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Preservation of Embryonic Stem Cells

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1. Introduction

With recent advances in tissue engineering, regenerative medicine, cell transplantation, stem cell therapy, and assisted reproduction, the living cell is becoming increasingly important as a tool for drug screening and therapy in modern medicine (Gearhart 1998; Langer and Vacanti 1993). As a result of their capability of differentiating into any type of cells, the pluripotent embryonic stem (ES) cells are of particular importance to the modern cell-based medicine (Gearhart 1998). However, ES cells may differentiate gradually during passaging when cultured at 37 °C. Therefore, for the eventual success of using ES cells in the emerging cell-based medicine, it is of great importance to maintain their pluripotency in the long term without passaging and in a cost effective way so that the cells can be widely distributed and readily available to end users in both research and clinical settings. This can be done by cell preservation to put the cells in a state of suspended animation, which can be achieved by either cooling the cells to preserve (cryopreservation) at a cryogenic temperature and/or drying the cells to preserve (lyopreservation) at ambient temperature (Acker 2004; Blow 2009; Coger and Toner 2000; Toner and Kocsis 2002). In either case, the cells must enter (before being damaged) an amorphous (or glassy) phase, a thermodynamically metastable state with an extremely high viscosity and low molecular mobility and activity to arrest any biophysical and biochemical activities within the cells. Although contemporary methods for cell preservation still rely on the use of cryogenic temperature (cryopreservation), cell lyopreservation at ambient temperature is gaining more and more attention, due to the relatively high cost of maintaining and difficulty of transporting cryopreserved cells in cryogenic fluids such as liquid nitrogen (Acker 2004; Blow 2009; Deb 2009; Kanas and Acker 2006; Meyers 2006). In this chapter, the fundamentals and recent advancement of both cryo and lyopreservation are first summarized, followed by a critical review of the progress and challenges in applying the various cell preservation strategies to maintain the pluripotent properties of embryonic stem cells in the long term. This chapter is concluded with an outlook of the future directions of embryonic stem cell preservation.

2. Cryopreservation at cryogenic temperatures

Cell cryopreservation can be achieved by either slow-freezing or vitrification (Coger and Toner 2000; Fahy et al. 1984; Mazur 1984; Rall and Fahy 1985). The former relies on the formation of extracellular ice (the crystallized state of water) to freeze concentrate the

extracellular solution in the presence of a low concentration ($< \sim 2$ M) of cryoprotectants (or cryoprotective agents, or CPAs for short) such as glycerol, ethylene glycol, PROH (1,2-propanediol or propylene glycol), and DMSO (dimethylsulfoxide). As a result, the cells gradually dehydrate due to osmosis during freezing and enter a glassy phase suitable for long-term storage. While slow-freezing is to vitrify what is within the cells in the presence of extracellular ice, the goal of vitrification is to have both intra and extracellular water enter the amorphous, glassy phase to eliminate any potential damaging (either mechanical or physicochemical) effect of ice formation. Conventionally, vitrification is achieved by using an unusually high concentration (up to 7 M) of CPAs, which can cause significant metabolic and osmotic injury to living cells. As a result, various approaches have been investigated to achieve vitrification of living cells at a low concentration of CPAs such as that used for slow-freezing, which is called low-CPA vitrification. The three approaches mentioned above for cell cryopreservation can be best demonstrated in an extended phase diagram of temperature versus solute concentration (Fig. 1), which consists of four regimens representing four probable phases (liquid, subcooled liquid, supersaturated liquid, and the solid-like amorphous or glassy phase) of an aqueous solution. The four lines that separate the four regimens are called liquidus (between liquid and subcooled liquid), extended

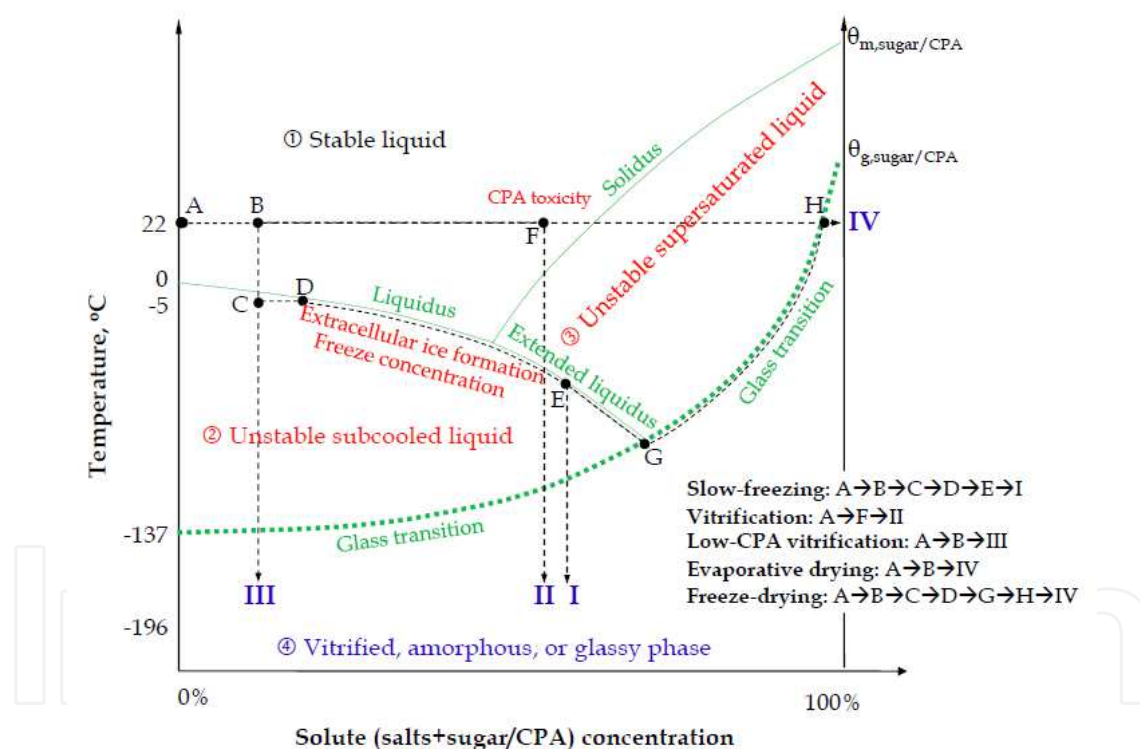


Fig. 1. An illustration on the extended phase diagram of the various methods for cell preservation at either cryogenic or ambient temperature from an initial (stable) liquid state (A) to a final glassy phase (I, II, III, or IV): The phase diagram is divided into four thermodynamic regimens by the liquidus, extended liquidus, solidus, and the line of glass transition; the four regimens are the liquid, subcooled liquid, supersaturated liquid, and the glassy phase; cells must enter the glassy phase for long-term storage; CPA represents cryoprotectant, and θ_g and θ_m represent the glass transition and melting temperature of pure sugar/CPA used as lyoprotectant or CPA, respectively. Of note, the diagram is not to scale (for example, the melting temperature of pure CPAs is usually below 20 °C)

