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# Emerging technologies in medical applications of minimum volume vitrification

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## Abstract

Cell/tissue biopreservation has broad public health and socio-economic impact affecting millions of lives. Cryopreservation technologies provide an efficient way to preserve cells and tissues targeting the clinic for applications including reproductive medicine and organ transplantation. Among these technologies, vitrification has displayed significant improvement in post-thaw cell viability and function by eliminating harmful effects of ice crystal formation compared to the traditional slow freezing methods. However, high cryoprotectant agent concentrations are required, which induces toxicity and osmotic stress to cells and tissues. It has been shown that vitrification using small sample volumes (i.e., <1 μl) significantly increases cooling rates and hence reduces the required cryoprotectant agent levels. Recently, emerging nano- and micro-scale technologies have shown potential to manipulate picoliter to nanoliter sample sizes. Therefore, the synergistic integration of nanoscale technologies with cryogenics has the potential to improve biopreservation methods.

### Keywords

cryopreservation; cryoprotectant agents; droplet vitrification; minimum volume vitrification

Biopreservation refers to extended conservation of cellular life at ultra-low temperatures to store cells and tissues. Cryopreservation has extensive applications in modern medicine, including human fertility preservation [1–4], conservation and transport of cells/tissues for transplantation [5] and storage of cells for tissue regeneration and cell therapy [6–8]. There have been significant efforts to develop cryopreservation techniques and to optimize cryopreservation protocols for various cell and tissue types, such as embryos [9], hepatocytes [10], stem cells [11] and blood vessels [12,13].

During cryopreservation, cells/tissues undergo cooling to sub-zero temperatures at which biological activity is slowed down or completely stopped. At the end of the cryopreservation

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process, biopreserved cells are thawed, and ideally resume biological activity. During the cryopreservation process, cryoprotectant agents (CPAs) are needed to protect cells from cryoinjury by decreasing the temperature at which intracellular ice formation occurs. CPAs act as osmotic buffers [14–16] and prevent harmful critical electrolyte concentration gradients [17,18]. Moreover, CPAs stabilize cell membranes and maintain macromolecules in their native form [19,20]. Some examples of widely used CPAs include Dimethylsulphoxide (DMSO), 1,2-propanediol (PROH) and ethylene glycol (EG) [21,22], sucrose, trehalose and mannitol.

Slow freezing and vitrification are currently available methods in laboratories and clinics [23]. Slow freezing is an established technique pioneered in the early 1970s, which cryopreserves biological samples at controlled freezing rates to avoid intracellular ice formation and minimizes structural damage to the cell membrane [24] and cytoskeleton [25]. CPAs are used at relatively low concentrations (e.g., 1.5 M) [21] in slow freezing, which has become a standard method for cell and tissue cryopreservation [26–29]. However, cells undergoing slow freezing processes still suffer injury, due to the formation of ice crystals, extreme hyperosmolarity and dehydration [30].

As an alternative cryopreservation method, vitrification offers improved outcomes [23,31–34] by preventing the formation of ice crystals and the increase in ionic strength of unfrozen concentrated solutions [35,36]. Vitrification refers to conversion of the liquid phase directly into a glass-like solid. Vitrification techniques require higher CPA concentrations (e.g., 6–8 M) and higher cooling rates (e.g., −1500°C/min) compared with slow freezing methods. Rapid cooling rates can be achieved by immersing cells/tissues directly into liquid nitrogen (−196°C) or liquid nitrogen vapor (−160°C). High CPA levels used in vitrification lower the freezing point and increase the medium viscosity. However, it is known that these high CPA levels can cause osmotic shock and toxicity to cells resulting in alterations in the cytoskeleton [37–39], spindle disassembly and chromosome dispersal [40].

Minimum sample volume (<1 μl) methods have been developed to reduce the adverse effects of high CPA concentrations needed for vitrification. These methods have been effective to increase the cooling rates and reduce the required CPA levels. These approaches include open pulled straws (OPS) [41], glass capillaries [42], electron microscopy grids (EMGs) [43,44], Cryoloop™ [45], Cryotop [46], gel loading tip [47,48] and droplet-based vitrification [10,49–51]. Even though cryobiological efficacy of minimum volume vitrification methods has been demonstrated, these methods are operationally demanding and require high technical skills limiting their widespread utilization. Recent advances in nano- and micro-scale technologies allow the manipulation of nanoliter sample volumes. Examples include microfluidics for CPA loading/unloading to minimize osmotic shock [52], quartz capillary microchannels as vitrification carriers to increase cooling rates [11,53], and ejector-based droplet generation systems for vitrification of cells at high throughput [10,49,51,54,55]. There are other droplet generation methods including acoustics [51], inkjet [56] and microfluidics [57].

Nano- and micro-scale technologies have already demonstrated the potential to transform modern medicine with alternative approaches in diagnostics, surgical and therapeutic practices [58–61]. These technologies, when applied to biopreservation, could enable the widespread use of minimum volume vitrification approaches in the biopreservation realm. In this article, we describe the existing vitrification-based cryo-preservation approaches with an emphasis on minimum volume droplet-based methods. We highlight the potential applications of these technologies in biopreservation of cells, tissues and tissue-engineered constructs.

