases [10]. This phenomenon, known as "ice recrystallization", causes mechanical injuries both in slow-freezing and

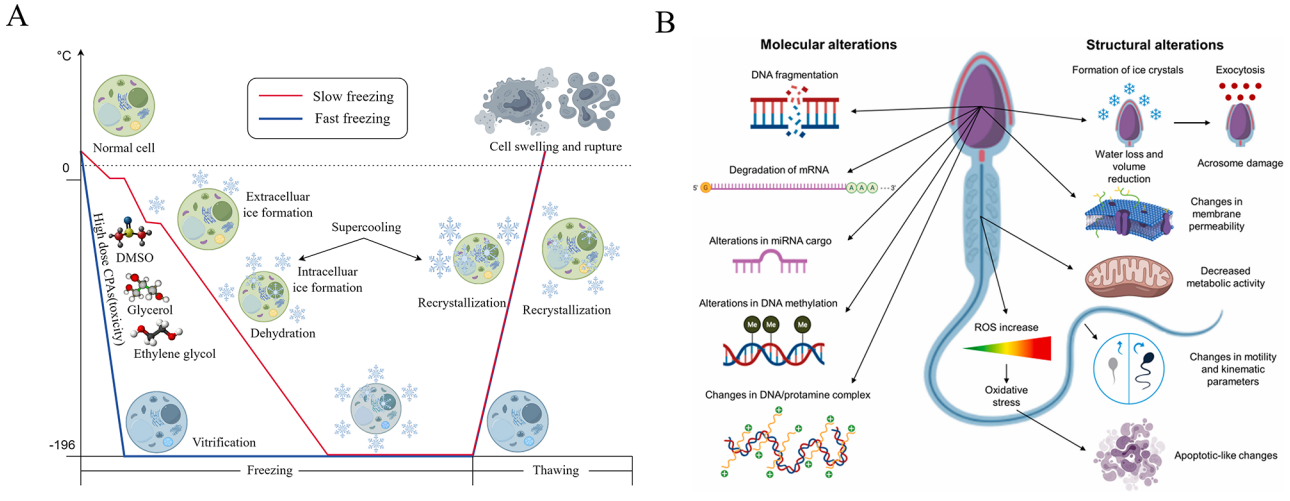


Fig. 1 Fundamental challenges occur in the freezing–thawing process. (**A**) The potential mechanisms of cryoinjury. In the slow freezing process, cooling causes dehydration of the cell from extracellous ice crystal osmosis. Supercooling increases the risk of intracellular ice. Fast cooling and CPAs aid vitrification, but high concentrations of CPAs may be toxic. During the rewarming process, ice recrystallization leads to cell swelling and potential rupture. By Figdraw. (**B**) Schematic illustration of structural and molecular alterations in cells. Reproduced with permission from Elsevier, 2021 [6]

fast-freezing process [16]. As the temperature increases near the melting point, the extracellular space becomes rapidly hypotonic as large ice crystals melt into water. Consequently, hyperosmotic stress drives an influx of water into the cells, leading to cell swelling and potential rupture.

Oxidative stress induced by cryopreservation

Oxidative stress is a significant concern during cryopreservation. Oxidative stress is caused by the generation of excessive reactive oxygen species (ROS), which can lead to cellular damage through lipid peroxidation, protein oxidation, and DNA damage [17, 18], thus leading to application failure [19]. The level of ROS, such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), is increased during cryopreservation, primarily due to the disruption of cellular metabolism and the activation of various enzymatic pathways.

While cellular metabolic activities are suppressed during cryopreservation, some biochemical reactions continue even at low temperatures, and the mitochondrial electron transport chain remains active in generating ROS. Additionally, cell dehydration, increased ion concentration, and changes in pH can further promote the production of free radicals. Solute damage, particularly from high concentrations of electrolytes, can alter the redox potential, leading to protein denaturation and lipid peroxidation. Low temperatures can also impair the activity of endogenous antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and the formation of ice crystals can directly damage cell membranes and organelles, causing the leakage of intracellular components (e.g., lysosomal enzymes) and triggering secondary oxidation reactions. Moreover, rapid temperature changes during cryopreservation can induce the formation of free radicals, further exacerbating oxidative stress. For a more detailed understanding of the different characteristics of ROS produced in cells, published review articles [20, 21] provide comprehensive insights.

Common cryoprotectants and their limitations

CPAs are used to moderate ice during the freezing–thawing process. Owing to their outstanding ability to form hydrogen bonds (HBs) with water, the most widely used CPAs, DMSO and glycerol, are exceptionally effective in controlling crystals and improving the outcomes of cryopreservation [22]. As the CPA concentration increases, the proportion of CPA-water hydrogen bonds increases, leading to a decrease in the nucleation temperature and an increase in the glass transition temperature, which reduces the opportunity for the formation and growth of ice. Meanwhile, their toxicity becomes apparent and can even dominate. For example, DMSO induces dehydration near lipid membrane surfaces [23]; has a synergistic effect with vanadium to delay the development of zebrafish embryos and cause pericardial edema [24]; inhibits osteoclast formation, differentiation and function in vitro [25]; induces drastic changes in human cellular processes and the epigenetic landscape in vitro [26]; dramatically decreases the percentage of oocytes with normal actin microfilaments by EG and DMSO treatment [7]; glycerol causes hemolysis or alters red blood cell shape [27]; and glycerol residues remain inside the cells even after multiple complex washing steps and thus occasionally cause complications [28]. The categories and affecting factors involved in CPA toxicity are documented in Benjamin P. Best's review [29]. Notably, CPAs prevent ice formation via the interference of hydrogen bonds between water molecules, but they also cause nonspecific toxicity at high doses. That is, "two sides of the same coin" or "double blades sword".

To date, cryopreservation strategies are divided into slow freezing and fast freezing strategies (also called vitrification, in which, at exceptionally high cooling rates, the crystalline phase can be bypassed to achieve ultrahigh-viscosity glass), according to Mazur's hypothesis [30]. The slow freezing method allows low CPA loading, but the challenges of exposure of cells to CPAs (potentially toxic) for an extended period of time and ice injuries are still not easy to overcome. For the vitrification mothod, high-dose CPA toxicity and achieving a completely vitrified state are the main problems. Taken together, the search for biocompatible cryoprotective materials and methods to control ice formation, ice growth and ice recrystallization during freeze–thaw cycles are still major tasks. In the following sections, we introduce recent developments, focusing on innovations in materials and methods.

Natural and synthetic materials that interact with ice

Polumers, surfactants, or other molecules can affect the nucleation, growth, and melting processes of ice through physical or chemical means. For example, some molecules interact with water by forming hydrogen bonds, whereas others may do so through van der Waals forces or electrostatic interactions. Certain polymers can adsorb onto the surface of ice crystals, forming a protective layer that inhibits the growth of ice crystals, whereas some surfactants can promote the melting of ice by reducing the surface tension of water. Some of these materials that interact with ice have been developed as CPAs for years, which has provided great opportunities to improve the outcomes of cryopreservation.

Sugars and polymers

Sugars and polymers, nonpermeating agents such as trehalose [31], *Rhodiola rosea* polysaccharides [32], fucose-conting polysaccharide(FucoPol) [33], poly(ethylene

glycol) [34] and poly(vinyl alcohol) [35], have been reported to be effective at recovering the composition of cells after freeze–thaw cycles. In addition, polysaccharides and their derivatives are used as scaffolding materials since they contain many negatively charged functional groups [36, 37]. Some of these biobased, biodegradable and noncytotoxic biomaterials have been used in commercial cryopreservation cocktails.

Efforts have been made to deliver trehalose, a nonreducing disaccharide cryoprotective agent, into cells because of its inability to be synthesized by mammalian cells or to enter cells directly through diffusion or endocytosis. Microinjection is used for large mammalian cells such as oocytes. Hypochlorite treatment is an effective method to permeabilize oocysts and enable the intracellular uptake of cocktail solutions, including trehalose, for the cryopreservation of infectious *Cryptosporidium parvum* oocysts [38]. Nanoparticle microencapsulation-based platforms are exploited for successful delivery of trehalose into small cells, which are often needed in large quantities to avoid cryoinjury [39–41]. Although it is the central dogma that regulates intra- and extracellular ice during freezing and thawing, according to the results of Robert C. Deller and coworkers, there is significant ice formation in the cryopreservation solution, whereas PVA enables nonvitreous cellular cryopreservation only by limiting extracellular ice growth, resulting in increased cell recovery [35]. This could be because the suspension system was already optimized or because the cryopreserved cells were freeze-tolerant. A deeper look into the evidence is needed.

Antifreeze proteins and their mimics

Antifreeze proteins (AFPs) are produced by various organisms, such as some vertebrates, polar fish, insects, and bacteria, that live in extremely cold environments, to allow them tolerence towards frozen (Fig. 2A). They can be classified into several structural categories based on their sequence and structural features. The most common classification includes type I, type II, and type III AFPs. Type I AFPs are typically found in polar fish and have a simple helical structure with repetitive alanine and threonine residues [42]. Type II AFPs, also found in fish, have a more complex beta-sheet structure with multiple disulfide bonds. Type III AFPs, present in insects, possess a unique sequence with a high content of glycine and aspartic acid, forming a compact and stable tertiary structure.

AFPs functions primarily include adhesion and ice inhibition. AFPs adsorb to the surface of ice crystals, preventing their growth and recrystallization. This binding is facilitated by the presence of specific ice-binding sites on the AFPs, which have complementary structures to the ice lattice. For instance, type I AFPs have a helical structure that aligns with the ice lattice, allowing for effective binding and inhibition of ice growth. As shown in Fig. 2B, AFPs comprise two distinct faces, the ice-binding face (IBF) and the nonice-binding face (NIBF). The ice-like hydration layer structure is due to the synergetic effects of the orderly arrangement of hydrophobic methyl groups and hydrophilic hydroxyl groups in the residues of the IBF, which consists of a flat array of β-sheets. In contrast, the water molecules in the hydration layer on the NIBF display a disordered arrangement. The specific arrangement of methyl groups and hydroxyl groups leads to an enhanced heterogeneous ice nucleation capability, whereas bulky hydrophobic groups and charged groups result in a depressing effect [43].

By binding as discrete proteins, AFPs cause microcurvature of the ice surface that depresses the freezing temperature below the melting point. This is the so called "Thermal hysteresis" phenomenon, which is a key mechanism of AFPs [44]. The thermal hysteresis effect is crucial for preventing the formation of large ice crystals that can damage cells and tissues, as it maintains the solution in a supercooled state without freezing. The extent depends on the concentration and type of AFPs present in the solution.

Recrystallization inhibition is another important mechanism of AFPs. During the freezing and thawing process, small ice crystals tend to grow into larger ones, which can cause significant damage to cells and tissues. AFPs prevent this recrystallization by adsorbing to the surface of ice crystals and inhibiting their growth. This results in the maintenance of smaller and more stable ice crystals throughout the freezing and thawing cycles.

AFPs are promising candidates for CPAs since they show excellent ability to modulate ice crystals; however, their wider application remains limited because of their drawbacks, including high cost, potential immunogenicity and toxicity effects, as well as the difficulties of large-scale production.

Currently, researchers focus on simple designs and syntheses of ice modulators with ice tuning functions by mimicking AFPs. Polymer-based nanomaterials are biocompatible and can be easily functionalized to interact with ice crystals. Wang's team designed biocompatible L-proline oligomers (L-Pro_n), which have the same polyproline II helical structure as AFPs do, for the cryopreservation of mouse oocytes by reducing the amount of DMSO and EG to 1.8 M, and the survival rate increased to 99.11% (Fig. 2C). Rami EI reported the preservation of human NK cell viability and antitumor function via a cocktail of CPAs based on dextran and carboxylated L-lysine, which act in a way similar to AFPs (Fig. 2D). PVA nanoparticles, for example, can form hydrogen bonds with water molecules, preventing the formation of large ice crystals. PEG-coated nanoparticles can also