liquidus (between subcooled liquid and supersaturated liquid), solidus (between liquid and supersaturated liquid), and the glass transition line (between the glassy phase and the unstable liquid that is either subcooled or supersaturated). A detailed description of the three processes of cell cryopreservation is given below.

2.1 Slow-freezing

For the conventional slow-freezing approach, the following steps are typically used (A→B→C→D→E→I in Fig. 1): (1), cells in an aqueous solution (state A) are first loaded with CPAs at a concentration usually up to 15 wt% (or up to ~ 2 M, state B); (2), the cells are then subcooled usually down to between -2 and -7 °C (C) to seed ice in the extracellular space by touching the sample contained usually in a cryovial with a deeply cooled (e.g., in liquid nitrogen) object (C→D); (3), the cells are further cooled slowly (typically, < 10 °C/min) along the liquidus and (if necessary) extended liquidus to between -40 and -100 °C, a process called freeze concentration (D→E); and (4), the cells in the frozen sample are transferred into liquid nitrogen for long-term storage (E→I).

During the slow-freezing process, the formation of extracellular ice after ice-seeding leads to freeze concentration of the unfrozen solutions by ejecting solutes and cells from the frozen to unfrozen phase (note: unlike pure water that becomes frozen at a specific temperature, solutions become frozen over a temperature range that is dependent on the types of solutes in the solution). As a result, dehydration of cells in the unfrozen phase driven by osmosis ensues, which minimizes intracellular water available for ice formation inside the cells so that the cells can enter the glassy phase (I) when transferred into liquid nitrogen. This approach typically requires a specialized machine usually called controlled rate freezer (CRF) to achieve freezing in a controllable manner. The time required for the slow-freezing process is typically in hours.

2.2 Conventional vitrification

Vitrification by definition is ice free. In other words, no (or negligible) ice formation or freezing will occur in the sample during cooling (Fahy et al. 1984; Fahy et al. 2004b; Rall and Fahy 1985). Conventional vitrification (A→F→II in Fig. 1) has also been studied for cryopreservation of both cells and tissue. In this approach, biological samples (state A) are first loaded with a very high concentration of CPAs (up to ~ 7 M, state F) (Fahy et al. 1984; Fahy et al. 2004b; Rall and Fahy 1985). The samples are then cooled directly from ambient temperature to a cryogenic temperature usually in liquid nitrogen (state II) and stored there for future use.

Although the conventional vitrification approach can be used to eliminate the detrimental effect of ice formation altogether, the unusually high CPA concentration required by the approach is toxic to most mammalian cells even in a short period of exposure (ranging from seconds to minutes dependent on the specific cells and tissues) (Chen et al. 2000; Chen et al. 2001a; Fahy et al. 2004a; Fowler and Toner 2005; Heng et al. 2005; Hunt et al. 2006). Therefore, the samples should be cooled as soon as possible after loading with CPAs. Oftentimes, a mixture of multiple CPAs is used to reduce the cytotoxicity of the high CPA concentration required (Fuller 2004). In addition, large, membrane impermeable molecules such as sugars (typically sucrose and trehalose) have been used to dehydrate the cells somewhat before cooling and protect cell membrane from injury during cooling (Beattie et al. 1997; He et al. 2008b). Vitrification can be done without a specialized machine and the time required is much shorter than that for slow-freezing.

2.3 Low-CPA vitrification

Low-CPA vitrification (A→B→III in Fig. 1) is a further advancement of the conventional vitrification with the goal to reduce the CPA concentration (e.g., at state F versus B) required for vitrification to a low, nontoxic level (similar to that used in slow-freezing). This can be done by creating an ultrafast cooling rate to cool the cells for cryopreservation. This is because the higher the cooling rate, the less the amount of cryoprotectants is required for achieving vitrification (Berejnov et al. 2006; Boutron 1986; He et al. 2008b; Karlsson et al. 1994; Toner et al. 1990; Yang et al. 2009). For example, even pure water can be vitrified without any ice formation when the cooling rate is approximately one million degree Celsius per second (Bhat et al. 2005; Bruggeller and Mayer 1980; Yang et al. 2009).

Various devices have been utilized to achieve fast cooling rates (up to $\sim 20,000$ °C/min) such as the traditional French type straw (Fig. 2), open pulled straw, electron grid, and cryoloops (Fowler and Toner 2005; Gardner et al. 2007; Vajta and Nagy 2006; Yavin and Arav 2007). As a result, the amount of cryoprotectant required for vitrification can be reduced to around 4 M. To achieve an ultrafast cooling rate, two recent studies reported the use of a micro-fabricated oscillating heat pipe (OHP) device (Han et al. 2008; Jiao et al. 2006). Although their theoretical analysis shows that a cooling rate of $\sim 10^6$ °C/min could be achieved, testing of the device for low-CPA vitrification using living cells has not been reported to date. Another recent study reported that a cooling rate as high as $\sim 200,000$ °C/min can be achieved by plunging an ultra-thin walled (10 μm) quartz microcapillary (QMC, 180 μm inner diameter that is slightly bigger than a human oocyte, Fig. 2) into liquid nitrogen (He et al. 2008b). As a result, the CPA concentration required for vitrification of mouse ES cells and mouse oocytes can be reduced to as low as 2.5 M altogether (He et al. 2008b; Lee et al. 2010), which is close to the highest CPA concentration usually used for slow-freezing.