## Vitrification

The vitrification phenomenon was first described and investigated back in the 1890s [62]. Application of vitrification for cryopreservation with structurally ceased state was not recognized until 1937 by Luyet [63,64]. In vitrification, as the glass transition temperature is reached during cooling, the elevated viscosity of the glass-like solid conserves the natural disorder of molecules existing in liquid and stops all chemical reactions that require molecular diffusion, leading to metabolic inactivity and stability over time [65,66]. Thus, cells/tissues survive the cooling process without significant damage resulting from ice-crystal formation.

Vitrification usually requires addition of high levels of CPAs (permeable and/or nonpermeable) (i.e., 6–8 M) prior to cooling. The use of nonpermeable CPAs, such as sugars (e.g., sucrose and trehalose) and polymers (e.g., polyvinylpyrrolidone and polyvinyl alcohol), can reduce the required permeable CPA concentration and enhance the glass transition process [67–69]. Another strategy is to use stepwise loading of CPAs, in which a pretreatment step with lower concentrations can be employed to minimize the osmotic shock and toxicity [23].

An increase in cooling rate facilitates cells to pass through the phase transition temperature rapidly, thereby decreasing cryoinjury to the cell membrane [70]. In addition, an increase in cooling and warming rates leads to vitrification at lower CPA concentrations, thus alleviating the detrimental toxic and osmotic effects to the cells [71]. To address these challenges, vitrification carrier systems have been developed to increase cooling and warming rates by using minimum volume approaches. However, most minimum volume vitrification systems have low throughput, which fits well with the cryopreservation of reproductive cells (i.e., oocytes) that are collected in small quantities during the clinical process. However, throughput becomes a limitation for vitrification of cells/tissues with larger process volumes, such as whole blood, blood components and stem cells.

### Carrier-based vitrification systems

A variety of carrier systems have been developed for vitrification using minimum sample volumes (Table 1) [67,72,73]. Conventional plastic straw is one of the initial vitrification carriers used for cryopreservation of embryos [9], which provides a cooling rate of 2500°C/min and a warming rate of 1300°C/min. However, since this carrier system encompasses a large sample volume (i.e., 45 μl) [74], it is hard to achieve an ultra rapid cooling rate, and high CPA concentrations are required to realize vitrification. To reduce the vitrification sample volume, several other methods have been developed; such as OPS (~1 μl) [41] and hemi-straw systems (~0.3 μl) (Figure 1a) [75]. Using these systems, cryoinjury was reduced as the cooling and warming rates (OPS: 16,700 and 13,900°C/min; hemi-straw: >20,000°C/min) were higher than conventional straws.

Glass capillaries were proposed as a vitrification carrier, offering controllable cooling and warming rates using different diameters. For example, capillaries of 440 μm–2 mm in diameter could provide cooling rates of 12,000–2000°C/min and warming rates of 62,000 to 5000°C/min, respectively [42]. The capillary approach has been applied for cryopreservation of bovine oocytes [42] and embryos [76]. This method resulted in comparable hatching rates compared with those using the OPS technique (glass capillary: 19%; OPS: 27%; control: 80%), suggesting no critical difference in the cooling/warming rates relative to OPS. Closed pulled straws have been developed to avoid potential cross-contamination from direct contact with liquid nitrogen. Improved survival (79%) and spindle morphology conservation [77] have been shown with this method compared with OPS (63%) for the vitrification of mouse oocytes.

Quartz microcapillaries have been recently utilized for vitrification [11,53,78] given that higher heat transfer rates (cooling rate of 250,000°C/min) can be achieved compared to glass and plastic capillaries. In this approach, thin-wall quartz micro capillary (outer diameter: 0.2 mm; wall thickness: 0.01 mm) has led to ultrafast cooling rates of up to 250,000°C/mm [11]. Therefore, this method lowered the CPA levels needed for vitrification. Accordingly, quartz microcapillaries have recently proved to be superior in terms of cell survival (murine embryonic stem cells [11] and mouse oocytes [79]) compared to conventional straws, OPS and EMGs.

Electron microscopy grid was developed to vitrify samples with a volume less than 1 μl, with theoretical cooling and warming rates of approximately 150,000°C/min [43,44]. This system has been used for vitrification of bovine blastocysts [80] and showed similar embryo survival rates compared with nonvitrified blastocysts and an increased hatching rate (68%) compared with plastic straw vitrification (53%). A simplified EMG method has been used for mouse oocytes with survival and fertilization rates of 90.0% and 56.7%, respectively [81].

Cryoloop is one of the most clinically employed approaches to vitrify embryos and oocytes, which has demonstrated increased cell survivability compared to straw-based approaches after thawing [45]. In this approach, the carrier consists of a small nylon loop (0.7–1.0 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial (Figure 1b). The sample is first placed on the Cryoloop, which has a thin layer of CPA film. The loop is then sealed in a cryovial, which is subsequently filled with liquid nitrogen. Since the sample volume is limited to the loop size, cooling rates as high as 700,000°C/min can be achieved due to the absence of a solid support and the minimum sample volume (<1 μl) that is used [46,82].