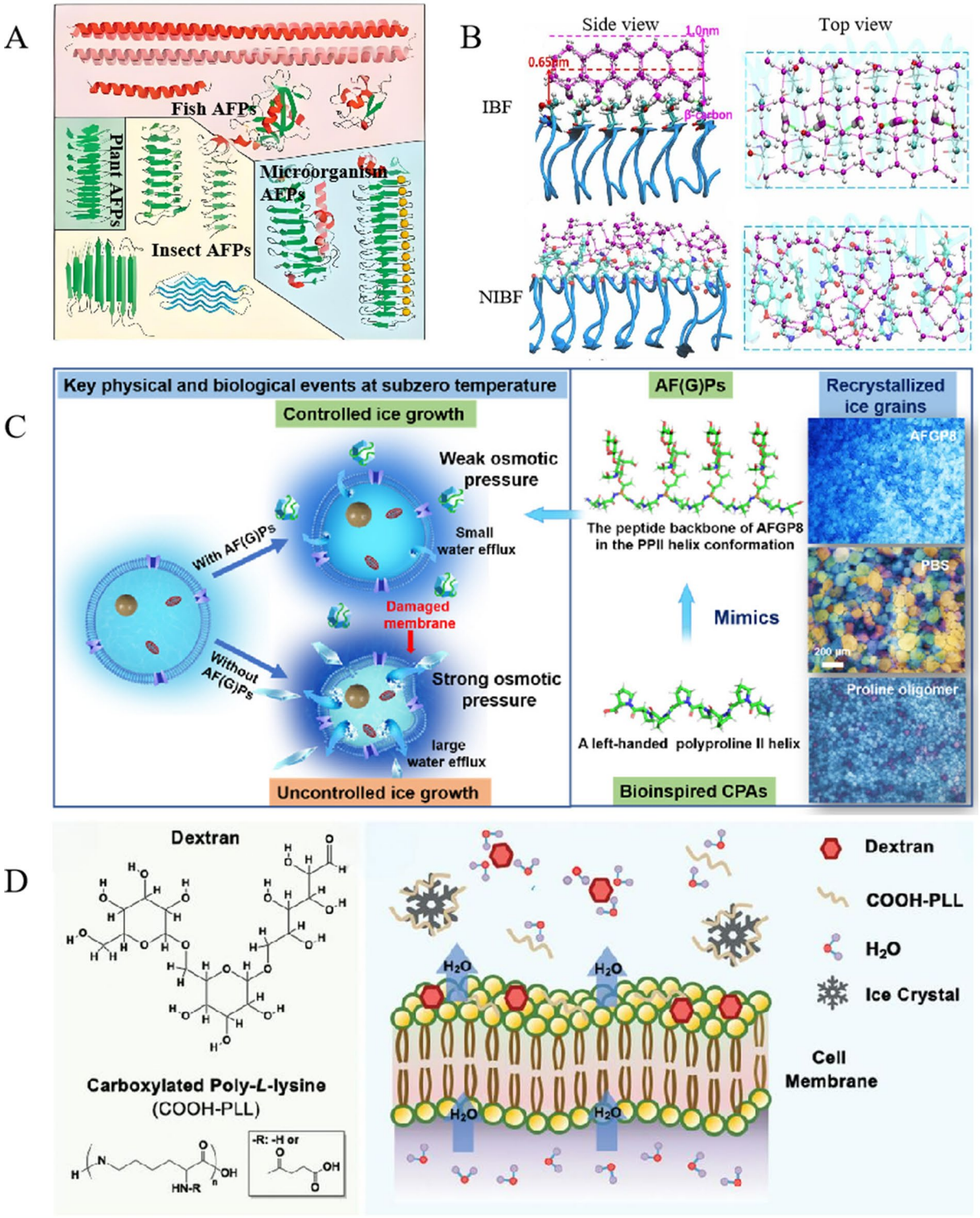


Fig. 2 Potential effects of AFPs and their mimics on trolling ice. (**A**) X-ray crystal structures of several typical AFPs. Reproduced with permission from Elsevier, 2021 [45]. (**B**) Molecular dynamic simulation of AFPs with ice. Reproduced with permission from the American Chemical Society, 2016 [43]. (**C**) Proposed mechanism of bioinspired L-proline oligomers on controlling the ice. Reproduced with permission from the American Chemical Society, 2020 [46]. (**D**) Potential mechanism of the use of bioinspired dextran and CPLL for the cryopreservation of NK cells. Reproduced with permission from Wiley, 2019 [47]

inhibit ice crystal growth by creating a physical barrier between the ice crystals and the surrounding water molecules. These polymers are often used in biomedical applications due to their non-toxicity and biocompatibility.

Carbon-based nanomaterials and metal-based nanomaterials have also been shown to have ice-regulating properties similar to those of AFPs and can be used as cryopreservation agents to inhibit ice. Graphene oxide (GO) is a typical example. Graphene oxide (GO) preferentially adsorbs on the surface of ice crystals in liquid water. This adsorption leads to the formation of curved ice crystalsand lowers the freezing temperature, and contributes to the suppression of ice growth (Fig. 3A). The zirconium (Zr)-based metal-organic framework (MOF) nanoparticles (NPs) are used for the cryopreservation of red blood cells (RBCs). These nanoparticles inhibit ice recrystallization. They also act as "catalysts" to accelerate the melting of ice crystals.

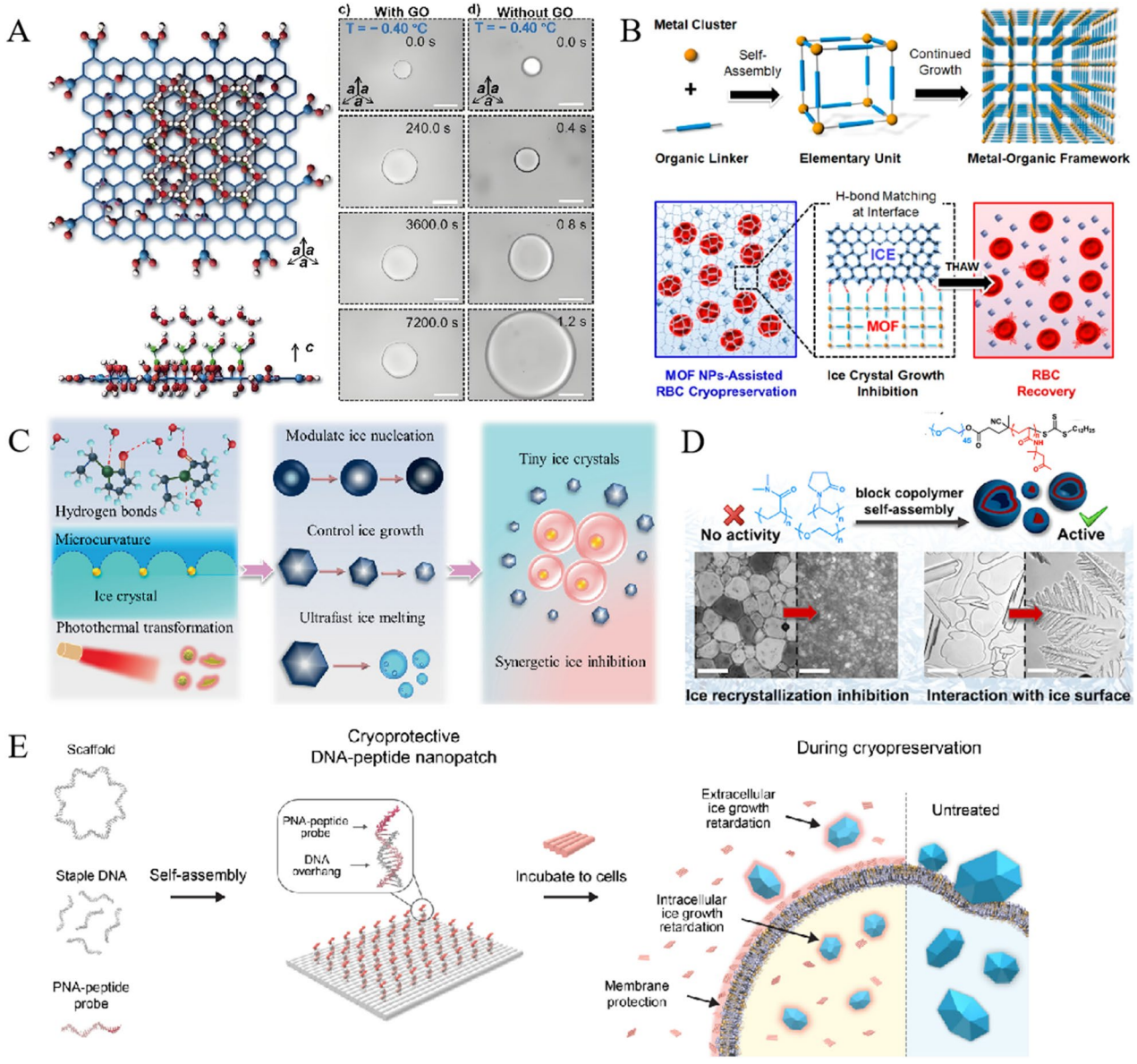


Fig. 3 Ice modulation activity of NPs with specific structures. (**A**) Ice crystal growth and shape are affected by the arrangement of oxidized groups on the basal plane of GO. Reproduced with permission from Wiley, 2017 [53]. (**B**) Schematic illustration of the structure and utilization of MOF NPs for ice recrystallization inhibition. Reproduced with permission from the American Chemical Society, 2019 [54]. (**C**) Proposed mechanism of bioinspired L-proline oligomers on controlling the ice. Reproduced with permission from Wiley, 2021 [55]. (**D**) Proposed mechanism of bioinspired L-proline oligomers on controlling the ice, Reproduced with permission from the American Chemical Society, 2021 [56]). (**E**) Schematic illustration of the assembly of cryoprotective DNA-peptide nanopatches and their preservation mechanisms. Reproduced with permission from the American Association for the Advancement of Science, 2022 [57]

(Fig. 3B). The key point was the periodic arrangement of organic linkers on the MOF outer surface, which provides precise spacing of hydrogen donor groups to recognize and match the prism/basal plane of ice crystals. Compared with GO NPs, the transition metal dichalcogenide (TMDC) family has great potential in this field. For example, tungsten diselenide (WSe2) is a good candidate because of its excellent photothermal transformation performance and tunable local surface. The WSe2-PVP NPs combined with the laser showed synergetic ice regulation ability in both the freezing and thawing processes (Fig. 3C).

Another promising work involves using antifreeze peptide-functionalized DNA origami as a cryoprotectant for cell preservation (Fig. 3E). In this study, M13mp18 single-stranded DNA, staple DNA strands, and PNA strands were used. The hightlight is that these strands were decorated with antifreezing Thr-peptides which can self-assembled onto DNA-peptide nanopatch structures. After incubation with cells, these nanopatches retrardated ice growth both inside and outside the cells. DNA-peptide nanopatch structures attached to the cell membrane additionally provided protection ability during the cryopreservation process. Owing to their biocompatibility and biodegradability, DNA-based nanomaterials are potentially superior to traditional cytotoxic CPAs for cryopreservation.

The structural properties of nanomaterials play a crucial role in their ability to regulate ice formation. Nanomaterials with high surface area and surface energy can interact more effectively with water molecules, inhibiting ice crystal growth. For example, graphene has a large surface area that allows it to adsorb a significant number of water molecules, preventing them from forming ice crystals. Similarly, metal oxide nanoparticles with high surface energy can adsorb onto ice crystal surfaces, disrupting their growth.

Interestingly, ice recrystallization inhibition (IRI) activity can be introduced into polymer nanoparticles from components that themselves have no ice binding or associated activity. Examples of such components include poly(ethylene glycol), poly(dimethylacrylate), and poly(vinylpyrrolidone) (Fig. 3D). Observations indicate that ice-binding domains may not be necessary for IRI activity at the macroscopic level; instead, polymer size and density could enhance IRI activity in polymers that are not typically active.

What, then, is important for an antifreeze particle? Some studies have focused on profiling the effects of nanomaterial morphology on ice suppression [48]; other efforts explorehave explored the impact of nanosize on regulating ice crystal formation [49]. Additionally, there is a focus on finding nanomaterials or platforms that have multifunctional ice inhibition capabilities and are easy to handle [50–52]. In-depth research is necessary to elucidate the mechanism of nanoparticles.

Ice nucleators

Unlike AFPs, ice nucleators are a different class of ice regulators that facilitate the phase transition of solutions at relatively high subzero temperatures, thereby inhibiting severe supercooling. Endogenous ice nucleators include proteins, lipoproteins, and inorganic crystals. Several bacterial species are known to produce ice-nucleating proteins; for example, Snomax, a commercial product used as an additive in the production of artificial snow, is from *Pseudomonas syringae*. In addition, ice mimics such as inorganic and mineral particles used to induce nucleation at temperatures close to zero have been shown to increase both cell recovery and reproducibility. Rao's team developed chitosan-decorated cellulose nanocrystals for enhancing the elimination of CD 44 high-expression cells in the phase-change process of cryoablation [58]. More interestingly, sand-madiated ice seeding has been shown to be useful for the cryopreservation of human iPSCs with less cryoproteectant and without serum, while retained high cell survival and high pluripotency [59].

Nucleants and antifreeze proteins have opposite effects on ice modulation, but both have tandem arrays of amino acids as β-helices and can bind to the ice surface effectively. The issue becomes whether it is inhibition or promotion. Some researchers have indicated that the determining factor is size [49], whereas others have emphasized the optimum freezing point when nucleators are added [59]. Considering the impacts of particles on cryopreservation, determining how freezing occurs and the exact link between antifreezing and freezing is necessary.

Hydrogels

Hydrogels are novel wet and soft materials that have been widely applied in tissue engineering, clinical medicine, and drug delivery owing to their superior softness and flexibility, wettability and lubricity, biocompatibility, and optical transparency. Recently, a series of hydrogel materials with anti-icing functions have been developed [60–62]. The underlying mechanisms of hydrogels are mainly attributed to the following. Strong hydrogen bond interactions exist between water molecules and the hydrogel network. These interactions reduce the free water fraction within the hydrogel and decrease the ice formation [63]. Alginate, a polysaccharide that can be crosslinked by divalent cations to form hydrogel networks, is the most commonly used polysaccharide. For example, alginate hydrogels have been shown to improve the survival of human ES cells during slow freezing; alginate hydrogels with ion crosslinks can also facilitate vitrification and

reduce CPA concentrations. Moreover, their low cost, mild crosslinking conditions, and cytocompatibility make them popular for cell encapsulation and suitable for 3D bioprinting ink. A detailed description of the hydrogels is provided in subsection "5.2 Cell encapsulation".