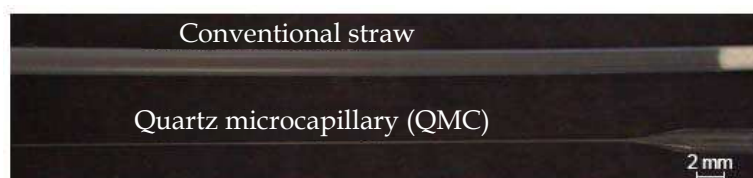


Fig. 2. (Adapted from (He et al. 2008b)) A comparison of the conventional French-type straw (top) used today for cell vitrification at an unusually high CPA concentration and the 200 μm (outer diameter), thin-walled (10 μm) quartz microcapillary (QMC, bottom) used to achieve ultrafast cooling to minimize the CPA concentration required for vitrification

Another way to improve cell vitrification is to confine cells in a small space such as sub-millimeter (in diameter) sized liquid droplets of aqueous cell suspension (Berejnov et al. 2006; Edd et al. 2008; Franks et al. 1983). A major disadvantage of using small liquid droplets to confine cells is that the droplets will merge with each other unless they are dispersed in an oil phase, which makes it difficult to retrieve cells from the droplets.

The hydrogel microcapsule (~ 250 -1000 μm) of natural, biocompatible polymers such as alginate has been widely explored to confine or encapsulate a variety of living cells for transplantation and cell-based therapy (Chang 1996; Maguire et al. 2006; Magyar et al. 2001; Orive et al. 2003; Orive et al. 2004; Orive et al. 2006; Rohani et al. 2008; Torre et al. 2007; Wang et al. 2006a; Wang et al. 2006b). Recently, living cells have been encapsulated in even smaller (~ 100 μm) microcapsules for better cell survival and transplantation efficacy (Zhang and He 2009). These microcapsules can well retain their morphology for an extended period of time in physiologic solutions both *in vitro* and *in vivo*. However, cryopreservation of cells

encapsulated in the large ($\geq \sim 250 \mu\text{m}$) microcapsules by slow-freezing has been challenging because the inevitable ice formation always results in significant damage to the microcapsules, which in turn can damage the encapsulated cells (Heng et al. 2004; Herrler et al. 2006; Stensvaag et al. 2004; Wu et al. 2007). Although the conventional vitrification approach can overcome this problem, the unusually high concentration CPA needed is detrimental to stress-sensitive cells such as the ES cells (Fahy et al. 1987; Fahy et al. 1984; Fahy et al. 2004b; Rall and Fahy 1985; Wu et al. 2007).

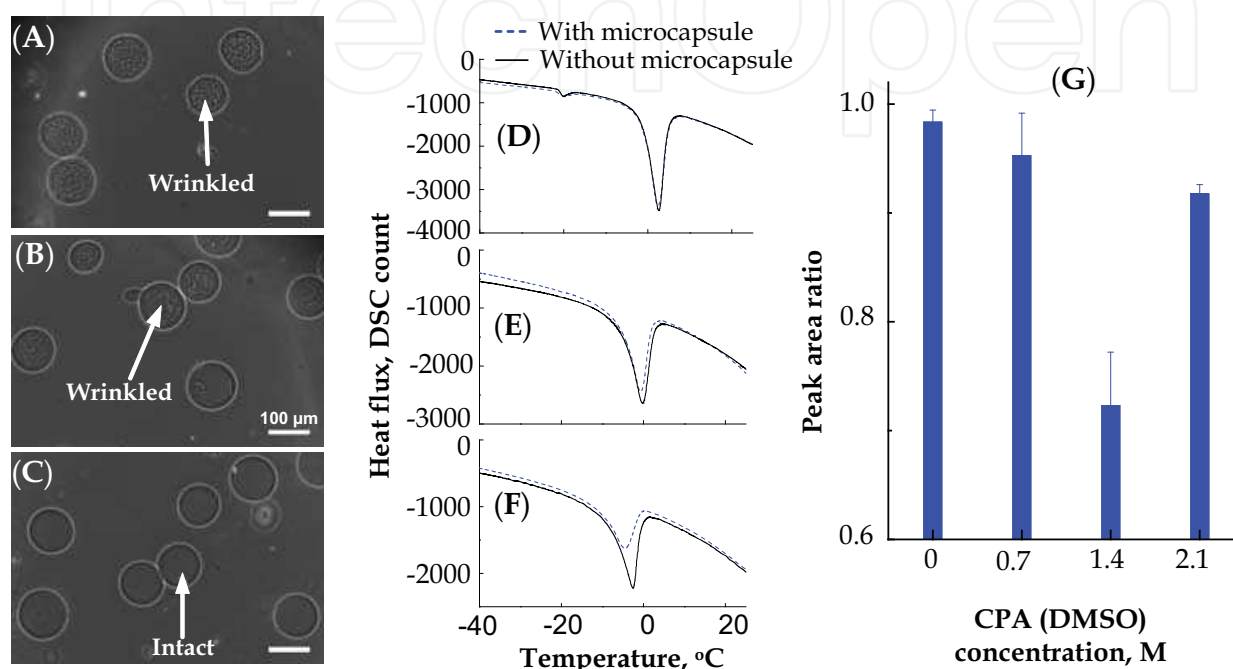


Fig. 3. (Adapted from (Zhang et al. 2010)) Typical morphology of alginate microcapsules after cryopreservation in physiological saline with (A) 0, (B) 0.7, and (C) 1.4 M DMSO (dimethylsulfoxide); typical scanning calorimetry data for samples with (D) 0, (E) 0.7, and (F) 1.4 M DMSO; and (G) the ratio of peak area on the scanning calorimetry data for samples with microcapsules ($\sim 30\%$ in volume) to that of samples without microcapsules at various CPA concentrations: Cooling rate, $100 \text{ }^\circ\text{C}/\text{min}$

By careful cryomicroscopy and scanning calorimetry studies, it was identified in a recent publication that water enclosed in $\sim 100 \mu\text{m}$ (in diameter) alginate microcapsules can be preferentially vitrified over the bulk water (where the microcapsules are suspended) with only 1.4 M DMSO at a cooling rate of $100 \text{ }^\circ\text{C}/\text{min}$ (Zhang et al. 2010). Typical results from the cryomicroscopy studies are shown in Fig. 3 for microcapsules cryopreserved with (A) 0, (B) 0.7, and (C) 1.4 M DMSO. The microcapsules appeared intact post cryopreservation when the DMSO concentration was 1.4 M (C) (or higher) while they were damaged (wrinkled) when $\leq 0.7 \text{ M}$ DMSO was used (A and B). The wrinkled appearance of microcapsules in (A) and (B) presumably was a result of significant ice formation in the microcapsules during freezing. Since water in the bulk solution was frozen under all the conditions, the data suggest that water enclosed in the microcapsules was preferentially vitrified in the presence of 1.4 M DMSO resulting in the intact morphology in (C).