Cryotop is a recently developed vitrification approach, which uses a polypropylene strip attached to a holder accompanied with a protective cap (Figure 1C). In this method, sample (<0.1 μl) is loaded with a glass capillary on top of the film strip. Then, the solution is removed, leaving behind a thin layer sufficient to cover the cells to be cryopreserved. The minimum sample volume used for this approach enhances the cooling rate up to 23,000°C/min, and warming rate up to 42,100°C/min [46]. Consequently, the Cryotop method has demonstrated higher efficiency for bovine oocyte vitrification compared to plastic straws and OPS with improved fertilization and blastocyst development, and with higher percentage of typical spindle and chromosome morphology [83,84]. On the other hand, Cryotip constitutes a narrow capillary that can be heat-sealed at both ends after cell loading to provide a closed system. Although lower cooling and warming rates (12,000 and 24,000°C/min, respectively) were reported with this system compared with the Cryotop method, survival of human blastocysts, pregnancy and delivery rates following vitrification did not display a significant difference [85].

In the gel loading tip method, the vitrification solution volume is controlled by the pipette tip size (typically 0.6–0.7 μl) (Figure 1D) [47,48]. Using this vitrification method, the cleavage rate of bovine embryos and rabbit zygotes were lower than the rates achieved by the Cryotop technique [48]. This difference can be attributed to high CPA concentrations and the inferior cooling rates offered by the gel loading tip approach.

## Limitations of carrier-based vitrification approaches

Carrier-based minimum volume vitrification systems described here have successfully improved the survivability and functionality of cells undergoing cryopreservation compared with conventional plastic straw systems. However, a major challenge associated with these systems is the need for manual handling, skilled technicians, hence, leading to a low

throughput process. Nano- and micro-scale droplet-vitrification offers a carrier-free approach, which involves generation of cell encapsulating CPA droplets followed by direct injection into liquid nitrogen [10]. Since no vitrification carrier or container is needed in this approach, higher cooling and warming rates can be achieved through direct contact with liquid nitrogen. More importantly, recent advances in nano- and micro-droplet generation and ejection technologies allowed effective vitrification of droplets in a continuous manner, which can potentially attain throughput levels required for most biopreservation applications, such as blood cryopreservation [86]. Recent developments in carrier-free, droplet-based vitrification methods are discussed in the next section.

## Carrier-free, droplet-based vitrification approaches

Droplet-based vitrification involves loading of samples with vitrification solution, followed by deposition of the solution cell suspension in the form of microdroplets into liquid nitrogen [10,49,52]. Alternatively, droplets are deposited onto a surface with high thermal conductivity (e.g., aluminum), followed by rapid immersion into liquid nitrogen. The main advantage of this technique is the potential to achieve higher cooling and warming rates due to lower heat capacity of a microdroplet at the absence of a bulky carrier. Carrier-free techniques reduce the likelihood of ice crystal formation and reduces the CPA concentrations [49]. Encouraging results have been achieved by droplet-based vitrification techniques with various plant species [87,88]. The promising outcomes achieved with this technology in plant biopreservation have paved the way for applications with mammalian cells, such as oocyte and embryo crypreservation [46,50,89,90].

Landa *et al.* has employed this technique for cryopreservation of mouse embryos [46,89]. In this study, after pre-equilibration in medium with 10% glycerol, embryos in 5–20 μl volume droplets were directly immersed into liquid nitrogen. High survivability (greater than 90%) and developmental potential was demonstrated with eight-cell embryos frozen in microdroplets, in which greater than 83% of embryos developed to blastocysts and more than 73% of embryos underwent implantation after 48 and 96 h of *in vitro* culture [89]. However, in this study, introducing a droplet (5–20 μl) directly into liquid nitrogen led to the suspension and hovering of the droplet on the surface surrounded by liquid nitrogen vapor blanket (Figure 2), which is known as the Leidenfrost phenomenon [49]. The vapor blanket becomes the bottleneck for effective heat transfer. Despite this drawback, carrier-free vitrification systems can still provide higher heat transfer rates than carrier-based systems, if smaller size droplets are used. Papis [50] and Dhali [90] utilized a similar direct droplet immersion method with reduced vitrification volume (i.e., 6 μl) for bovine oocyte and mouse zygote vitrification, and higher cleavage and blastocyst rates were observed compared with those droplet vitrification methods that used larger droplets. The enhancement in cryopreservation outcomes could be attributed to the increased cooling rates achieved with smaller droplets.