Biochemical regulation for tolerating-freezing

Antioxidants for reducing oxidative stress

Excessive ROS can lead to cellular damage through lipid peroxidation, protein oxidation, and DNA damage. One way for scavenging ROS is by providing redox cofactors, such as nicotinamide nucleotides and flavins, which are crucial for most redox reactions and necessary for maintaining proper redox balance. Another approach is to supply antioxidants, including small bioactive molecules like vitamins, micronutrients, and metal ions, as well as components of enzymes like redoxins (thioredoxin, glutaredoxin, and peroxiredoxin), glutathione peroxidase (GPx), and superoxide dismutases (SODs).

Enzymatic antioxidants function due to their ability to directly neutralize ROS. SOD is a key enzyme that catalyzes the dismutation of superoxide radicals (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Catalase is another essential enzymatic antioxidant that decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), thereby preventing the formation of highly reactive hydroxyl radicals (OH^-). GPx utilizes GSH as an electron donor to convert H_2O_2 to H_2O. Non-enzymatic antioxidants play a vital role in cryopreservation through the following ways: scavenge free radical, protect of cell membranes and organelles, regulate antioxidant enzyme expression, inhibit oxidative stress-induced apoptosis, and repair cryoinjury.

Direct scavenging of ROS

Vitamin C, Vitamin E, and glutathione (GSH) can neutralize free radicals by donating electrons, converting them into less reactive molecules, thus preventing the chain reactions initiated by free radicals [64]. Some lipid-soluble antioxidants, such as Vitamin E, can embed in the phospholipid bilayer of cell membranes, preventing lipid peroxidation and thus protecting the membrane's integrity and function.

Activating endogenous antioxidant systems

The Nrf2/ARE pathway is a key cellular defense mechanism against oxidative stress. Nrf2 (nuclear factor erythroid 2-related factor 2) regulates the expression of antioxidant enzymes such as SOD and GPx. Under normal conditions, Nrf2 is bound to Keap1 (Kelch-like ECH-associated protein 1) in the cytoplasm. When cells are exposed to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it binds to the antioxidant response element ARE and activates the transcription of antioxidant enzymes. Antioxidants can activate the Nrf2/ARE pathway, enhancing the expression of antioxidant enzymes and reducing oxidative damage. For example, melatonin has been shown to reduce oxidative stress and apoptosis in cryopreserved ovarian tissue (Fig. 4A) and mouse germinal vesicle oocytes [65] by activating the Nrf2/HO-1 signaling pathway.

Regulating apoptosis-related signaling pathways

Oxidative stress can induce apoptosis. Apoptosis is a major factor leading to cell death during cryopreservation [66]. Inhibition of apoptosis by caspase is one of the main functions of antioxidant substances. By inhibiting apoptosis-related signaling pathways, the release of pro-inflammatory factors and apoptotic signals can be blocked.

The MAPK (mitogen-activated protein kinase) pathway and the AMPK (5′AMP-activated protein kinase) pathway are two distinct but interconnected signaling pathways in cells, each playing crucial roles in regulating various cellular processes. Activation of such pathways lead to the phosphorylation of downstream substrates, triggering a cascade of cellular responses, including apoptosis. Mitoquinone (MitoQ), a mitochondria-targeted antioxidant, has been shown to regulate the expression of mitochondrial dynamics proteins (Drp1 and Mfn2) and inhibit the p38 MAPK pathway, thereby reducing apoptosis in mouse ovarian tissue vitrification [67]. Resveratrol, a polyphenol compound, improves boar sperm quality by promoting AMPK (5′AMP-activated protein kinase) phosphorylation. This process decreases ROS production and enhances the post-thaw sperm's antioxidative defense system during cryopreservation [68]. Similarly, carboxylated C_{60} could effectively protect boar sperm against oxidative injury by inhibiting the ROS-induced protein dephosphorylation via the cAMP-PKA signaling pathway (Fig. 4B). Pretreatment of IL-15 and IL-18 improves natural killer cell recovery and function after cryopreservation by upregulating antiapoptotic genes, especially BCL2L1 (Bcl-XL), from granzyme B-mediated cell death [69].

Facilitating the recovery post-thaw

Furthermore, certain substrates contribute to recovery following cryopreservation. Experiments using GS and human HLC have shown that the metabolism of 5-aminolevulinate (5-ALA) is crucial for limiting free radical generation during the rewarming process. Supplemented 5-ALA enhances Complex III activity and improves mitochondrial respiration, thereby promoting both anabolic and catabolic activities while reducing cell death, inflammation, hypoxia, and other stress responses in isolated perfused rat livers (Fig. 4C). Adding RGD-REP (a ligand for integrin protein) to the cryopreservation solution

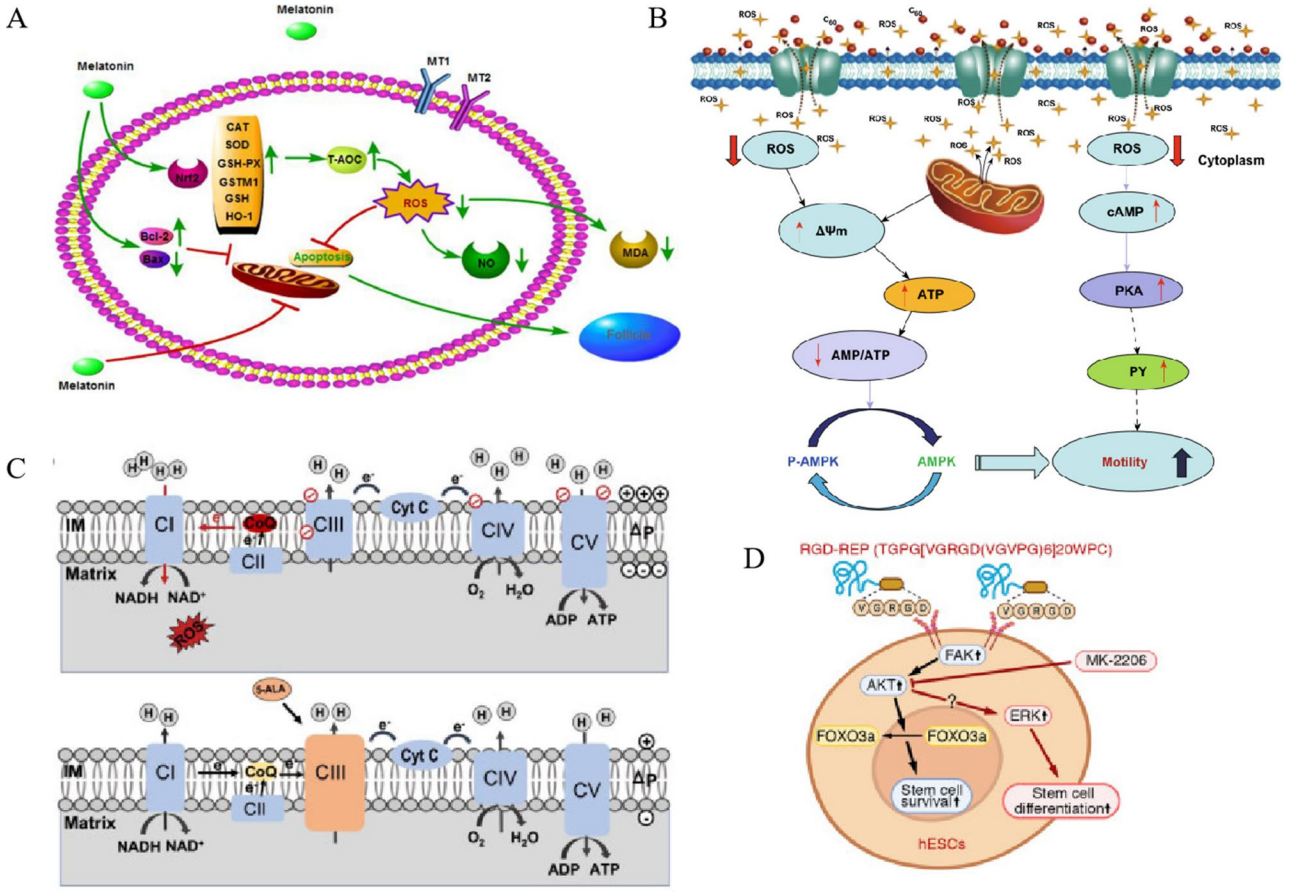


Fig. 4 Schematic illustration of antioxidants function during cryopreservation. (**A**) Schematic mechanisms by which melatonin ameliorates oxidative stress via modulation of the Nrf2 pathway. Reproduced with permission from Frontiers, 2020 [70]. (**B**) Putative mechanisms by which carboxylated C60 protects boar sperm from ROS-induced functional damage. Reproduced with permission from Spring Nature, 2019 [71]. (**C**) Schematic diagrams of a possible protective effect of 5-ALA via Complex III (down) during rewarming. Reproduced with permission from vyspring International Publisher, 2022 [72]. (**D**) Schematic illustration of the underlying mechanism of the RGD-REP-induced improvement of hESC viability. Reproduced with permission from Springer Singapore, 2023 [73]

boosts cell survival and the expression of pluripotency markers. This improvement is attributed to the activation of the FAK-AKT signaling cascade by RGD-REP binding to the integrin protein on the surface of hESCs, which subsequently inhibits FoxO3a. The inactivation of FoxO3a decreases the expression of apoptosis-related genes such as BIM, leading to enhanced survival of PSCs in a suspension state (Fig. 4D).

The pleiotropy of ROS in physiological signalling is well-known [74]. Redox signaling influences protein function, thereby causing alterations in signaling pathways, enzyme activities, transporter, receptor and transcription factor regulatory sites, as well as allosteric and macromolecular interaction sites. Although individual bioactive molecules and their combinations have been demonstrated to be effective in reducing the oxidative stress associated with cryopreservation, the list of redox signaling targets in cryopreservation is too extensive to cover exhaustively. We present here a few prototypical examples in Table 1.

Ions for stabilizing cell membrane

Cell membrane plays a crucial role in maintaining the integrity and functionality of cells. The protection effect of cell membrane is important for preventing damage caused by ice crystals when the cells are frozen. Studies have shown that certain ions can stabilize the cell membrane during cryopreservation.

Zwitterions, also known as dipolar ions, are neutral compounds that contain both positive and negative charges within the same molecule. Their unique physicochemical properties, including low volatility, tunable hydrophobicity, and strong hydrogen-bonding capacity, enable them to mitigate freezing-induced cellular damage. Some structures of zwitterions were shown in Fig. 5A. Unlike other materials, zwitterions have electric charges. Owing to strong electrostatic interactions with

Table 1 Representative antioxidants in cryopreservation

Antioxidants	Biosamples	Effects	References
Melatonin	Sperm	Up-regulate SOD, catalase and GPx activity	[75, 76]
	Sperm	Up-regulate BCL−2, SOD2, GSTM1, NRF2, HSP90AA1, catalase and HO−1 gene expression	[77]
	Oocyte	Up-regulate oocyte maturation rate, MMP, ATP MMP、ATP	[65]
	Embryo	Down-regulate ROS levels, DNA fragmentation and apoptotic gene expression Up-regulate telomere maintenance genes expression Up-regulate embryonic stem cell derivation and implantation rate	[78]
Vitamin C (ascorbate)	Sperm	Up-regulate post-thaw motility and viability Up-regulate acrosomal integrity and hypoosmotic swelling positivity	[79]
Vitamin E (α-tocopherol)	Sperm	Down-regulate malondialdehyde (MDA) levels Up-regulate the activities of SOD and GSH-PX	[80]
	Sperm	Down-regulate MDA levels	[81]
Trolox(Vitamin E analog)	Ovarian tissue	Up-regulate BMP4, BMP15, CTGF, GDF9, KL expression	[82]
Glutathione (GSH)	Sperm	Up-regulate fertilization rate and % cells with ability to undergo acrosome reaction Down-regulate lipid peroxidationg, mitochondrial ROS, total ROSs and intracellular ROS	[83]
L-carnitine(LC)	Sperm	Up-regulate viability and motility	[84]
	COC complexes	Up-regulate SOD1 gene expression	[85]
Combination of L-carnitine, N-acetyl-L-cysteine and α-lipoic acid	Embryo	Down-regulate H3K9 and H3K27 levels Up-regulate blastocysts developmental potential in vitro and viability	[86]
L-proline	Oocyte	Up-regulate survival rate (%) Up-regulate MMP Down-regulate ROS levels	[87]
Metformin	Adipose tissue	Activate the AMPK signaling pathway Up-regulate Bmal1, Nrf2 and SOD	[88]
Resveratrol	Oocyte	Down-regulate cell apoptosis	[89]
	Sperm	promoting AMPK phosphorylation	[68]
Quercetin	Oocyte	Up-regulate oocyte maturation rate and embryo developmental ratio	[90]
Astaxanthin	Oocyte	Down-regulate ROS levels, cathepsin B	[91]
Pterostilbene	Sperm	Up-regulate AMPK phosphorylation Up-regulate ubiquitination Up-regulate LC3 conversion Up-regulate SQSTM1/p62 degradation	[92]
Catalase	Sperm	Down-regulate Apoptotic like changes, apoptotic and necrotic cells Down-regulate DNA damage Up-regulate MMP	[93]
Coenzyme Q10	COC complexes	Up-regulate cell survival after vitrification Up-regulate migration of cortical granule	[94]
MitoQ	Sperm	Attenuates the vitrification-induced ultrastructural changes and alterations in the key proteins involved in spermatozoa functions and fertilization	[95]
MitoTEMPO	Sperm	Up-regulate motility, membrane integrity, sperm vitality, MMP, SOD activity, catalase activity, GPx activity, GPI protein levels Down-regulate MDA level	[96]

water, zwitterion solutions are viscous and easily transform into a glassy state, thus resulting in the inhibition of ice crystallization. Yang reported that zwitterionic betaine could be ultrarapidly taken up by cells for intracellular protection during the freezing process [97]. Synthetic zwitterions are also efficient cryoprotectants. They can interact with lipid molecules on cell membranes and change the surface charge of the membrane, or embedded into the cell membrane by hydrophobic interactions and cover a large area of the cell membrane (Fig. 5B and D). Consequently, membrane stability is maintained during cryopreservation. In addition, they can inhibit the permeation of DMSO through the membrane, thus reducing toxicity. Similarly, zinc cations interact moderately with lipid molecules and promote the formation of small, dynamic lipid clusters. As a result, membrane fluidity is maintained, and cells become more resistant to osmotic and mechanical stresses (Fig. 5C).