The calorimetry data are also shown in Fig. 3 for samples with (D) 0, (E) 0.7, and (F) 1.4 M DMSO either in the absence or presence of $\sim 30\%$ (by volume) alginate microcapsules. The

area of the major peak on the heat flux curve for each sample is proportional to the amount of ice formed in the sample. Clearly, the amount of ice formed in samples with and without microcapsules was not significantly different when the DMSO concentration was ≤ 0.7 M (D and E), suggesting ice formed equally in the bulk solution and the microcapsules resulting in damage to the microcapsules shown in Fig. 3A and B. When the DMSO concentration was increased to 1.4 M, however, the peak area for samples with microcapsules was much smaller than that for samples without microcapsules (Fig. 3F), indicating much less ice formation in the samples with microcapsules. Presumably, the reduced ice formation in the samples with microcapsules was due to vitrification of water enclosed in the microcapsules resulting in the intact microcapsules shown in Fig. 3C.

The ratio of the peak area for samples with $\sim 30\%$ (by volume) microcapsules to that of samples without microcapsules was further quantified and is shown in Fig. 3G. The ratio was not different from 1, suggesting equal ice formation in the bulk solution and the microcapsule when the DMSO concentration was ≤ 0.7 M. When the DMSO concentration was 1.4 M, the ratio was ~ 0.7 suggesting that water enclosed in the microcapsules (30% by volume) were preferentially vitrified under this condition. When further increasing DMSO to 2.1 M, the ratio increased back to 0.91, presumably due to the vitrification of more bulk water. Therefore, water enclosed in the microcapsules can be preferentially vitrified in the presence of 1.4 M DMSO while more than 2.1 M DMSO is required to vitrify the same amount of water in the bulk solution, indicating the capability of alginate microcapsules in enhancing vitrification of the enclosed water even at a cooling rate of 100 °C/min. The preferential vitrification of water enclosed in the microcapsule is due to its higher viscosity (Ahearne et al. 2005; Qin 2008; Zhang et al. 2006) and small volume (sub-nanoliter) and is expected to be much more significant at much higher cooling rates (e.g., $> 10,000$ °C/min) (Chen and Li 2008; Karlsson et al. 1994; Yang et al. 2009; Zhang et al. 2010; Zhao et al. 2006).

The preferential vitrification of water enclosed in small alginate microcapsules demonstrated in Fig. 3 should be able to enhance vitrification of living cells encapsulated in the microcapsules at high cooling rates (e.g., $> 10,000$ °C/min). This is because it can not only depress ice formation and growth in the microcapsule but also prevent ice (if any) propagation into cells from the bulk solution where ice is usually formed first (because of its much bigger volume) (Berejnov et al. 2006; Fahy et al. 1987; Franks et al. 1983; He et al. 2008b; Karlsson et al. 1994; Mazur et al. 2005a; Mazur et al. 2005b; Toner 1993; Toner et al. 1990; Yavin and Arav 2007). This hypothesis is confirmed by a recent study where the C3H10T1/2 mouse mesenchymal stem cells encapsulated in ~ 100 μm alginate microcapsules were vitrified using a 400 μm , thin-walled quartz microcapillary at a low-CPA concentration (1.4 M DMSO) (Zhang et al. 2010). Typical images of the cells are shown in Fig. 4 for both before (A-D) and after (E-H) the low-CPA vitrification procedure.

Before vitrification, both the non-encapsulated (A and B) and microencapsulated (C and D) cells remained alive, indicating that the microencapsulation process did not result in any significant damage to the cells. After vitrification, many of the non-microencapsulated cells appeared swollen with damaged plasma membrane (E) and were significantly injured (red, F) with a cell viability of $42.0 \pm 4.4\%$. For the microencapsulated cells, however, most of them appeared intact (G) and viable (green, H) after vitrification. The viability of the microencapsulated cells post cryopreservation was determined to be $88.9 \pm 2.9\%$, which is more than twice of that of the non-encapsulated cells and is only $\sim 5\%$ less than that before vitrification.

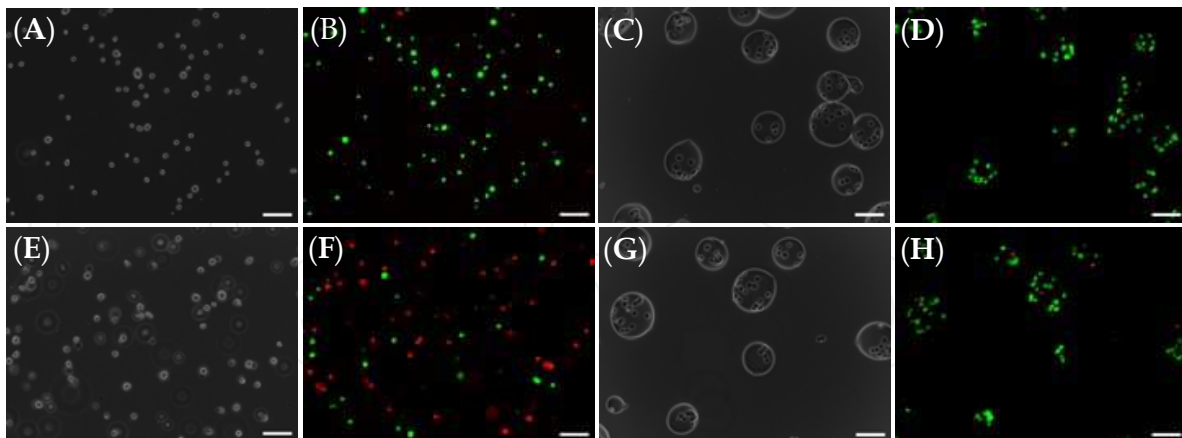


Fig. 4. (Adapted from (Zhang et al. 2010)) Typical phase and fluorescence images of non-encapsulated (A, B, E, and F) and microencapsulated (C, D, G, and H) cells before (A-D) and after (E-H) cryopreservation by low-CPA vitrification. In the fluorescence micrographs, live and dead cells were stained green and red, respectively. Scale bars: 100 μ m

After liquefying the microcapsules, the collected cells were found to attach well with an attachment efficiency (ratio of the number of cells attached in the cryopreserved samples to that in the control fresh samples at day 1, one day after seeding the cells) of \sim 85 and 37% for the microencapsulated and non-microencapsulated cells, respectively (Fig. 5). Moreover, the viable cells with low-CPA vitrification proliferate normally just like the control fresh cells (Fig. 5). These data clearly demonstrate the capability of the small alginate microcapsule in protecting cells from injury during low-CPA vitrification, presumably by minimizing ice formation (or enhancing vitrification) in the microencapsulated cells.