Solid surface vitrification (SSV) [91] method vitrifies droplets without direct contact with liquid nitrogen. In this approach, cells/tissues in a small droplet of CPA solution are placed with pipettes or glass capillaries onto a metal surface (e.g., aluminum foil) cooled by liquid nitrogen or liquid nitrogen vapor (Figure 3a). SSV has been utilized for cryopreservation of *in vitro*-matured oocytes derived from cattle [92], goat [93] and pig [94,95] by encapsulating 5–10 oocytes in a 1–2 μl droplet of the vitrification solution. However, these studies displayed low rates of developmental competence of vitrified oocytes after activation [92] or IVF [94,96]. In addition, the cleavage rate of vitrified oocytes was reduced (14%) compared with noncryopreserved oocytes (46%), suggesting that damage still existed even though the plasma membrane integrity of the vitrified oocytes was maintained. The decreased developmental competence could be attributed to compromised spindle and/or chromosome configuration caused by vitrification as suggested in previous reports [96,97]. This potential

cryoinjury could be resulting from inefficient heat transfer within a relatively bulky droplet that contains 5–10 oocytes. The heat transfer efficiency can be improved by reducing the droplet volume through encapsulating a single oocyte.

Minimizing the volume of vitrification solution containing cells/tissues not only offers the obvious benefit of increasing both cooling and warming rates, but also decreases the likelihood of ice crystal nucleation/formation [98]. Although there is no clear definition of what volume can be defined as 'minimum' for vitrification, it usually refers to a volume around, or less than, 1 μl [46]. Based on this principle, Arav *et al.* [99–101] developed the minimum drop size (MDS) technique, in which nanoliter (0.1–0.5 μl) volumes of vitrification solution was used. The MDS technique has been successfully used to vitrify porcine [99,100] and bovine [101] oocytes, and bovine and sheep embryos [54]. The MDS method was recently modified by depositing 0.1–0.5 μl droplets on glass coverslips followed by immersion into liquid nitrogen or nitrogen slush (Figure 3b). This technique was shown to maximize the cooling rates up to 130,000°C/min, allowing up to a 50% reduction in CPA concentrations compared with conventional vitrification protocols (Figure 3C–3F) [54].

Improved cryopreservation outcomes have been demonstrated with droplet-vitrification methods for oocyte and embryo cryopreservation compared to slow freezing and conventional straw-based vitrification methods with increased survivability and function. However, the labor- and time-intensive procedures often limit the throughput for cell vitrification. In addition, the limited control over the droplet size prevents a uniform size distribution among the generated droplets, which causes inconsistencies in droplet vitrification. Thus, application of the technologies listed above to vitrify cells in a bulk processing volume, such as red blood cells (RBCs), stem cells and hepatocytes, has not been practical.

Recently, Demirci *et al.* demonstrated ejection-based droplet-vitrification, with which a single to few cells could be encapsulated in reduced droplet volumes (1.5–500 nl) with a negligible reduction in cell viability [10,51]. This droplet generation and ejection technology has been demonstrated to generate droplets at high-throughput with rates up to 1000 droplets per second [10,49,51,102–104]. Moreover, there are other droplet generation technologies that offer extremely small droplet volumes (<1 nl) based on acoustic ejection [51,105–107], which has the capability to generate smaller droplets than the methods described above. Smaller droplets could allow the vitrification to occur at even higher cooling and warming rates than the MDS technique, while using significantly reduced CPA concentrations (~1.5 M), and hence minimizing the osmotic and mechanical stress to cells. It was also experimentally demonstrated that there is a relationship between droplet size and CPA concentrations, suggesting that small droplets vitrify at lower CPA concentrations [10]. With this method, droplets maintain their spherical shape when ejected and dropped into liquid nitrogen. This may provide even cooling of droplets from surface to center compared to other droplet-vitrification approaches discussed above, such as solid surface vitrification, in which hemispheres were generated instead of spheres. Various mammalian cell types (AML-12 hepatocytes, NIH-3T3 fibroblasts, HL-1 cardiomyocytes, mouse embryonic stem cells and RAJI cells) were ejected using droplets with high viability (>89%) after recovery [10,49].

Although most of the minimum volume vitrification approaches were initially designed for cryopreservation of reproductive cells (e.g., embryos and oocytes), advances in biomedical sciences and regenerative medicine will broaden the applications of vitrification technologies to find broad applications, such as preserving high quality cell/tissue sources for transplantation and cell therapy, and extending shelf-life of tissue engineered constructs for tissue regeneration. For future clinical applications, the ejection systems are sterilized

and operated in sterile hoods [108,109]. Furthermore, sterile liquid nitrogen can be used to avoid potential contamination [110]. Sterilization of liquid nitrogen has been shown by methods using sterile polytetrafluoroethylene (PTFE) cartridge filteration [111] or ultraviolet (UV) radiation [112].

## Applications of vitrification in cryopreservation of cells & tissues

The procedures used to cryopreserve cells or tissues depend on several factors, including cell size, cytoplasm volume and types of cells in the population. Most mammalian cells can be cryopreserved by using the slow-freezing method with a cooling rate of approximately −1°C/min and rapid warming, which results in more than 90% retained cell viability. However, there is a challenge to cryopreserve cells that have a large cytoplasm (e.g., oocytes) due to the harmful effects of intracellular ice crystal formation. There are also challenges associated with cryopreservation of tissues with mixed cell populations of varying sizes, since the optimal freezing procedure for each cell type is different. Thus, cryopreservation methods preserving the utmost cell viability after recovery are desired. As discussed earlier, vitrification has presented great potential to address the particular challenges listed above since it has demonstrated improved viability after both freezing and thawing compared with other methods (Table 2).