It is necessary to understand the structure-property relationships of ionic liquids and their interactions with

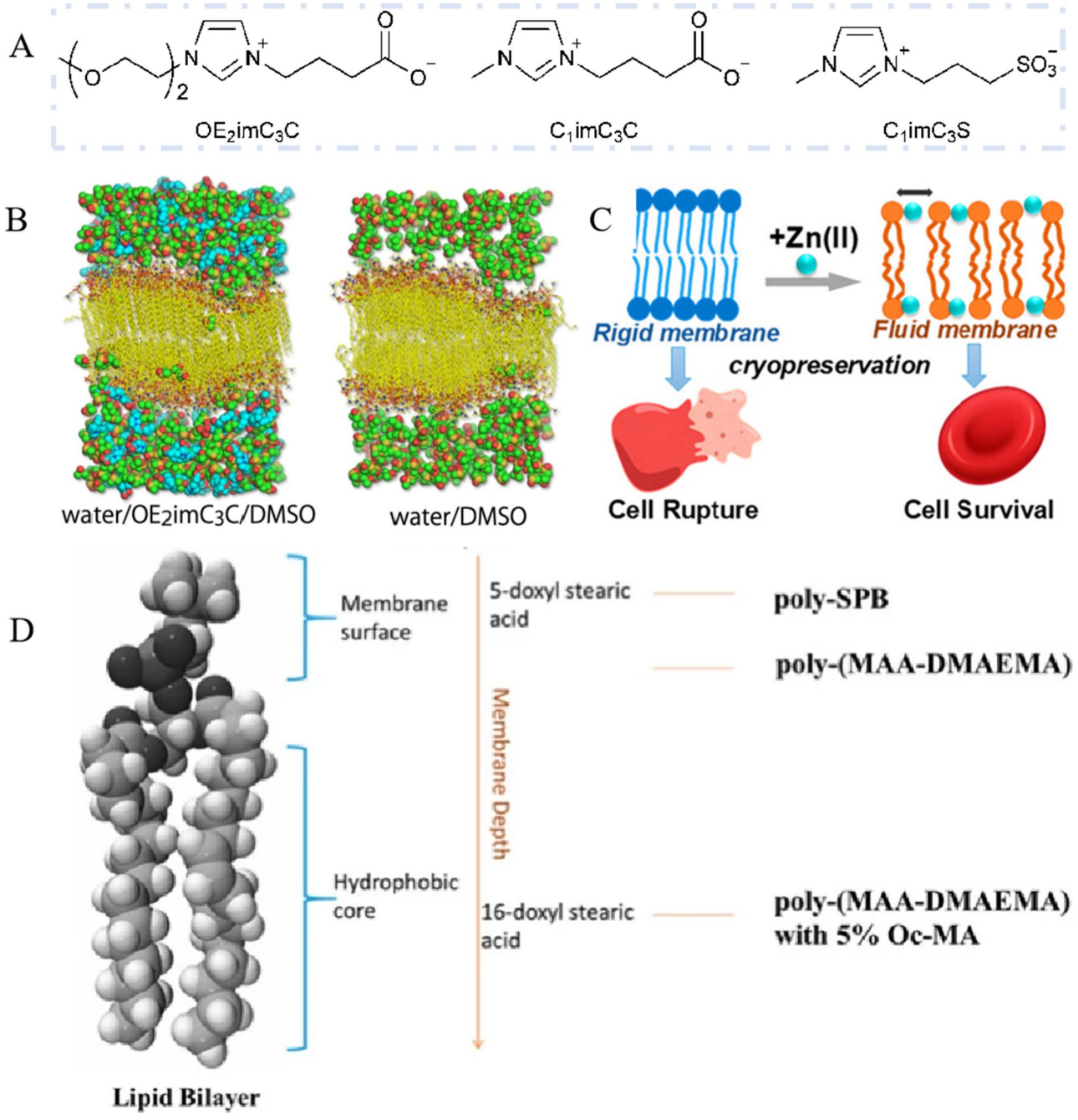


Fig. 5 Schematic illustration of ions for cryopreservation. (**A**) Some structures of zwitterions. Reproduced with permission from Royal Society of Chemistry, 2020 [98]. (**B**) Images of the cell membrane in the water/OE2imC3C/DMSO and water/DMSO mixtures. Reproduced with permission from Spring Nature, 2021 [99]. (**C**) Schematic illustration of zinc cations for stabilizing cell membrane. Reproduced with permission from the American Chemical Society, 2023 [100]. (**D**) Schematic illustration of ionic liquids–membrane interaction/localization. Reproduced with permission from American Chemical Societ, 2016 [101]

cells. Zwitterions indirectly inhibit intracellular ice formation by dehydrating cells due to the high osmotic pressure, which can damage the cells [102]. To develop better zwitterionic cryoprotectants, a structure that can reduce osmotic pressure after freeze concentration is required. It is important to note that all of the ionic liquids were significantly more toxic than the current standard cryoprotective agent, DMSO [103]. Introduction of polar groups at the end of alkyl chain, composed of bio-derived ions(such as choline cations), or zwitterionization, can result in very low toxicity [98]. A synthetic zwitterion-type ionic liquid containing histidine-like module, ZIL, possesses suitable affinity to the plasma membrane and acts as a biocompatible cryoprotectant [104].

Scaled-up approaches for cryopreservation

Nanowarming

There are two aspects of the application of nanotechnology in cryopreservation. One is the use of nanomaterials that mimic AFPs, which possess ice-regulating properties and can be added as CPAs in the process of freezing and thawing. Such contents have been presented in subsection "3.2 Antifreeze proteins and their mimics". The other application uses nanomaterials as self-heating seeds accompanied by external physical fields to achieve rapid and uniform rewarming during the thawing process, leading to ice recrystallization inhibition (Fig. 6).

The rewarming rate is a crucial factor for the outcome of cryopreservation. The ability to remove ice can be greatly enhanced by the heating effect. To this end, nanowarming can not only greatly increase rewarming rates but also lower the requirements for CPAs and eliminate temperature nonuniformity, which is very important, especially for large-volume biosamples. Such nanowarming involves two main physical processes: magnetothermal conversion and photothermal conversion. Radiofrequency radiation and laser illumination are the main external physical fields used in this technique. Currently, nanowarming has been shown to facilitate the rewarming of cells, tissues, and even organs, both physically and biologically. The preparation of nanoparticles was also optimized to be scalable [107]. The representative studies that used nanomaterials as rewarming promoters are summarized in Table 2.

Although nanomaterials have made great progress in cryopreservation, limitations such as how to load uniform nanomaterials in samples, how to optimize the parameters for better rewarming efficiency, and the potential safety concerns of residual nanoparticles should be evaluated.

Cell encapsulation

Cell microencapsulation is now widely used to facilitate off-the-shelf materials for biomedical applications [111]. Biocompatible hydrogels are utilized to enclose living cells in capsules. Encapsulated cells perform better than nonencapsulated cells in cryopreservation [112]. The mechanisms of the cell encapsulation strategy for cryopreservation may be attributed to the following factors: the inhibition of mechanical damage and shear stress from extracellular ice formation, the promotion of cell attachment and cell–matrix interactions in three-dimensional environments [113], and the stabilization of cell

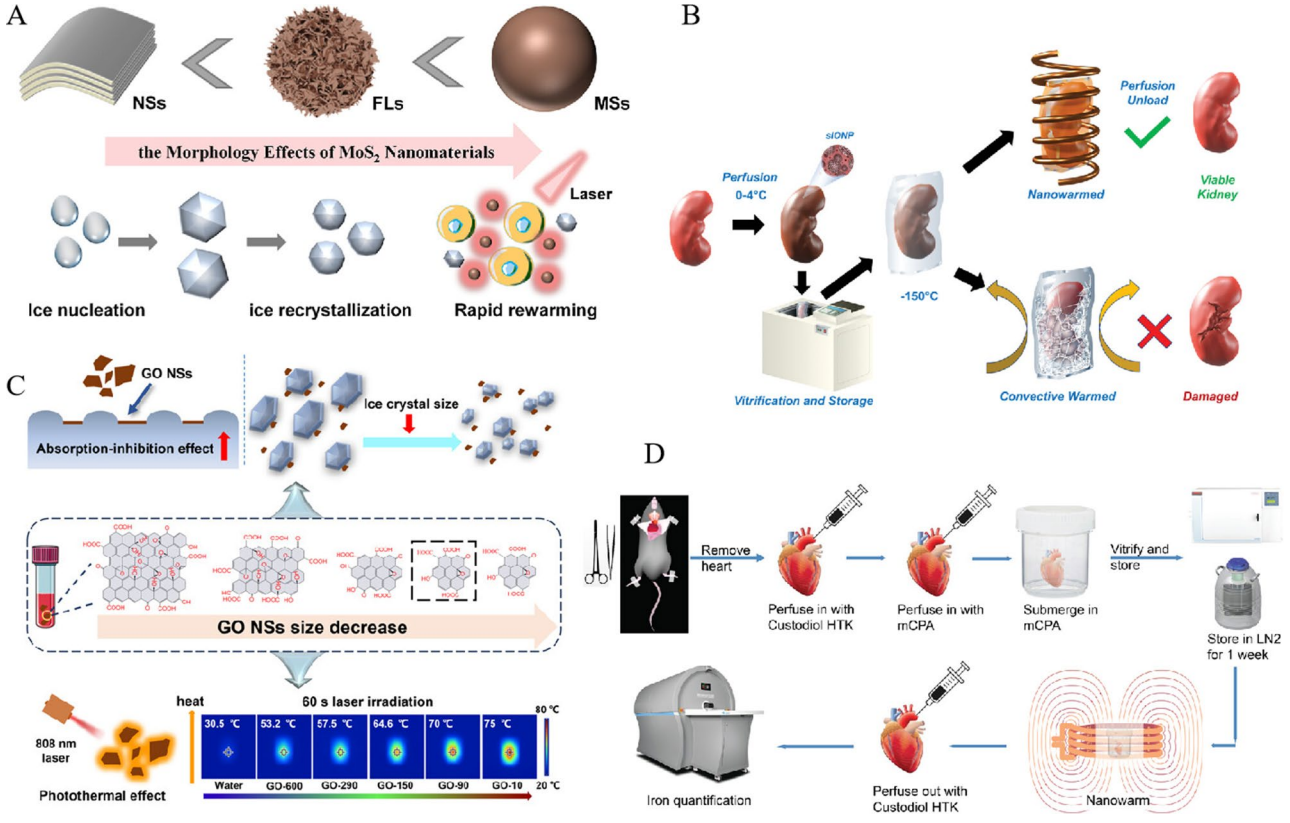


Fig. 6 Schematic illustrations of cryopreservation and nanowarming. (**A**) Morphology effects of MoS2 Nps on ice suppression and rapid rewarming. Reproduced with permission from American Chemical Society, 2024 [48]. (**B**) Schematic flow of kidney nanowarming. Reproduced with permission from Wiley, 2021 [105]. (**C**) Size effect of GOs on ice crystal regulation and rewarming. Reproduced with permission from American Chemical Society, 2024 [49]. (**D**) Procedure for nanowarming of whole hearts. Reproduced with permission from American Association for the Advancement of Science, 2021 [106]

Table 2 Representative nanowarming for some biosamples

Nanowarming methods	Warming parameters	Nanoparticles	Biosamples	References
Radiofrequency magnetic field	20 kA/m, 360 kHz, > 130 °C/min	Mesoporous silica-coated iron oxide nanoparticle(mslONPs)	Human dermal fibroblast cells, porcine arteries and porcine aortic heart valve leaflet tissues(1–50 ml)	[108]
Radiofrequency magnetic field	63 kA/m, 180 kHz, mean rate 63.7 °C/min	mslONPs	First time successful recovery of a rat kidney from the vitrified state using nanowarming	[105]
Radiofrequency magnetic field	63 kA/m, 180 kHz, mean rate 72 ± 8 °C/min	mslONPs	Life-sustaining kidney transplantation in a rat model	[109]
Radiofrequency magnetic field	42.5 kA/m, 278 kHz, up to 321 °C/min	Superparamagnetic iron oxide nanoparticles (SPIONs)	Whole heart	[106]
Radiofrequency magnetic field	Melting time about 30 s to 1 min	Cold- responcive Fe_3O_4 NPs	Rat β cells	[52]
Radiofrequency magnetic field and laser	\	GO-Fe_3O_4 nanocomposites (NPs)	Mesenchymal stem cells (MSCs), 80.1% survival	[51]
Laser	800 nm, 2 W/cm^2, recrystallization time duration from 4.5s reduce to 1.4s	2D Ti_3C_2Tx MXene	MSCs, 80.9% survival	[50]
Laser	808 nm, 1.5 W/cm^2	Molybdenum disulfide (MoS2)	A549 cells, 85.3% survival	[48]
Laser	808 nm, 2 W/cm^2	2D GO	A549 cells, 95.2% viability; Hela cells, 93% survival	[49]
Radiofrequency magnetic field and laser	15 A, 3 W/cm^2	GO NPs and Fe_3O_4 NPs	Mouse preantral follicles(PAFs), 90% survival, gained healthy mouse pups	[110]

morphology, which is often linked to functional performance [114]. The advances in slow freezing cryopreservation of microencapsulated cells have been reviewed by Gurruchaga H and coworkers [115]. Here, we pay more attention to microencapsulation cells for vitrification.