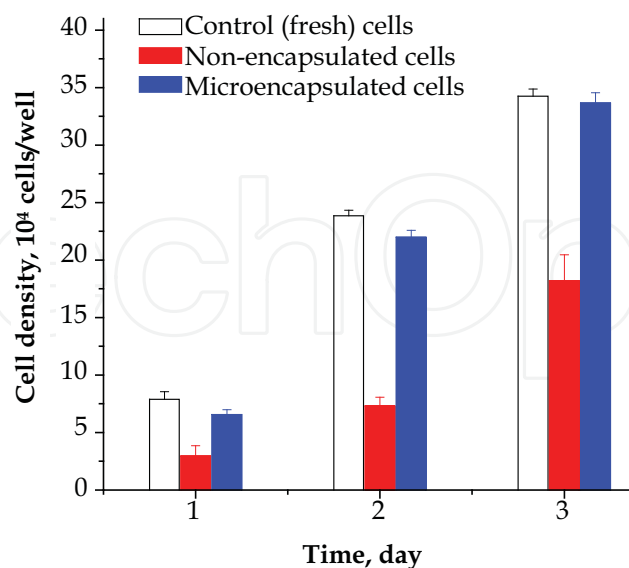


Fig. 5. Proliferation of the non-microencapsulated and microencapsulated mesenchymal stem cells in 3 days after vitrification using 1.4 M DMSO in the 400 μ m quartz microcapillary together with control fresh cells without cryopreservation: The total number of cells seeded for each of the three conditions were the same

3. Lyopreservation at ambient temperature

The idea of dry or lyopreservation at ambient temperature is actually not new as many lower organisms, resurrection plants, and seeds can survive extreme drought in nature upon rehydration, a phenomenon called anhydrobiosis or life without water (Armstrong July, 1996; Browne et al. 2002; Clegg 2001; Crowe and Cooper 1971; Crowe and Crowe 2000; Crowe et al. 2004; Crowe et al. 1992; Perry 1999). A high concentration of small sugars (disaccharides typically sucrose for plants and trehalose for lower organisms) have been found in these organisms and plants when they are in the anhydrobiotic (or desiccated) state. Learning from nature, both sucrose and trehalose have been investigated as the protective agent (also called lyoprotectant) in protocols of lyopreservation (Crowe and Crowe 2000; Crowe et al. 2001; Crowe et al. 2005; Eroglu et al. 2000; Wolkers et al. 2002). Two methods of desiccation have been studied to dry cells in aqueous samples for lyopreservation: evaporative drying and freeze-drying (or lyophilization) which are illustrated in Fig. 1 as well.

3.1 Evaporative drying

During desiccation by evaporative drying (A→B→IV in Fig. 1), water in an aqueous sample with cells is removed by exposing the sample to a dry environment (e.g., dry air, inert gas such as nitrogen, or vacuum) without freezing (or ice formation) after loading with up to ~ 15 wt% lyoprotectants. Forced convection is usually used to increase the drying rate of natural convection. Desiccation by evaporative drying has been used to achieve lyopreservation of biomacromolecules such as proteins and lipids, pharmaceutical drugs,

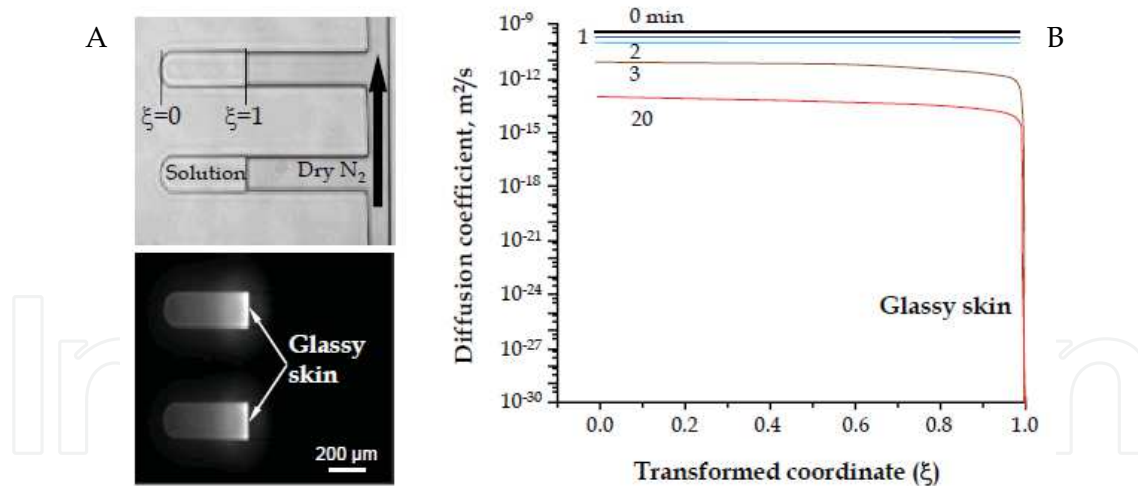


Fig. 6. (Adapted from (Aksan et al. 2006) and (He et al. 2008a)) (A) Evaporative drying of 0.2 M aqueous trehalose solution in microrchannels: The brightness in the solution in the lower panel indicate the viscosity of solution (the stronger the intensity, the higher the viscosity is in the solution), which clearly shows a glassy skin formed on the interface between the solution and the dry nitrogen gas (N_2) during drying and the heterogeneity of viscosity in the residual solution; and (B) predicted diffusivity in the trehalose solution during evaporative drying at various times: The glassy skin forms after 3 minutes drying and has an extremely low diffusivity while the diffusivity in the rest of the solution is much higher, indicating a significant residual water in the dried residual solution

prokaryotic cells (microbials), and blood cells (red cells and platelets), but not eukaryotic mammalian cells.