### Vitrification of reproductive cells

Cryopreservation remains a practical option to preserve and extend human fertility in modern clinical practice [113,114]. Embryo cryopreservation has become a routine procedure in IVF clinics [115]. Patients who might have ethical or religious concerns on embryo freezing potentially consider cryopreservation of oocytes as a viable option. This technology is particularly valuable for cancer patients, offering an opportunity to preserve fertility before chemotherapy or radiation treatment. However, oocytes cryopreserved using slow freezing or traditional vitrification methods have not led to birth rates that are acceptably high for clinical applications after implantation *in vivo* (<5%) [116,117]. This is mostly due to irreversible damage to oocytes, such as zona hardening and misalignment of the chromosomes [113]. The distinct advantages of vitrification have led to wide investigation for fertility preservation, especially as a possible means to increase oocyte survival, pregnancy and birth rates.

### Vitrification of oocytes

Vitrification of bovine, mouse and human oocytes have been studied using various vitrification carrier systems [74,77,118]. Vajta *et al.* used the OPS method for the vitrification of bovine oocytes, and reported that 50% of the vitrified oocytes cleaved and 25% of them developed into blastocysts after IVF, with pregnancies achieved following transfer at both oocyte and blastocyst stages [41]. Similar survival rates were observed when EMG and Cryoloop approaches were used. However the cleavage rate (EMG: 30%, Cryoloop: 26 vs fresh oocytes: 50–70%) and blastocyst development (EMG: 15 vs fresh oocytes: 40%) after IVF were significantly reduced [44,119]. Morato *et al.* compared Cryotop to OPS for vitrification of bovine oocytes, and showed that Cryotop was superior to OPS approach yielding higher cleavage (46 vs 32%) and blastocyst rates (5 vs no blastocyst), with uncompromised spindle and chromosome configurations (60 vs 38%) [84]. Higher survival rates and improved conservation of spindle morphology have been achieved with mouse oocytes by using close pulled straws (79 and 93%) rather than conventional straws (77 and 79%), microscopy grids (39 and 95%) and OPS (63 and 89%) [77]. When vitrifying mouse oocytes using electron microscopy grids, significant reduction in hatching rates (39 vs 85% for fresh oocytes) was observed [81]. The Cryoloop approach proved successful in vitrification of mouse oocytes, showing comparable survival, fertilization and

blastocyst development rates, intact spindles and similar chromosome morphology to fresh oocytes [120,121]. When quartz microcapillaries were used to vitrify mouse oocytes, similar survival, fertilization and blastocyst development rates to fresh oocytes were observed. However, with this method, when blastocyts derived from vitrified oocytes were transferred to surrogate mothers, lower birth rates were observed (19%) compared with those obtained from fresh oocytes (23%) [79].

In the case of human oocytes, when the OPS method was used for vitrification, high survival and fertilization rates (68.6 and 71.7%) were achieved. However, pregnancy (15.3%) and birth (4.6%) rates were still relatively low [122]. Cryoloop and hemi-straw systems were also tested for vitrification of human oocytes [123], and similar survival rates were observed with these two approaches (81 vs 85%, respectively). Another study [124] using the Cryoloop for vitrification of human oocytes reported similar results with moderate fertilization rate (72%), low pregnancy rates (5–6%), and reduced rates of normal meiotic spindles and chromosomes [125]. On the other hand, encouraging results have been reported with the use of the Cryotop method for human oocyte vitrification, with higher survival (90–99%), fertilization (81–93%) and pregnancy (32–41%) [46,83,113,126] rates. However, there is still need for improvement to achieve higher success rates.

## Vitrification of embryos

**Early-stage embryos—**Vitrification of bovine early-stage embryos was demonstrated using 0.25 ml conventional straws with post-thaw cleavage rates less than 5% [127]. By contrast, higher survival rates and better conservation of normal cell morphology were achieved when OPS (88%) and close pulled straws (85%) were used [128]. For human early-stage embryo vitrification, the hemi-straw method resulted in comparable survival rates with higher development potential than Cryoloop (38 vs 29%) [123]. However, higher survival (85%) and pregnancy (44%) rates were observed in a separate study, which also utilized Cryoloop for vitrification of human embryos leading to births [129]. Successful results were also demonstrated with the Cryotop method for vitrification of early-stage human embryos, with higher levels of survival (100%), cleavage (93%) and blastocyst (52%) rates compared with the results obtained with other approaches detailed above [46].

**Blastocysts—**Both OPS and EMGs have been used for vitrification of bovine blastocysts. Using OPS, pregnancies were achieved following blastocyst transfer, although low survival rate was observed [41]. When EMGs were used for vitrification of bovine blastocysts, no significant difference was observed in survival rates between vitrified and nonvitrified blastocysts. However, survival rate was reduced 48 h after the vitrification procedure compared with nonvitrified cells (73 vs 84%) [80].