In general, three main methods are used to produce encapsulated cells: electrostatic spraying, air-flowing and microfluidics (Fig. 7A). Electrostatic spraying technology is typically used to produce microcapsulated hydrogel beads. Although emulsion droplet vitrification has emerged as a promising cryopreservation method, the upper sample size is limited by the nozzle of the inkjet head, which makes it inapplicable for mammalian oocytes, organoids and spheroids [116]. Even when a combination of droplet printing, conduction cooling and laser warming is used to dramatically increase the droplet vitrification volume(best for 4 uL)(Fig. 7C), the device is complex and not amenable to storage and thawing. Notably, Zhao's group introduced a simple small centrifugal microfluidic device to microencapsulate mouse preantral follicles (PAFs) in alginate hydrogels, and healthy mouse pups were successfully born after IVF and embryo transfer from the vitrified oocytes (Fig. 7B).

Another type of cell encapsulation is the core-shell structure. Zhao developed capillary microfluidics-based large-volume core-shell alginate hydrogel encapsulation technology to successfully facilitate vitreous cryopreservation of encapsulated porcine adipose-derived stem cells (pADSCs) with low concentrations of CPAs (2 M penetrating CPAs). The microfluidics device was fabricated by using three tube-in-tube patterned glass capillaries with different diameters, and it provides a cost-effective approach for "ready-to-use" cell-laden material [115]. There is room for improvement. Oleksandr Gryshkov evaluated the structural properties and swelling behavior of core–shell capsules and demonstrated that core–shell capsules produced from low-viscosity high-G alginate are superior to high-M capsules and that the coaxial electrospraying process can be scaled up to potentially produce the required number of core–shell capsules for clinical applications [117].

More recently, a core–shell microfiber encapsulation system based on the integration of 3D printing, microfluidics, and core–shell encapsulation technology has become more attractive for clinical applications. A 3D cell printing system supplemented with a UV treatment system, microfluidic channels and a core–shell nozzle connected to a three-axis robot was developed to fabricate a cryopreservable cell-laden GelMa-based scaffold. Furthermore, freeform shelf-ready tissue fabrication and storage were successful by cryobioprinting (Fig. 7D). These methods are perfect techniques except for the safety concern of UV exposure to living cells. More safe, nontoxic and glycerol-free cryopreservation methods for human red blood cells (RBCs) with high final hematocrits were investigated by Zhao's group. They used nonpermeating trehalose as the sole CPA to dehydrate RBCs and core-shell alginate microfibers, which were produced by a coaxial needle to increase heat transfer (Fig. 7E). This method can be easily scaled up for practical meaningful

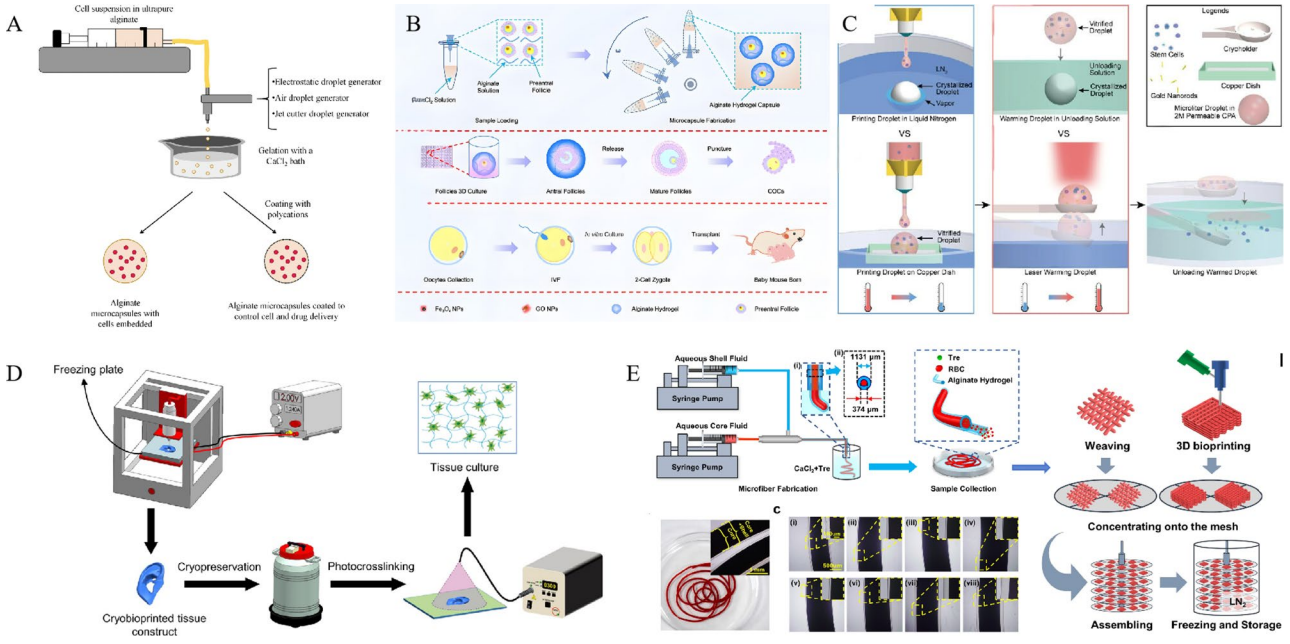


Fig. 7 Schematic illustration of different microencapsulation methods for cryopreservation. (**A**) Scheme of the microencapsulation process. Reproduced with permission from Elsevier, 2018 [115]. (**B**) Oocyte microencapsulation via centrifugal microfluidic technology, in vitro culture, and the generation of mouse pups from vitrified oocytes. Reproduced with permission from Springer Nature, 2022 [110]. (**C**) Capillary microfluidic encapsulation of a biosample in core–shell microcapsules. Reproduced with permission from Wiley, 2021 [118]. (**D**) Cryobioprinting of tissue constructs for simultaneous cryopreservation. Reproduced with permission from Elsevier, 2022 [119]. (**E**) Microfluidic system used to generate core–shell microfibers. Reproduced with permission from Springer, 2024 [120]

throughput by using 3D bioprinting or woven fibers and coupling with cryomeshes for batch cooling and thawing operations.

Cryomesh

Directly printing microfibers on a substrate or into liquid nitrogen is a good approach for small-volume cells. However, bioprinting is not suitable for large biosystems, and achieving scalable cooling and rewarming in throughput operations is still challenging. Cryomesh may be a promising alternative for embryo and organism vitrification. Li Zhan developed a nylon mesh attached to a thin polystyrene holder for simple and robust cryopreservation for *Drosophila melanogaster* embryos (Fig. 8A) and islets (Fig. 8B). They further developed a rapid and scalable rewarming platform for adherent cells, *Drosophila melanogaster* embryos, and rat kidney slices. The platform was made of a stainless steel sheet or mesh as an electrical conductor, connected to a pulse generator, and generated heat via jouke heating (Fig. 8C and D). No doubt, wicking the CPA solution off the cryomesh prior to vitrification results in a "CPA solution-free" method and maximizes the cooling and warming rates while allowing the processing of a large number of samples within a short time. However, varying the mesh wire size, pore size, and materials (nylon, stainless steel or copper) may lead to different outcomes for vitrification when using cryomesh [121].

Isochoric freezing

Cryopreservation, as practiced today, is mostly an isobaric (constant pressure) process that takes place at a pressure of 1 atm. The concept of 'isochoric cold storage' was first developed by Rubinsky and his group in 2005 [125]. An isobaric (constant volume) cryopreservation system is in which ice and the solution exist in thermodynamic equilibrium at a constant temperature and constant volume. It requires a constant volume chamber capable of withstanding the pressures that develop in the system. In a constant-volume container, when freezing begins, the lower-density ice cannot expand freely. Consequently, the still-unfrozen portion of the liquid is compressed, forcing the freezing process to follow the "liquid line". It can reduce mechanical damage related to the formation of dendritic crystals. A container with excellent pressure resistance and sealing performance, as well as various sensor devices, is needed. Obviously, biobanking and other fields may also benefit from isochoric freezing. For example, mammalian hearts were preserved for the first time at subzero centigrade temperatures without chemical cryoprotectants [126]; autonomously beating genetically engineered cardiac tissue was preserved for the first time, even for several days [127]; mammalian

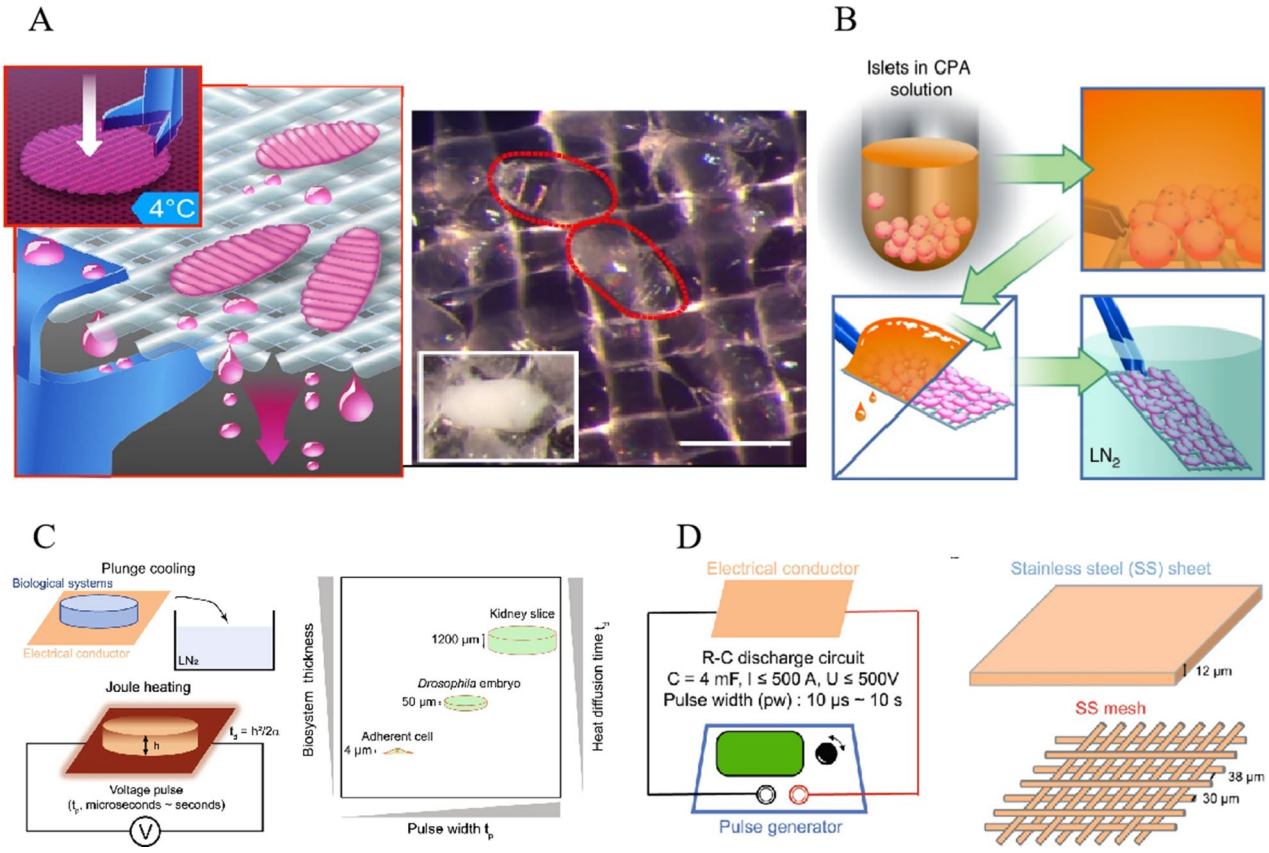


Fig. 8 Schematic illustration of cryopreservation with cryomesh. (**A**) Cryopreservation procedures for *Drosophila melanogaster* embryos with cryomesh. Reproduced with permission from Springer Nature, 2021 [122]. (**B**) Pancreatic islet cryopreservation with cryomesh. Reproduced with permission from Springer Nature, 2022 [123]. (**C** and **D**) A rapid and scalable rewarming platform using cryomesh and joule heating. Reproduced with permission from Springer Nature, 2022 [124]

pancreatic islets were preserved for several days [128]; tand cm-scale coral fragments were successfully cryopreserved and revived via mL-scale isochoric vitrification [129]. Moreover, by altering the fundamental thermodynamics of freezing, significant energy savings can be achieved [130].

Conclusion and future perspectives

Table 3 summarizes the machanism, cost, operational complexity, and biocompatibility of different materials and methods used in cryopreserving various biological samples.