A major engineering difficulty to dry the glass-forming disaccharide solution for cell lyopreservation by evaporative drying is that a thin glassy skin can easily form on the interface between the solution and the dry environment (Fig. 6A). The glassy skin has an extremely high viscosity and low diffusivity (Fig. 6B) leading to incomplete drying (up to 20% residual water after hours drying) and heterogeneity in the evaporatively dried sample. This problem might be alleviated by breaking up the solution into micron or submicron sized droplets or thin-films (He et al. 2008a).

3.2 Freeze-drying

A typical freeze-drying process is illustrated in Fig. 1 as A→B→C→D→G→H→IV: (1), cells in an aqueous solution (state A) is first supplemented with lyoprotectants at a concentration of up to ~ 15 wt% (state B); (2), the sample is subcooled to usually between -2 and -7 °C (C) to seed extracellular ice in the sample by touching the sample with a deeply cooled object (C→D); (3), the sample is further cooled to between -30 and -50 °C slowly at a cooling rate usually less than 10 °C/min (D→G); (4), the ice formed in the sample during freezing is then sublimated (i.e., from solid ice crystal to vapor directly without going through the liquid water phase) by exposing the sample to a vacuum usually less than 10 Pa at the phase G (primary drying); and (5) a secondary drying process is then done by heating the sample in vacuum slowly to ambient temperature to further dehydrate the sample for additional hours to days (G→H→IV). The samples are then sealed and preserved in the dry phase (IV) at ambient temperature for future use. Freeze-drying has been used successfully in achieving lyopreservation of many biomacromolecules such as proteins and lipids and many pharmaceutical drugs. It has also been used for lyopreservation of prokaryotic cells such as microbials and blood cells including the red cells and platelets, but not eukaryotic cells at this time.

For freeze-drying, it is crucial to keep the temperature below the so-called collapse temperature (T_C) (Abdul-Fattah et al. 2007; Bellows and King 1972; Felix 2007; Fonseca et al. 2004a; Fonseca et al. 2004b; Gieseler et al. 2005; Kramer et al. 2009; MacKenzie 1966; Meister and Gieseler 2006; Meister et al. 2006; Nail et al. 2002; Pikal 1985; Pikal and Shah 1990; Pikal et al. 1983; Rey and May 1999) during primary drying (at the phase G). Otherwise, the sample may collapse during primary drying (Fig. 7A) and blow up during secondary drying (Fig. 7B), resulting in incomplete drying and heterogeneity (Fig. 7C versus D) in the freeze-dried product as that in evaporatively dried sample. Consequently, the biostability of the freeze-dried biologicals could be significantly compromised (Hancock et al. 1995; He et al. 2008a; He et al. 2006b). More importantly, a recent study reported that the collapse temperature of cell culture medium-based trehalose solutions important for freeze-drying mammalian cells can be much lower than that of a simple binary trehalose-water solution ($T_C = \sim -30$ °C) and trehalose solutions used for freeze-drying pharmaceuticals and prokaryotes (Yang et al. 2010a), as shown in Fig. 7E.

Beside the engineering challenge to effectively dry the trehalose solutions, effective delivery of the small hydrophilic lyoprotectants (trehalose and sucrose) into mammalian cells has been challenging as the first step toward cell preservation at ambient temperature. This is because lyoprotectants such as trehalose must be present both intra and extracellularly to provide the maximum protection during drying, but mammalian cells lack a mechanism to synthesize trehalose endogenously and their plasma membrane is impermeable to the

sugars (Acker 2004; Chen et al. 2001b; Eroglu et al. 2002). Over the past decades, a number of approaches have been explored to introduce trehalose into living cells for preservation purposes. The most straightforward approach is to deliver exogenous trehalose into the cytosol of living cells by direct microinjection. This approach has been successfully used for intracellular delivery of trehalose to cryopreserve mammalian oocytes that have a large size ($\sim 100 \mu\text{m}$ in diameter) and are generally limited in quantity (less than a few hundred) (Bhowmick et al. 2002; Eroglu et al. 2005; Eroglu et al. 2003; Eroglu et al. 2002).