Higher survival rate (82%) was achieved with vitrification of mouse blastocysts using OPS compared with conventional plastic straw method (69%) [130], which can be attributed to reduced vitrification volume of OPS. On the other hand, closed pulled straws showed higher pregnancy and birth rates (70 and 45%) compared with the OPS (20 and 8%) and conventional straws (10 and 5%) obtained after transferring vitrified expanded blastocysts to the uterus of a pseudo pregnant mouse [131]. The Cryoloop approach also resulted in high implantation (80%) and fetal development (55%) rates of vitrified mouse blastocysts after being transferred to pseudopregnant recipients, which were comparable to those observed with nonvitrified cells [45].

A number of approaches have been used for vitrification of human blastocysts, including EMGs, hemi-straw, Cryoloop and Cryotop. EMGs yielded high survival (83–90%), hatching (49%), implantation (29%) and pregnancy rates (34–48%) [132,133], which were comparable to those obtained using Cryoloop [45,134–137] and Cryotop [46,138] (90 and

53% for survival and pregnancy rates, respectively). The results obtained using the hemi-straw method resulted in lower survival, hatching (19%), implantation (13%) and pregnancy (19%) rates compared with other approaches described above [75].

Vitrification of oocytes and embryos using various minimum volume carriers all showed improvements compared with conventional straws. However, it should be noted that the results achieved with these methods are highly dependent on the skills of the embryologist, and hence comparisons between different studies have to take this as a consideration for future biopreservation technologies. The variations due to manual processes could potentially be eliminated, if automated systems are applied. It should also be noted that the efficiencies of these methods depend on the species and developmental stages of embryos in preservation.

## Vitrification of stem cells

Regenerative medicine has shown potential to repair or replace tissue/organ function lost due to disease, damage or congenital defects [139]. The strategies presently under development include transplantation of stem cells, manipulation of a patient's own stem cells, and the use of scaffold materials that provide biochemical signals to stimulate stem cells to start differentiation. Therefore, it is crucial to be able to preserve stem cells for future regenerative therapies.

Biopreservation of stem cell lines is often challenging due to the difficulty in conservation of undifferentiated phenotypes through cycles of cell division. Hence, it is hard to provide persistent and qualified cell sources for clinical use [140]. Cryopreservation provides an affordable, fast, and efficient solution for stem cell banking for both research and clinical use. Vitrification has been successfully used to preserve various types of stem cells, such as embryonic stem cells [141], mesenchymal stem cells [142] and hemopoietic stem cells [143]. Current vitrification protocols for stem cells consist of stepwise addition of vitrification solution to minimize osmotic shock, in which a small volume of the CPA solution and cells are immersed directly into liquid nitrogen. Upon warming, a series of stepwise dilutions is sufficient to completely remove the vitrification solution. After vitrification, cell viability in higher than 80% was achieved [144]. On the other hand, cryopreservation of stem cells using slow freezing resulted in a survival rate of only 10% [140].

Both Cryotip and quartz microcapillaries have been tested for the vitrification of mouse embryonic stem cells. When Cryotip approach was used, more than 99% of the cells survived after warming [145]. By using the quartz micro-capillary approach, murine embryonic stem cells were successfully cryopreserved without compromising their undifferentiated phenotype expression postvitrification [11]. OPS approach was used to vitrify human embryonic stem cells derived from the inner cell mass of human blastocysts [146,147]. The achieved cell attachment postwarming was comparable to those of nonvitrified stem cells, although a small percentage of nonspecific differentiation was observed (24%). However, the pluripotency of stem cells was retained after warming [147]. The efficiency of other vitrification approaches for human embryonic stem cell preservation needs further investigation.

Vitrification approaches used for stem cell cryopreservation discussed above are time consuming and require a high level of skill to prevent cell loss. In addition, given the large number of cells to be preserved, a high throughput approach is needed. Therefore, the ejector-based carrier-free droplet-vitrification technique described above could be a suitable candidate technology for stem cell vitrification with its high-throughput processing capability and the use of lower CPA concentrations with small droplet size.

### Vitrification of tissue engineered constructs

Tissue engineering technologies have the potential to develop regenerative therapies for currently incurable diseases and untreatable conditions, such as diabetes, heart disease, renal failure, osteoporosis and spinal cord injuries [148–153]. The ability to cryogenically preserve tissue engineered constructs (TECs) would be indispensable for successfully transferring replacement tissues from the laboratory setting to the clinic. This would allow distributing cell-based products to patients on a demand basis by increasing the shelf life of the products.