Scientists have made significant progress with innovative materials and strategies for cryopreservation. However, until now, even the best cryopreservation strategy has not guaranteed the success of application. The biochemical pathways of different biosamples differ; thus, cryopreservation is unlikely to be a one-size-fits-all process. Freeze-tolerant creatures, such as arctic woolly bear caterpillars, marine intertidal snails, wood frogs, hatchling painted turtles, and Alaskan beetles have developed different strategies to overcome freezing in nature, even undergo several freeze–thaw cycles. Understanding the natural cold tolerance mechanisms of creatures that survive at cold temperatures clearly offers perfect solutions for protecting biological samples during freezing and thawing. The prospects for achieving this goal have become increasingly plausible due to swift advancements in imaging technology, DNA sequencing, omics techniques, and other related fields. Although different cryopreservation methods are discussed separately here, it is essential to integrate a rational scientific mechanism with multidisciplinary techniques to translate cold tolerance strategies into cryopreservation practices for biosamples.

The biophysics of the cryopreservation process need to be clearly understood. Currently, the issue of 'what is the exact link between antifreeze and freezing' is still a mystery. Even the janus effect of AFPs on ice nucleation and the existence and temperature-dependent size of the critical ice nucleus have been provided by experimental information [43, 131], there is ongoing exploration surrounding the universal mechanism by which natural AFPs inhibit ice formation. Molecular modeling techniques and computational complexity, such as biomimetic crowding coacervates and regioisomerism [132, 133], for determining the molecular interactions between

Table 3 Summary of the different materials and methods used in cryopreservation

Materials/methods	Machanisms	Biosamples	Cost	Operation	Toxicity
Sugars and polymers	Induce cell dehydration and reduce intracellular ice formation, IRI. Need high concentration.	Sperm, oocyte, embryo, stem cell, etc.	Low	Easy	Biodegradable
Antifreeze proteins(AFPs)	IRI and adhesion to ice by orderly arrangement of hydrophobic methyl groups and hydrophilic hydroxyl groups.	Oocyte, embryo, certain types of stem cells, etc.	High	Purification difficulty	Potential immunogenicity
Nanoparticles(NPs)	Adsorb on the surface of crystals by honeycomb structure, curve ice crystalsand, lowers the freezing temperature, and suppress ice growth.	Cells, tissue, and organ	High	Hard	Potential toxicity
Ice nucleators	Provide localized deep supercooling to induce ice nucleation at high subzero temperatures.	Cell	Low	Difficult to standardize	\
Hydrogels	Interact water with hydrogel network, and decrease the ice formation. Poor mechanical properties.	Engineering cell and tissue, stem cells, etc.	Low	Easy	Biocompatable
Antioxidants	Scavenge ROS. Effect differs according to cell type.	Samples with poor ROS tolerance: sperm, oocyte	Low	Easy	Excessive is harmful.
Zwitterions	Interact with water through strong electrostatic charges, easily transform into a glassy state, thus inhibit ice crystallization.	Cell	Low	Easy	Ionic liquids are toxic, need modifications.
Nanowarming	Self-heating by external physical fields to achieve rapid and uniform rewarming, leading to IRI	Embryo, tissue, organ	High	Hard	Potential toxicity
Cell encapsulation	Use hydrogels to enclose living cells. Microfluidic devices and 3D printing devices.	Cell, oocyte	High	Hard	Biodegradable
Cryomesh	Utilize good thermal conductivity materials to create a mesh, robust cryopreservation.	Large-volume samples: embryo, tissue, organ	Low	Easy	Copper is toxic.
Isochoric freezing	Use a constant volume chamber to inhibite ice growth freely.	A variety of samples	High	Hard	Non- toxic

ice-inhibition materials and water/ice molecules should be studied. High-throughput discovery methodologies are progressing, and phage display in conjunction with ice-affinity selection can be utilized to discover novel ice-binding peptides [134]. In addition, machine learning/AI tools are crucial for dissecting complex datasets and nonlinear trends.

Traditionally, cryopreservation has been viewed as merely a physical process that can be solved by controlling and modulating ice growth and nucleation, addressing osmotic changes, and altering freezing rates. Currently, a biochemical perspective is increasingly being taken into account when considering cryopreservation, since biochemical pathways can be modulated to mitigate cold stress. In addition to genomic analysis, transcriptomics, proteomics, metabolomics, and other relevant omics analyses can provide more information about cold tolerance.

To effectively implement cold tolerance strategies in cryopreservation, a multidisciplinary approach involving the use of cutting-edge materials and the integration of various techniques are imperative. Recombinant protein fibers produced through synthetic biology may serve as a promising alternative to natural AFPs and other ice regulators [135]. Searching for multifunctional CPAs is worthwhile [136]. For example, soft, strong, tough, durable and antifreeze hydrogels [137–139] for cryopreservations. Even more, finding some materials that are beneficial for cryopreservation and suitable for in vitro cultivation and sustained use in vivo. The rational design of all-in-one protection may promote the development of a competent platform [140, 141].

In addition to their use in cell encapsulation with 3D bioprinting, microfluidic technology/lab-on-a-chip systems can also be harnessed for cryopreservation, encompassing various aspects such as CPAs uptake and elimination, cell membrane characteristics, and the implications of temperature reduction and increase patterns [142, 143]. DNA origami structures and cryosilicification may be promising new protection methods for cryopreservation [144–146]. Liquid helium, slush nitrogen, or other cryogens, instead of liquid nitrogen, may be adopted to achieve high cooling rates for vitrification [147]. Hypothermic storage, including supercooling and deep supercooling, is also crucial in sample preservation [148]. Automated and standardized operational processes should be developed to reduce human error and ensure the repeatability and reliability of the preservation process.

In light of these, key research areas for cryopreservation are as follows.

(1) Elucidating mechanisms of the natural cold tolerance mechanisms of creatures by imaging technology, multi-omic techniques, and other related techniques.
(2) Investigating signal pathway targets involved in cryoinjury repair for specific samples.
(3) Developing multi-functional platform that is antioxidant, anti-freezing, biocompatible, and scalable, by computational chemistry, molecular dynamics simulations, and AI from a multidisciplinary perspective.

Abbreviations

CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
EG	Ethylene glycol
AFP	Antifreeze protein
HB	Hydrogen bond
NIBF	Nonice-binding face
SOD	Superoxide dismutase
RNS	Reactive nitrogen species
GPx	Glutathione peroxidase
GO	Graphene oxide
MOF	Metal-organic framework
NP	Nanoparticle
IRI	Ice recrystallization inhibition
ROS	Reactive oxygen species
RBCs	Red blood cells

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Author contributions
MH: Conceptualization, Investigation, Writing-original draft, Writing-review & editing, Visualization. MH: Investigation, Resources, Writing- original draft. GC: Resources, Project administration, Funding acquisition. HW: Investigation, Writing-review & editing. SH: Project administration. EZ: Writing-review & editing, Funding acquisition. ZW: Resources, Writing- review & editing, Supervision, Project administration, Funding acquisition.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]State Key Laboratory of Swine and Poultry Breeding Industry, National Engineering Research Center for Breeding Swine Industry, College of Animal Science, South China Agricultural University, Guangzhou 510642, China
[2]National Regional Gene Bank of Livestock and Poultry, Gene Bank of Guangdong Livestock and Poultry, South China Agricultural University, Guangzhou 510642, China
[3]National Canine Laboratory Animal Resources Center, Guangzhou General Pharmaceutical Research Institute Co., Ltd, Guangzhou 510240, China

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References

1. Zuchowicz N, Liu Y, Monroe WT, Tiersch TR. An automated modular open-technology device to measure and adjust concentration of aquatic sperm samples for cryopreservation. SLAS Technol. 2023;28:43–52.
2. Han Z, Sharma A, Gao Z, Carlson TW, O'Sullivan MG, Finger EB, et al. Diffusion limited cryopreservation of tissue with radiofrequency heated metal forms. Adv Healthc Mater. 2020;9:2000796.
3. Onofre J, Baert Y, Faes K, Goossens E. Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation. Hum Reprod Update. 2016;22:744–61.
4. Sun Y, Li Y, Zong Y, Mehaisen GMK, Chen J. Poultry genetic heritage cryopreservation and reconstruction: advancement and future challenges. J Anim Sci Biotechnol. 2022;13:115.
5. Ryu D-Y, Song W-H, Pang W-K, Yoon S-J, Rahman MS. Pang M-G. Freezability biomarkers in bull epididymal spermatozoa. Sci Rep. 2019;9:12797.
6. Yánez-Ortiz I, Catalán J, Rodríguez-Gil JE, Miró J, Yeste M. Advances in sperm cryopreservation in farm animals: cattle, horse, pig and sheep. Anim Reprod Sci. 2022;246:106904.
7. López A, Ducolomb Y, Casas E, Retana-Márquez S, Betancourt M, Casillas F. Effects of Porcine immature oocyte vitrification on actin microfilament distribution and chromatin integrity during early embryo development in vitro. Front Cell Dev Biol. 2021;9:636765.
8. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update. 2015;21:209–27.
9. Murray KA, Gibson MI. Chemical approaches to cryopreservation. Nat Rev Chem. 2022;6:579–93.
10. Liu Z, Zheng X, Wang J. Bioinspired Ice-Binding materials for tissue and organ cryopreservation. J Am Chem Soc. 2022;144:5685–701.
11. Argyle CE, Harper JC, Davies MC. Oocyte cryopreservation: where are we now? Hum Reprod Update. 2016;22:440–9.
12. Truong TT, Gardner DK. Antioxidants increase blastocyst cryosurvival and viability post-vitrification. Hum Reprod. 2020;35:12–23.
13. Arav A. Cryopreservation by directional freezing and vitrification focusing on large tissues and organs. Cells. 2022;11:1072.
14. Dou M, Lu C, Rao W. Bioinspired materials and technology for advanced cryopreservation. Trends Biotechnol. 2022;40:93–106.
15. Petersen A, Schneider H, Rau G, Glasmacher B. A new approach for freezing of aqueous solutions under active control of the nucleation temperature. Cryobiology. 2006;53:248–57.
16. Briard JG, Poisson JS, Turner TR, Capicciotti CJ, Acker JP, Ben RN. Small molecule ice recrystallization inhibitors mitigate red blood cell Lysis during freezing, transient warming and thawing. Sci Rep. 2016;6:23619.
17. Casillas F, Ducolomb Y, López A, Betancourt M. Effect of Porcine immature oocyte vitrification on oocyte-cumulus cell gap junctional intercellular communication. Porc Health Manag. 2020;6:37.
18. Trapphoff T, Dieterle S. Cryopreservation of ovarian and testicular tissue and the influence on epigenetic pattern. IJMS. 2023;24:11061.
19. Mark C, Czerwinski T, Roessner S, Mainka A, Hörsch F, Heublein L, et al. Cryopreservation impairs 3-D migration and cytotoxicity of natural killer cells. Nat Commun. 2020;11:5224.