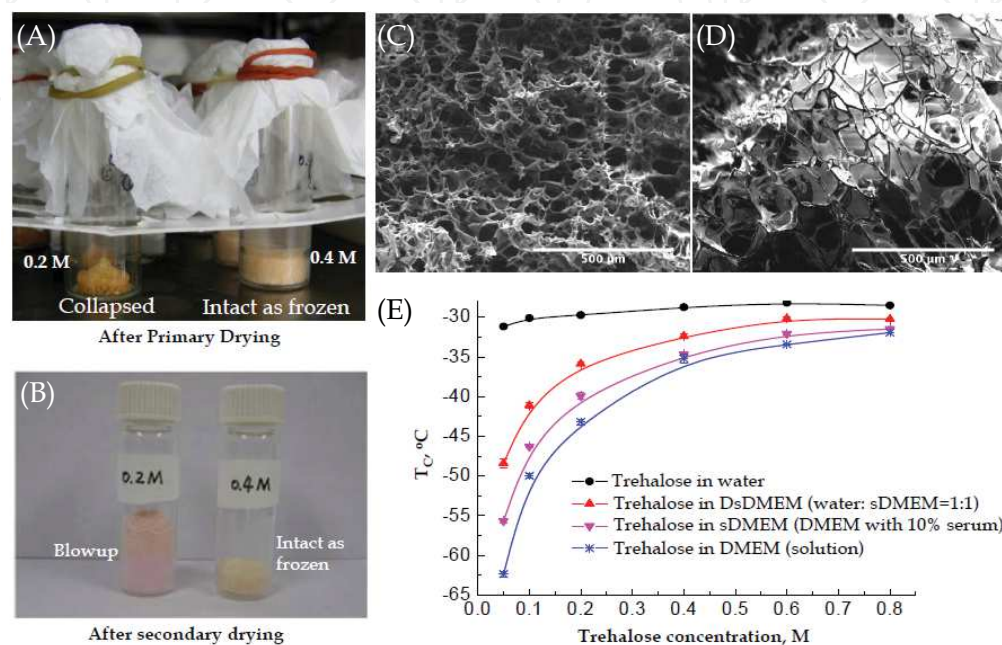


Fig. 7. (Adapted from (Yang et al. 2010a)) (A) Typical photograph showing collapsed vs. intact sample after primary drying (at $-38 \text{ }^\circ\text{C}$) of 0.2 versus 0.4 M trehalose in DMEM with 10% fetal bovine serum; (B) the collapse sample blew up during secondary drying (heating at $0.5 \text{ }^\circ\text{C}/\text{min}$ to room temperature) as a result of the evaporation of the significant residual water after primary drying while the non-collapsed sample appeared intact; SEM (scanning electron microscopy) images showing homogeneous microporous structure in the intact sample (C) and heterogeneous microstructure in the collapsed and blew-up sample (D) after secondary drying; and the collapse temperature (T_c) as a function of trehalose concentration in various solutions: DMEM, Dulbecco's modified eagle medium (aqueous) widely used for culturing mammalian cells

However, the microinjection approach is difficult (if not at all impossible) to apply for most living cells that are generally much smaller ($< \sim 20 \mu\text{m}$) than mammalian oocytes and usually present in a large quantity (millions). Small living cells have been genetically engineered to synthesize trehalose endogenously. This approach requires the constant production of adenoviral vectors that exhibit significant cytotoxicity, particularly at high multiplicities of infection (Gordon et al. 2001; Guo et al. 2000; Puhlev et al. 2001). Trehalose has also been introduced into mammalian cells or their organelles through engineered or native transmembrane pores (Acker et al. 2003; Chen et al. 2001b; Elliott et al. 2006; Eroglu et al. 2000; Liu et al. 2005), electroporation (Reuss et al. 2004; Shirakashi et al. 2002), fluid-phase endocytosis (He et al. 2006a; Oliver 2004; Wolkers et al. 2003), and lipid phase transition (Beattie et al. 1997; He et al. 2006a).

In spite of the various approaches being explored, a consistent report of cell preservation using trehalose for small eukaryotic living cells is still absent (Acker 2004; Crowe 2007; Crowe et al. 2005; Kanas and Acker 2006). This could be due to the inability to deliver a sufficient amount of intracellular trehalose ($\geq \sim 0.1$ M) for cell preservation using some of the approaches (e.g., fluid phase endocytosis). In addition, cells could be too severely compromised during the delivery step to withstand further freezing/dehydration stresses during preservation, considering the highly invasive nature of some of the approaches (e.g., electroporation). Recently, research has been sought to use nanoparticles (liposome and polymeric nanocapsules) as the intracellular delivery vehicles of the small hydrophilic lyoprotectants with promising outcomes (Holovati and Acker 2007; Holovati et al. 2009; Scott 2006; Zhang et al. 2009).

Besides the non-reducing disaccharides (trehalose and sucrose), small stress proteins particularly, the late embryogenesis abundant (LEA) proteins have been suggested to be an important part of the molecular repertoire that renders desiccation tolerance in anhydrobiotic organisms and are attracting more and more research attention (Browne et al. 2002; Clegg 2001; Crowe and Crowe 2000; Crowe et al. 2004; Crowe et al. 1992; de Castro et al. 2000; Hand et al. 2007; Huang and Tunnacliffe 2007; Iturriaga 2008; Li and He 2009; Perry 1999; Tunnacliffe et al. 2001).

4. Biophysics in cell preservation

As mentioned above, one or more protective agents (cryo and lyoprotectants for cryo and lyopreservation, respectively) are required to protect cells from being damaged during preservation. Although it has not been well established, the mechanism of protection provided by these agents is usually hypothesized to be three folds: 1) acting as water to form hydrogen bonds with proteins and lipids so that their functional conformations can be preserved during water deficit (Clegg et al. 1982; Crowe 1993a; Crowe 1993b; Crowe et al. 1998), 2) promoting preferential hydration of the biomacromolecules in cells during water loss (Cottone 2007; Cottone et al. 2005; D'Alfonso et al. 2003; Roche et al. 2006), and 3) forming a stable glassy matrix with extremely low molecular mobility to prevent the 3D intracellular structure from collapse and to suspend any degradative and metabolic reactions in response to water loss (Crowe et al. 1998; Crowe et al. 2001; He et al. 2006b; Sun et al. 1996).

At the cellular level, two biophysical events (cell dehydration and intracellular ice formation (IIF)) have been well established to be the major causes of cell injury. During slow-freezing, these two biophysical events result in the classical inverted U-curve of cell survival as a function of cooling rate during freezing with the cell survival being the highest at the optimal cooling rate (CR_{SF}), as demonstrated in Fig. 8. At a very slow cooling rate ($< CR_{SF}$), cell dehydration induced biochemical/biophysical alterations are the dominant mechanism of cell injury while at a not-so-high cooling rate ($< CR_V$), cells are mainly damaged by IIF. With the further increase of cooling rate to higher than CR_V , the kinetics of cooling is faster than that of both IIF and cell dehydration and cell injury due to both events is minimized. As a result, the cell survival increases with the increase of cooling rate till it reaches 100%. Both CR_{SF} and CR_V are dependent on the cell type, the CPA type (propylene glycol has been reported to be superior to ethylene glycol in terms of the capability of vitrification (He et al. 2008b)), and the CPA concentration. Of note, the damaging (both osmotic and metabolic) effect of an unusually high concentration of CPAs required by the conventional vitrification is not considered in the figure.