With the recent developments in cryogenic science, cryopreservation of tissues and TECs for extended periods of time has become achievable. The tissues that have been found incompatible with freezing protocols are generally highly vascularized due to endothelium damage. So far, animal intestines and ovaries have been successfully recovered from the cryopreservation process, showing function after transplantation. The primary issue with tissue and TEC cryopreservation is preventing ice crystal formation and maintaining the integrity and mechanical properties of both the cells and extracellular matrix (ECM) during cooling and thawing steps. In addition, dehydration that occurs during the freezing process results in a stiffer tissue due to increased crosslinking between collagen fibers [140]. Thus, slow freezing is not optimal for cryopreservation of tissues or TECs due to the difficulties in preserving the structural stability of biomaterials and regenerative tissue integrity during liquid-ice phase transition. Vitrification appears to be a more promising modality for TEC cryopreservation, since it can maintain tissue integrity by preventing ice crystal formation [140].

Vitrification of various TECs, such as tissue engineered (TE) bones (TEBs) [154], TE pancreatic substitutes (TEPS) [155,156], and TE blood vessels (TEBVs) [157], have shown encouraging results. Song *et al.* demonstrated the vitrification of TEPS using DMSO and 1,2-propanediol, showing comparable results with the nonfrozen control [156]. In the case of TE cartilage cryopreservation, chondrocytes showed survival rates of up to 93% after vitrification [158]. Similarly, vitrification of rabbit cartilage has resulted in high viability (>80%) after thawing [159]. In addition, improved cryopreservation outcomes were observed by vitrification of native vascular tissues compared to those by slow freezing [160,161]. More than 80% of the maximum contractions were maintained in vitrified vessels with similar responses to drug stimulation, while less than 30%, were retained in frozen vessels [160]. In a separate study, a vitrification solution consisting of DMSO, propylene glycol and formamide was used for a collagen-based TEBVs, and negligible ice formation was observed. By contrast, extensive ice formation was observed in frozen specimens using the slow freezing method. Thus, vitrification seems to be an appropriate approach for TEBV cryopreservation. However, a similar reduction in viability was observed in both frozen and vitrified constructs (38 vs 39%) compared with fresh samples (63%) [140].

Compared with slow freezing, enhanced outcomes have been shown for TEC cryopreservation using vitrification. However, challenges still exist with current existing approaches. For instance, it is not practical to achieve ultra-rapid vitrification using minimum volume approaches given the large TEC size, and high CPA concentrations have to be used for vitrification to occur. Moreover, it is important to ensure that vitrification solution is present throughout the construct and cells. Additionally, toxicity and cellular osmotic shock of high levels of CPAs is another challenge for TEC vitrification, so it is necessary to minimize CPA concentrations to reduce damage to cells. Furthermore, optimizing protocols for CPA removal is also vital to avoid devitrification since construct viability has been found to decrease during the warming process [13].

## Conclusion & future perspective

In this article, we presented the minimum volume vitrification approaches and their applications in medicine. Minimum volume vitrification approaches reduce CPA concentrations required for vitrification since they can attain increased cooling and warming rates. Hence, this process enables reduced toxicity and osmotic shock to cells and tissues that undergo cryopreservation processes. Among these methods, droplet-vitrification offers further potential advantages, allowing vitrification at even higher cooling rates using smaller droplet sizes. However, challenges still remain, such as throughput limitations and demand for skilled operators. Therefore, future research efforts need to focus on improving current vitrification approaches by developing novel vitrification systems that can minimize both the dependence on skilled operators as well as variations due to manual handling process steps. Emerging nano- and micro-scale technologies enable sample manipulation in small scales. The successful integration of these technologies with the current know-how in cryobiology has the potential to address these needs by offering automation and high throughput operation in biopreservation, leading to an increase in successful clinical biopreservation outcomes.

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▪ of interest

▪▪ of considerable interest


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Executive summary

- Existing and emerging applications of vitrification include cryopreservation of reproductive cells, stem cells, blood and tissue engineered constructs.
- Vitrification displays significant improvement in post-thaw cell viability and function compared with the traditional slow freezing methods by eliminating ice crystal formation.
- Vitrification using minimum sample volume enables increased cooling and warming rates and the utilization of lower cryoprotectant agent concentrations, thus reducing toxicity and osmotic shock to cells/tissues.
- Minimum volume vitrification systems are divided into two categories: carrier-based and carrier-free (droplet-based vitrification) systems.
- Droplet-based vitrification offers potential advantages over carrier-based vitrification methods, allowing vitrification at even higher cooling rates using smaller sample sizes.
- Emerging nano- and micro-scale technologies have the potential to improve throughput limitations and reduce skill dependence of the current vitrification systems by enabling sample manipulation in a small scale.

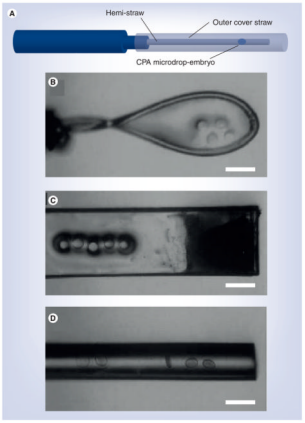


**Figure 1. Minimum volume vitrification carriers**

**(A)** Hemi-straw, which was inserted into a 0.5 ml straw for storage, **(B)** Cryoloop [48], **(C)** Cryotop [48] and **(D)** gel loading tip [48]. Scale bar = 400 μm.