20. Len JS, Koh WSD, Tan S-X. The roles of reactive oxygen species and antioxidants in cryopreservation. Biosci Rep. 2019;39:BSR20191601.
21. Liu X, Xu Y, Liu F, Pan Y, Miao L, Zhu Q, et al. The feasibility of antioxidants avoiding oxidative damages from reactive oxygen species in cryopreservation. Front Chem. 2021;9:648684.
22. Lee E, Baiz CR. How cryoprotectants work: hydrogen-bonding in low-temperature vitrified solutions. Chem Sci. 2022;13:9980–4.
23. Cheng C-Y. DMSO induces dehydration near lipid membrane surfaces. Biophys J. 2015;109:330–9.
24. Kim K, Lee S-E. Combined toxicity of dimethyl sulfoxide (DMSO) and vanadium towards zebrafish embryos (Danio rerio): Unexpected synergistic effect by DMSO. Chemosphere. 2021;270:129405.
25. Lemieux JM, Wu G, Morgan JA, Kacena MA. DMSO regulates osteoclast development in vitro. Vitro CellDevBiol-Animal. 2011;47:260–7.
26. Verheijen M, Lienhard M, Schrooders Y, Clayton O, Nudischer R, Boerno S et al. DMSO induces drastic changes in human cellular processes and epigenetic landscape in vitro. Sci Rep. 2019;9.
27. Valeria P, Cristian DSF, Fiammetta P. B. Effect of Media with Different Glycerol Concentrations on Sheep Red Blood Cells'Viability In Vitro. Animals [Internet]. 2021;11. Available from: https://kns.cnki.net/kcms2/article/abstract?v=PAev8JwjQit9zKjQLAnKmu1JeMq75O-zCIlw529gnHamaTmW2jBlmB_LgLr2TKYhX5OHhAyl4YAV1U5b8EvvIA48pNNyfLIZtL0aDxskuJ1iLVWFNWP-ClPlyzliH7tiiQxJdh-S90imBoAxOeuCFg==%26;uniplatform=NZKPT%26;language=gb
28. Zou CG, Agar NS, Jones GL. Haemolysis of human and sheep red blood cells in glycerol media: the effect of pH and the role of band 3. Comp Biochem Physiol Mol Integr Physiol. 2000;127:347–53.
29. Best BP. Cryoprotectant toxicity: facts, issues, and questions. Rejuven Res. 2015;18:422–36.
30. Peter Mazur. Cryobiology: the freezing of biological systems. Science. 1970;168:939–49.
31. Chen F, Zhang W, Wu W, Jin Y, Cen L, Kretlow JD, et al. Cryopreservation of tissue-engineered epithelial sheets in Trehalose. Biomaterials. 2011;32:8426–35.
32. Yang S, Wang T, Wen D, Hou J, Li H. Protective effect of Rhodiola rosea polysaccharides on cryopreserved Boar sperm. Carbohydr Polym. 2016;135:44–7.
33. Guerreiro BM, Freitas F, Lima JC, Silva JC, Dionísio M, Reis MAM. Demonstration of the cryoprotective properties of the fucose-containing polysaccharide FucoPol. Carbohydr Polym. 2020;245:116500.
34. Patel M, Park JK, Jeong B. Rediscovery of poly(ethylene glycol)s as a cryoprotectant for mesenchymal stem cells. Biomater Res. 2023;27:17.
35. Deller RC, Vatish M, Mitchell DA, Gibson MI. Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing. Nat Commun. 2014;5:3244.
36. Diaz-Dussan D, Peng Y-Y, Sengupta J, Zabludowski R, Adam MK, Acker JP, et al. Trehalose-Based polyethers for cryopreservation and Three-Dimensional cell scaffolds. Biomacromolecules. 2020;21:1264–73.
37. Chopra P, Nayak D, Nanda A, Ashe S, Rauta PR, Nayak B. Fabrication of poly(vinyl alcohol)-Carrageenan scaffolds for cryopreservation: effect of composition on cell viability. Carbohydr Polym. 2016;147:509–16.
38. Jaskiewicz JJ, Sandlin RD, Swei AA, Widmer G, Toner M, Tzipori S. Cryopreservation of infectious Cryptosporidium parvum oocysts. Nat Commun. 2018;9:2883.
39. Stefanic M, Ward K, Tawfik H, Seemann R, Baulin V, Guo Y, et al. Apatite nanoparticles strongly improve red blood cell cryopreservation by mediating Trehalose delivery via enhanced membrane permeation. Biomaterials. 2017;140:138–49.
40. Yao X, Jovevski JJ, Todd MF, Xu R, Li Y, Wang J, et al. Nanoparticle-Mediated intracellular protection of natural killer cells avoids cryoinjury and retains potent antitumor functions. Adv Sci. 2020;7:1902938.
41. Zhang Y, Wang H, Stewart S, Jiang B, Ou W, Zhao G, et al. Cold-Responsive nanoparticle enables intracellular delivery and rapid release of Trehalose for Organic-Solvent-Free cryopreservation. Nano Lett. 2019;19:9051–61.
42. Yeh Y, Feeney RE. Antifreeze proteins: structures and mechanisms of function. Chem Rev. 1996;96:601–18.
43. Liu K, Wang C, Ma J, Shi G, Yao X, Fang H, et al. Janus effect of antifreeze proteins on ice nucleation. Proc Natl Acad Sci USA. 2016;113:14739–44.
44. Farag H, Peters B. Engulfment avalanches and thermal hysteresis for antifreeze proteins on supercooled ice. J Phys Chem B. 2023;127:5422–31.
45. Davies PL. Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth. Trands Biochem Sci. 2014;39:548–55.
46. Qin Q, Zhao L, Liu Z, Liu T, Qu J, Zhang X, et al. Bioinspired L -Proline oligomers for the cryopreservation of oocytes *via* controlling ice growth. ACS Appl Mater Interfaces. 2020;12:18352–62.
47. El Assal R, Abou-Elkacem L, Tocchio A, Pasley S, Matosevic S, Kaplan DL, et al. Bioinspired preservation of natural killer cells for Cancer immunotherapy. Adv Sci. 2019;6:1802045.
48. Han W, Zhong L, Zhang J, Li Y, Li N, Wang Z, et al. Profiling the morphology effects of MoS_2 nanomaterials on ice suppression and rapid rewarming for cryopreservation. ACS Appl Nano Mater. 2024;7:12783–94.
49. Li L, Zhang J, Li Y, Liu B, Yu J, Li N, et al. Probing the size effect of graphene oxide nanosheets on ice crystal regulation and Laser-Assisted rapid rewarming. ACS Appl Mater Interfaces. 2024;16:33149–58.
50. Cao Y, Chang T, Fang C, Zhang Y, Liu H, Zhao G. Inhibition Effect of $Ti_3C_2T_x$ MXene on Ice Crystals Combined with Laser-Mediated Heating Facilitates High-Performance Cryopreservation. ACS Nano. 2022;16:8837–50.
51. Cao Y, Hassan M, Cheng Y, Chen Z, Wang M, Zhang X, et al. Multifunctional Photo- and magnetoresponsive graphene Oxide–Fe_3O_4 Nanocomposite–Alginate hydrogel platform for ice recrystallization Inhibition. ACS Appl Mater Interfaces. 2019;11:12379–88.
52. Cheng Y, Yu Y, Zhang Y, Zhao G, Zhao Y. Cold-Responsive nanocapsules enable the Sole-Cryoprotectant-Trehalose cryopreservation of B Cell–Laden hydrogels for diabetes treatment. Small. 2019;15:1904290.
53. Geng H, Liu X, Shi G, Bai G, Ma J, Chen J, et al. Graphene oxide restricts growth and recrystallization of ice crystals. Angew Chem Int Ed. 2017;56:997–1001.
54. Zhu W, Guo J, Agola JO, Croissant JG, Wang Z, Shang J, et al. Metal–Organic framework Nanoparticle-Assisted cryopreservation of red blood cells. J Am Chem Soc. 2019;141:7789–96.
55. Chang T, Moses OA, Tian C, Wang H, Song L, Zhao G. Synergistic ice Inhibition effect enhances rapid freezing cryopreservation with low concentration of cryoprotectants. Adv Sci. 2021;8:2003387.
56. Georgiou PG, Marton HL, Baker AN, Congdon TR, Whale TF, Gibson MI. Polymer Self-Assembly induced enhancement of ice recrystallization Inhibition. J Am Chem Soc. 2021;143:7449–61.
57. Lee C, Lee Y, Jung WH, Kim T-Y, Kim T, Kim D-N, et al. Peptide-DNA Origami as a cryoprotectant for cell preservation. Sci Adv. 2022;8:eadd0185.
58. Hou Y, Sun X, Dou M, Lu C, Liu J, Rao W. Cellulose nanocrystals facilitate Needle-like ice crystal growth and modulate molecular targeted ice crystal nucleation. Nano Lett. 2021;21:4868–77.
59. Jiang B, Li W, Stewart S, Ou W, Liu B, Comizzoli P, et al. Sand-mediated ice seeding enables serum-free low-cryoprotectant cryopreservation of human induced pluripotent stem cells. Bioactive Mater. 2021;6:4377–88.
60. Hou LX, Ju H, Hao XP, Zhang H, Zhang L, He Z, et al. Intrinsic Anti-Freezing and unique phosphorescence of glassy hydrogels with ultrahigh stiffness and toughness at low temperatures. Adv Mater. 2023;35:2300244.
61. Wu S, Liu Z, Gong C, Li W, Xu S, Wen R, et al. Spider-silk-inspired strong and tough hydrogel fibers with anti-freezing and water retention properties. Nat Commun. 2024;15:4441.
62. Zhang X, Ma X, Hou T, Guo K, Yin J, Wang Z, et al. Inorganic salts induce thermally reversible and Anti-Freezing cellulose hydrogels. Angew Chem Int Ed. 2019;58:7366–70.
63. Yukun Jian S, Handschuh-Wang J, Zhang W, Lu X, Zhou T, Chen. Biomimetic anti-freezing polymeric hydrogels: keeping soft-wet materials active in cold environments. Mater Horiz. 2021;8:351–69.
64. Niki E. Lipid oxidation that is, and is not, inhibited by vitamin E: consideration about physiological functions of vitamin E. Free Radic Biol Med. 2021;176:1–15.
65. Guo S, Yang J, Qin J, Qazi IH, Pan B, Zang S, et al. Melatonin promotes in vitro maturation of Vitrified-Warmed mouse germinal vesicle oocytes, potentially by reducing oxidative stress through the Nrf2 pathway. Animals. 2021;11:2324.
66. Hai E, Li B, Zhang J, Zhang J. Sperm freezing damage: the role of regulated cell death. Cell Death Discov. 2024;10:239.
67. Du T, Su H, Cao D, Meng Q, Zhang M, Liu Z, et al. Mitochondria-targeted antioxidant mitoquinone mitigates vitrification-induced damage in mouse ovarian tissue by maintaining mitochondrial homeostasis via the p38 MAPK pathway. Eur J Med Res. 2024;29:593.
68. Zhu Z, Li R, Fan X, Lv Y, Zheng Y, Hoque SAM, et al. Resveratrol improves Boar sperm quality via 5 ′ AMP-Activated protein kinase activation during cryopreservation. Oxidative Med Cell Longev. 2019;2019:1–15.
69. Berjis A, Muthumani D, Aguilar OA, Pomp O, Johnson O, Finck AV, et al. Pretreatment with IL-15 and IL-18 rescues natural killer cells from granzyme B-mediated apoptosis after cryopreservation. Nat Commun. 2024;15:3937.
70. Sun TC, Liu XC, Yang SH, Song LL, Zhou SJ, Deng SL, et al. Melatonin inhibits oxidative stress and apoptosis in cryopreserved ovarian tissues via Nrf2/HO-1 signaling pathway. Front Mol Biosci. 2020;7:163.