**(B–D)** Reproduced with permission from [48] © (2011) Elsevier.

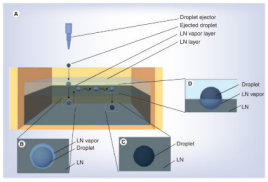


**Figure 2. Generation of droplets and levitation of droplets on top of liquid nitrogen**

**(A)** Experimental setup and the behavior of droplets over time. **(B)** Droplet swimming in the liquid nitrogen. The droplet in darker color is surrounded by the liquid nitrogen vapor as shown in the schematic drawing. **(C)** Droplet sunk in the liquid nitrogen. **(D)** Droplet floating on top of liquid nitrogen.

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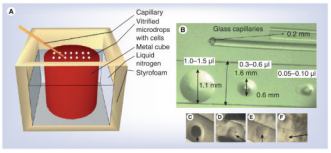


**Figure 3. Droplet vitrification**

**(A)** The solid surface vitrification device. A metal cube covered with aluminum foil was partially submerged into liquid nitrogen. Microdroplets of vitrification solution containing the oocytes were dropped onto the cold upper surface of the metal cube and are instantaneously vitrified. **(B)** The modified minimum drop size technique. Droplets of various sizes were placed on glass cover slips using capillaries. **(C–F)** Droplets generated by MDS technique during vitrification with **(C)** ice crystals, **(D & E)** with fractures, and **(F)** without crystallization and fractures using CPA concentration reduced by 50% of those used in **(C)**.

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**Table 1**

Summary of vitrification carrier systems.

| Vitrification carrier | Sample volume/size | Cooling rate (°C/min) | Warming rate (°C/min) | Clinically in use | Ref. |
|---|---|---|---|---|---|
| Conventional straw | 0.25 ml | 2500 | 1300 | Yes | [9] |
| Open pulled straw | ~1 μl | 16,700 | 13,900 | No | [41] |
| Hemi-straw | ~0.3 μl | >20,000 | N/A | No | [75,162] |
| Electron microscopy grid | <1 μl | 150,000 | 150,000 | | [43–44] |
| Cryoloop | 0.7–1.0 mm (inner diameter) | 700,000 | N/A | Yes | [45] |
| Cryotop | <0.1 μl | 23,000 | 42,000 | No | [46] |
| Cryotip | N/A | 12,000 | 24,000 | No | [85] |
| Gel loading tip | 0.6–0.7 μl | | | No | [47–48] |
| Quartz microcapillary | ~3 mm (inner diameter) | 250,000 | N/A | No | [11,53,78] |
| Minimum drop size | 0.1–0.5 μl | 130,000 | N/A | No | [54,99–101] |
| Ejector-based droplet | ~ 1 nl | N/A | N/A | No | [10,49,51,102–104] |

**Table 2**

Vitrification methods for cell and tissue cryopreservation.

| Cells/tissues | Challenges for cryopreservation | Commonly used vitrification methods | Survival/viability | Limitations | Ref. |
|---|---|---|---|---|---|
| Corneal grafts | Endothelium damage (intracellular freezing at high cooling rates, solution effect injury at low cooling rates) | Nonpermeating CPAs, Teflon-coated bag as vitrification carrier | <10% | CPA toxicity, devitrification during heating process | [163–165] |
| Human embryonic stem cells | Sensitive to CPA, requires extremely critical timing and high degree of skill | OPS (1–20 μl), Glass straw (20–130 μl) | >75% | Time consuming, labor intensive | [12,144] |
| Adult stem cells | Loss of undifferentiated state and viability | OPS | >80% | Time consuming, labor intensive | [12,140,144] |
| Tissue engineered constructs | CPA needs to permeate entire TEC to prevent ice formation | Glass vial | 95% | Organs are too large to be vitrified | [12–13] |
| Chondrocytes/cartilage | CPA concentration required to prevent ice formation | Glass vial | Chondrocytes: 93% Cartilage: 85% | High thermal processing required to be rapid enough | [12,161] |
| Tissue engineered blood vessels | Stiffening of biomaterials during freezing | CPA concentration >50% (v/v) | 50% | Viability loss during warming | [12–13] |
| Oocytes/zygotes/cleaved embryos | Extremely sensitive to CPAs | OPS, Cryoloops, Cryotop, 0.25 ml straw, EM copper grid, solid surface | Zygotes: 90% survival, 82% cleavage, 30% blastocyst | Hardening of the zona, misalignment of chromosomes | [67,113] |
| Sperm | Sensitive to osmotic shock, large volume | Multi-thermal gradient | 50% decrease in motility | Large sample volume, optimal cooling rate needed | [54,67] |
| Ovarian tissue | Large sample size | Straws or grids | Pyknosis, vacularization, cell swelling detected | Hard to preserve large samples, must be cut into pieces, crystallization during cooling | [67,166] |

CPA: Cryoprotectant agent; EM: Electron microscopy; OPS: Open pulled straws; TEC: Tissue engineered constructs.