71. Li X, Wang L, Liu H, Fu J, Zhen L, Li Y, et al. C60 fullerenes suppress reactive oxygen species toxicity damage in Boar sperm. Nano-Micro Lett. 2019;11:104.
72. Zhang X, Chen L, Liu W, Shen J, Sun H, Liang J, et al. 5-Aminolevulinate improves metabolic recovery and cell survival of the liver following cold preservation. Theranostics. 2022;12:2908–27.
73. Kim JH, Choi JI, Che YH, Sung SH, Lee H, Lee S, et al. Enhancing viability of human embryonic stem cells during cryopreservation via RGD-REP-Mediated activation of FAK/AKT/FoxO3a signaling pathway. Tissue Eng Regen Med. 2023;20:1133–43.
74. Sies H, Jones DP. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat Rev Mol Cell Biol. 2020;21:363–83.
75. Zhu Z, Li R, Lv Y, Zeng W. Melatonin protects rabbit spermatozoa from cryodamage via decreasing oxidative stress. Cryobiology. 2019;88:1–8.
76. Appiah MO, He B, Lu W, Wang J. Antioxidative effect of melatonin on cryopreserved chicken semen. Cryobiology. 2019;89:90–5.
77. Deng S-L, Sun T-C, Yu K, Wang Z-P, Zhang B-L, Zhang Y, et al. Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen. Free Radic Biol Med. 2017;113:347–54.
78. Lee AR, Hong K, Choi SH, Park C, Park JK, Lee JI, et al. Anti-apoptotic regulation contributes to the successful nuclear reprogramming using cryopreserved oocytes. Stem Cell Rep. 2019;12:545–56.
79. Gangwar C, Kharche SD, Ranjan R, Kumar S, Goel AK, Jindal SK, et al. Effect of vitamin C supplementation on freezability of barbari Buck semen. Small Ruminant Res. 2015;129:104–7.
80. Yue D, Yan L, Luo H, Xu X, Jin X. Effect of vitamin E supplementation on semen quality and the testicular cell membranal and mitochondrial antioxidant abilities in Aohan fine-wool sheep. Anim Reprod Sci. 2010;118:217–22.
81. Figueroa E, Farias JG, Lee-Estevez M, Valdebenito I, Risopatrón J, Magnotti C, et al. Sperm cryopreservation with supplementation of α-tocopherol and ascorbic acid in freezing media increase sperm function and fertility rate in Atlantic salmon (Salmo salar). Aquaculture. 2018;493:1–8.
82. Brito DC, Brito AB, Scalercio SRRA, Percário S, Miranda MS, Rocha RM, et al. Vitamin E-analog trolox prevents Endoplasmic reticulum stress in frozen-thawed ovarian tissue of capuchin monkey (Sapajus apella). Cell Tissue Res. 2014;355:471–80.
83. Gray JE, Starmer J, Lin VS, Dickinson BC, Magnuson T. Mitochondrial Hydrogen Peroxide and Defective Cholesterol Efflux Prevent In Vitro Fertilization by Cryopreserved Inbred Mouse Sperm1. Biology of Reproduction [Internet]. 2013 [cited 2025 Jan 27];89. Available from: https://academic.oup.com/biolreprod/article-lookup/doi/https://doi.org/10.1095/biolreprod.113.109157
84. Banihani S, Agarwal A, Sharma R, Bayachou M. Cryoprotective effect of L -carnitine on motility, vitality and DNA oxidation of human spermatozoa. Andrologia. 2014;46:637–41.
85. Xu H, Jia C, Cheng W, Zhang T, Tao R, Ma Y, et al. The effect of L-Carnitine additive during *In vitro* maturation on the vitrification of pig oocytes. Cell Reprogramming. 2020;22:198–207.
86. Truong TT, Gardner DK. Antioxidants increase blastocyst cryosurvival and viability post-vitrification.
87. Zhang L, Xue X, Yan J, Yan L-Y, Jin X-H, Zhu X-H, et al. L-proline: a highly effective cryoprotectant for mouse oocyte vitrification. Sci Rep. 2016;6:26326.
88. Deng Y, Liu X, Jian X, Zhang Y, Hou Y, Hou S, et al. A novel cryopreservation solution for adipose tissue based on Metformin. Stem Cell Res Ther. 2025;16:20.
89. Giaretta E, Spinaci M, Bucci D, Tamanini C, Galeati G. Effects of Resveratrol on vitrified Porcine oocytes. Oxidative Med Cell Longev. 2013;2013:1–7.
90. Davoodian N, Kadivar A, Ahmadi E, Nazari H, Mehrban H. Quercetin effect on the efficiency of ovine oocyte vitrification at GV stage. Theriogenology. 2021;174:53–9.
91. Xiang D-C, Jia B-Y, Fu X-W, Guo J-X, Hong Q-H, Quan G-B, et al. Role of Astaxanthin as an efficient antioxidant on the in vitro maturation and vitrification of Porcine oocytes. Theriogenology. 2021;167:13–23.
92. Chavas C, Sapanidou VG, Feidantsis K, Lavrentiadou SN, Mavrogianni D, Zaroulidou I, et al. Treatment with pterostilbene ameliorates the antioxidant status of bovine spermatozoa and modulates cell death pathways. Antioxidants. 2024;13:1437.
93. Li Z, Lin Q, Liu R, Xiao W, Liu W. Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. J Androl. 2010;31:437–44.
94. Ruiz-Conca M, Vendrell M, Sabés-Alsina M, Mogas T, Lopez-Bejar M. Coenzyme Q_{10} supplementation during in vitro maturation of bovine oocytes (*Bos taurus*) helps to preserve oocyte integrity after vitrification. Reprod Domest Anim. 2017;52:52–4.
95. Kumar P, Wang M, Isachenko E, Rahimi G, Mallmann P, Wang W, et al. Unraveling subcellular and ultrastructural changes during vitrification of human spermatozoa: effect of a Mitochondria-Targeted antioxidant and a permeable cryoprotectant. Front Cell Dev Biol. 2021;9:672862.
96. Lu X, Zhang Y, Bai H, Liu J, Li J, Wu B. Mitochondria-targeted antioxidant Mito-TEMPO improves the post-thaw sperm quality. Cryobiology. 2018;80:26–9.
97. Yang J, Cai N, Zhai H, Zhang J, Zhu Y, Zhang L. Natural zwitterionic betaine enables cells to survive ultrarapid cryopreservation. Sci Rep. 2016;6:37458.
98. Kuroda K. A simple overview of toxicity of ionic liquids and designs of biocompatible ionic liquids. New J Chem. 2022;46:20047–52.
99. Kato Y, Uto T, Tanaka D, Ishibashi K, Kobayashi A, Hazawa M, et al. Synthetic zwitterions as efficient non-permeable cryoprotectants. Commun Chem. 2021;4:151.
100. Zheng X, Zhang C, Cao H, Zhou X, Liu Z, Wang J. Zinc cations uniquely stabilize cell membrane for cell cryopreservation. Nano Lett. 2023;23:9920–7.
101. Rajan R, Hayashi F, Nagashima T, Matsumura K. Toward a molecular Understanding of the mechanism of cryopreservation by polyampholytes: cell membrane interactions and hydrophobicity. Biomacromolecules. 2016;17:1882–93.
102. Ishizaki T, Takeuchi Y, Ishibashi K, Gotoh N, Hirata E, Kuroda K. Cryopreservation of tissues by slow-freezing using an emerging zwitterionic cryoprotectant. Sci Rep. 2023;13:37.
103. Bryant SJ, Brown SJ, Martin AV, Arunkumar R, Raju R, Elbourne A, et al. Cryopreservation of mammalian cells using protic ionic liquid solutions. J Colloid Interface Sci. 2021;603:491–500.
104. Kuroda K, Komori T, Ishibashi K, Uto T, Kobayashi I, Kadokawa R, et al. Non-aqueous, zwitterionic solvent as an alternative for dimethyl sulfoxide in the life sciences. Commun Chem. 2020;3:163.
105. Sharma A, Rao JS, Han Z, Gangwar L, Namsrai B, Gao Z, et al. Vitrification and nanowarming of kidneys. Adv Sci. 2021;8:2101691.
106. Chiu-Lam A, Staples E, Pepine CJ, Rinaldi C. Perfusion, cryopreservation, and nanowarming of whole hearts using colloidally stable magnetic cryopreservation agent solutions. Sci Adv. 2021;7:eabe3005.
107. Gao Z, Ring HL, Sharma A, Namsrai B, Tran N, Finger EB, et al. Preparation of scalable Silica-Coated Iron oxide nanoparticles for nanowarming. Adv Sci. 2020;7:1901624.
108. Manuchehrabadi N, Gao Z, Zhang J, Ring HL, Shao Q, Liu F, et al. Improved tissue cryopreservation using inductive heating of magnetic nanoparticles. Sci Transl Med. 2017;9:eaah4586.
109. Han Z, Rao JS, Gangwar L, Namsrai B-E, Pasek-Allen JL, Etheridge ML, et al. Vitrification and nanowarming enable long-term organ cryopreservation and life-sustaining kidney transplantation in a rat model. Nat Commun. 2023;14:3407.
110. Tian C, Shen L, Gong C, Cao Y, Shi Q, Zhao G. Microencapsulation and nanowarming enables vitrification cryopreservation of mouse preantral follicles. Nat Commun. 2022;13:7515.
111. Li B, Zhang L, Yin Y, Chen A, Lou J, Mooney DJ et al. Stiff hydrogel encapsulation retains mesenchymal stem cell stemness for regenerative medicine. Matter. 2024.
112. Yao J, Shen L, Chen Z, Zhang B, Zhao G. Hydrogel microencapsulation enhances cryopreservation of red blood cells with Trehalose. ACS Biomater Sci Eng. 2022;8:2066–75.
113. Ahmad HF, Sambanis A. Cryopreservation effects on Recombinant myoblasts encapsulated in adhesive alginate hydrogels. Acta Biomater. 2013;9:6814–22.
114. El Assal R, Guven S, Gurkan UA, Gozen I, Shafiee H, Dalbeyler S, et al. Bio-Inspired Cryo-Ink preserves red blood cell phenotype and function during nanoliter vitrification. Adv Mater. 2014;26:5815–22.
115. Gurruchaga H, Saenz Del Burgo L, Hernandez RM, Orive G, Selden C, Fuller B, et al. Advances in the slow freezing cryopreservation of microencapsulated cells. J Controlled Release. 2018;281:119–38.
116. Akiyama Y, Shinose M, Watanabe H, Yamada S, Kanda Y. Cryoprotectant-free cryopreservation of mammalian cells by superflash freezing. Proc Natl Acad Sci USA. 2019;116:7738–43.
117. Gryshkov O, Mutsenko V, Tarusin D, Khayyat D, Naujok O, Riabchenko E, et al. Coaxial alginate hydrogels: from Self-Assembled 3D cellular constructs to Long-Term storage. IJMS. 2021;22:3096.
118. Zhan L, Guo S, Kangas J, Shao Q, Shiao M, Khosla K, et al. Conduction cooling and plasmonic heating dramatically increase droplet vitrification volumes for cell cryopreservation. Adv Sci. 2021;8:2004605.
119. Ravanbakhsh H, Luo Z, Zhang X, Maharjan S, Mirkarimi HS, Tang G, et al. Free-form cell-laden Cryobioprinting for shelf-ready tissue fabrication and storage. Matter. 2022;5:573–93.

120. Qin X, Chen Z, Shen L, Liu H, Ouyang X, Zhao G. Core–Shell microfiber encapsulation enables Glycerol-Free cryopreservation of RBCs with high hematocrit. Nano-Micro Lett. 2024;16:3.
121. Guo Z, Zuchowicz N, Bouwmeester J, Joshi AS, Neisch AL, Smith K, et al. Conduction-Dominated cryomesh for organism vitrification. Adv Sci. 2024;11:2303317.
122. Zhan L, Li M, Hays T, Bischof J. Cryopreservation method for Drosophila melanogaster embryos. Nat Commun. 2021;12:2412.
123. Zhan L, Rao JS, Sethia N, Slama MQ, Han Z, Tobolt D, et al. Pancreatic islet cryopreservation by vitrification achieves high viability, function, recovery and clinical scalability for transplantation. Nat Med. 2022;28:798–808.
124. Zhan L, Han Z, Shao Q, Etheridge ML, Hays T, Bischof JC. Rapid joule heating improves vitrification based cryopreservation. Nat Commun. 2022;13:6017.
125. Rubinsky B, Perez PA, Carlson ME. The thermodynamic principles of isochoric cryopreservation. Cryobiology. 2005;50:121–38.
126. Wan Lili PPMJ, Anshal LCG, Bradley W, Clemens Mark P. Preservation of rat hearts in subfreezing temperature isochoric conditions to– 8°C and 78 mpa. Biochem Biophys Res Commun. 2018;496:852–7.
127. Powell-Palm MJ, Charwat V, Charrez B, Siemons B, Healy KE, Rubinsky B. Isochoric supercooled preservation and revival of human cardiac microtissues. Commun Biol. 2021;4:1118.
128. Powell-Palm MJ, Zhang Y, Aruda J, Rubinsky B. Isochoric conditions enable high subfreezing temperature pancreatic islet preservation without osmotic cryoprotective agents. Cryobiology. 2019;86:130–3.
129. Powell-Palm MJ, Henley EM, Consiglio AN, Lager C, Chang B, Perry R, et al. Cryopreservation and revival of Hawaiian stony corals using isochoric vitrification. Nat Commun. 2023;14:4859.
130. Zhao Y, Powell-Palm MJ, Wang J, Bilbao-Sainz C, McHugh T, Rubinsky B. Analysis of global energy savings in the frozen food industry made possible by transitioning from conventional isobaric freezing to isochoric freezing. Renew Sustain Energy Rev. 2021;151:111621.
131. Bai G, Gao D, Liu Z, Zhou X, Wang J. Probing the critical nucleus size for ice formation with graphene oxide nanosheets. Nature. 2019;576:437–41.
132. Shao Y, Han D, Tao Y, Feng F, Han G, Hou B et al. Leveraging macromolecular isomerism for phase complexity in Janus nanograins. ACS Cent Sci. 2023.
133. Liu Z, Cao H, Fan Y, Wang Y, Wang J. Strong Inhibition of ice growth by biomimetic crowding coacervates. Angew Chem Int Ed. 2023;62:e202311047.
134. Stevens CA, Bachtiger F, Kong X-D, Abriata LA, Sosso GC, Gibson MI, et al. A minimalistic Cyclic ice-binding peptide from phage display. Nat Commun. 2021;12:2675.
135. Zhang P, Li J, Sun J, Li Y, Liu K, Wang F, et al. Bioengineered protein fibers with Anti-Freezing mechanical behaviors. Adv Funct Mater. 2022;32:2209006.
136. Tian J, Walayat N, Ding Y, Liu J. The role of trifunctional cryoprotectants in the frozen storage of aquatic foods: recent developments and future recommendations. Comp Rev Food Sci Food Safe. 2022;21:321–39.
137. Wang M, Xiao X, Siddika S, Shamsi M, Frey E, Qian W, et al. Glassy gels toughened by solvent. Nature. 2024;631:313–8.
138. Liu D, Cao Y, Jiang P, Wang Y, Lu Y, Ji Z, et al. Tough, transparent, and slippery PVA hydrogel led by syneresis. Small. 2023;19:2206819.
139. Wang M, Sun S, Dong G, Long F, Butcher JT. Soft, strong, tough, and durable protein-based fiber hydrogels. Proc Natl Acad Sci USA. 2023;120:e2213030120.
140. He Q, Liao Y, Zhang J, Yao X, Zhou W, Hong Y et al. All-in-One gel system for whole procedure of Stem-Cell amplification and tissue engineering. Small. 2020;16.
141. Gao S, Niu Q, Wang Y, Ren L, Chong J, Zhu K, et al. A dynamic Membrane-Active glycopeptide for enhanced protection of human red blood cells against Freeze-Stress. Adv Healthc Mater. 2023;12:2202516.
142. Özsoylu D, Isık T, Demir MM, Schöning MJ, Wagner T. Cryopreservation of a cell-based biosensor chip modified with elastic polymer fibers enabling ready-to-use on-site applications. Biosens Bioelectron. 2021;177:112983.
143. Farooq U, Haider Z, Liang XM, Memon K, Hossain SMC, Zheng Y, et al. Surface-Acoustic-Wave-Based Lab-on-Chip for rapid transport of cryoprotectants across cell membrane for cryopreservation with significantly improved cell viability. Small. 2019;15:1805361.
144. Kim M, Lee C, Jeon K, Lee JY, Kim Y-J, Lee JG, et al. Harnessing a paper-folding mechanism for reconfigurable DNA Origami. Nature. 2023;619:78–86.
145. Zhou L, Lei Q, Guo J, Gao Y, Shi J, Yu H, et al. Long-term whole blood DNA preservation by cost-efficient cryosilicification. Nat Commun. 2022;13:6265.
146. Xin Y, Kielar C, Zhu S, Sikeler C, Xu X, Möser C, et al. Cryopreservation DNA Origami Nanostruct Small. 2020;16:1905959.
147. Hanson BM, Kim JG, Suarez SI, Ackerman BK, Comito CE, Pangasnan R, et al. Embryology outcomes after oocyte vitrification with super-cooled slush nitrogen are similar to outcomes with conventional liquid nitrogen: a randomized controlled trial. Fertil Steril. 2022;117:106–14.
148. Huang H, He X, Yarmush ML. Advanced technologies for the preservation of mammalian biospecimens. Nat Biomed Eng. 2021;5:793–804.

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