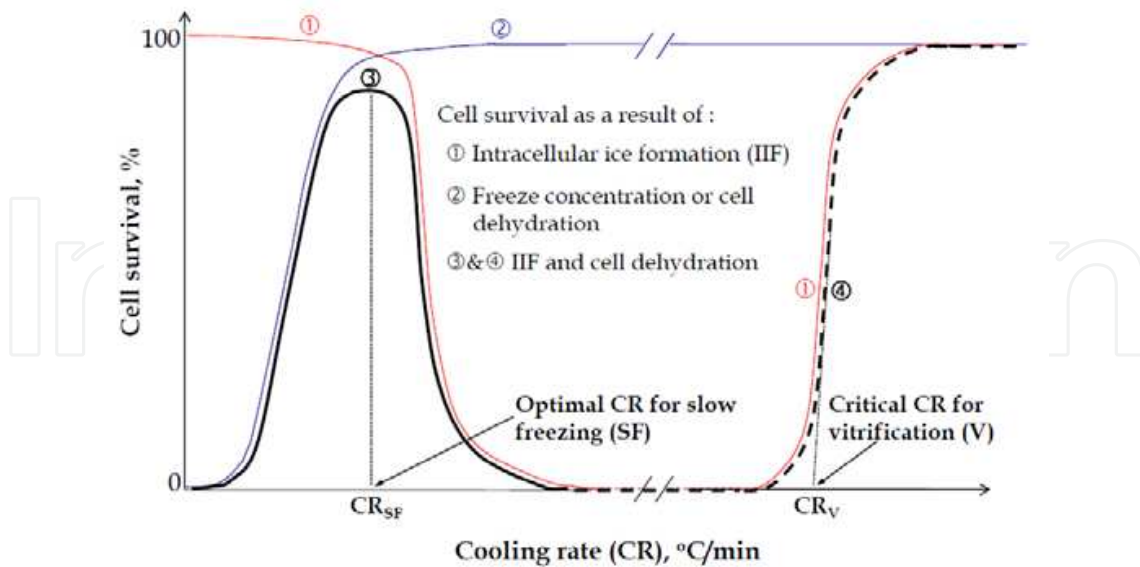


Fig. 8. Cell survival accounting for the effect of intracellular ice formation (IIF, ①), cell dehydration (②), the combination of IIF and cell dehydration at both low (for slow-freezing, ③) and high (for vitrification, ④) cooling rates. At a very slow cooling rate ($< CR_{SF}$), cell dehydration induced biochemical/biophysical alterations are the dominant mechanism of cell injury while at not-so-high cooling rates ($< CR_V$), cells are mainly damaged by IIF; with the further increase of cooling rates to higher than CR_V , cell injury due to both cell dehydration and IIF is negligible. Both CR_{SF} and CR_V are dependent on the cell type, the CPA type, and the CPA concentration. Of note, the damaging (both osmotic and metabolic) effect of an unusually high concentration of CPAs required by the conventional vitrification is not considered in the figure

Both cell dehydration and IIF can be quantified by modeling. IIF has been studied using both phenomenological and mechanistic models (Pitt 1990; Toner 1993). The mechanistic model has been widely used and delineates the ice formation process as two consecutive events: (1) Nucleation to form ice nuclei and (2) the subsequent growth of the nuclei (Hobbs 1974; Toner 1993). Nucleation of intracellular ice can be catalyzed by either a surface (surface catalyzed nucleation, SCN) such as the cell plasma membrane or a volume of subcooled solution (volume catalyzed nucleation, VCN) such as the cytoplasm (Hobbs 1974; Toner 1993). The rate of ice nucleation (I) due to either VCN or SCN can be estimated as follows (Toner et al. 1990; Toner et al. 1992):

$$I = \Omega_0 \frac{N}{N_0} \frac{\eta_0}{\eta} \left(\frac{T}{T_{f0}} \right)^{0.5} \exp \left[\frac{-\kappa_0 (T_f/T_{f0})^4}{(T - T_f)^2 T^3} \right] \quad (1)$$

where T_f is the equilibrium freezing point of the intracellular solution, N is the number of water molecules either in the cells (for VCN) or in contact with the cell plasma membrane (for SCN), η is viscosity, Ω and κ are two model parameters that are usually called the kinetic and thermodynamic model parameter, respectively, and the subscript 0 represents the isotonic solution state. The two model parameters (constants) under isotonic solution state (Ω_0 and κ_0) need to be determined *a priori* by experimental studies and have been

reported in the literature for a number of cell types as summarized elsewhere (He and Bischof 2003; Toner 1993; Yang et al. 2010b). The cumulative probability of intracellular ice formation (P_{IIF}) can then be calculated as follows (Toner 1993; Toner et al. 1990):

$$P_{IIF}^{VCN} = 1 - \exp\left(-\int_0^t VI^{VCN} dt\right) \quad (2)$$

$$P_{IIF}^{SCN} = 1 - \exp\left(-\int_0^t AI^{SCN} dt\right) \quad (3)$$

$$P_{IIF}^{Tot} = P_{IIF}^{SCN} + (1 - P_{IIF}^{SCN})P_{IIF}^{VCN} \quad (4)$$

where V and A are the cell volume and surface area available for catalyzing the nucleation of intracellular ice, respectively. Significant IIF is usually manifested as darkening of the cell cytoplasm when observed under a bright field of light microscopy, which has been used widely to quantify the kinetics of IIF (Diller 2005; Toner et al. 1991; Yang et al. 2010b).

Of note, the above IIF model is valid only when the ice nucleation (the first step of IIF) is the rate-limiting step of IIF which is often true when freezing cells/tissues at not very high cooling rates (e.g., less than a few hundred Celsius per minute) and in the absence of a high concentration of CPA (e.g., less than 10 wt%) (Toner 1993). When cooling cells/tissue with much higher cooling rates and/or a high concentration of intracellular CPA (e.g., during vitrification and after significant cell dehydration during slow-freezing), the rate limiting-step of IIF is the growth of the ice nuclei. The IIF under these conditions is said to be diffusion-limited for which more complicated models are needed to predict the amount of intracellular ice (Chen and Li 2008; Karlsson et al. 1993; Karlsson et al. 1994; Yang et al. 2009; Zhao et al. 2006). To better predict the diffusion-limited ice nucleation and growth, an advanced model such as the free volume model that can account for the effect of glass transition on solution viscosity and diffusion coefficient might be necessary. Such free volume models for several cryo and lyoprotectants have been reported in (He et al. 2006b).

In order to predict the probability of IIF using the above model during slow-freezing where freeze concentration induced cell dehydration is significant, information on the cell volume, V (or cell surface area, A , related to the diameter of the cells when the cells assume a spherical geometry), during freezing is required. The following model has been commonly used to predict the cell volume change during freezing (Karlsson et al. 1994; Mazur 1963):

$$\frac{dV}{dt} = -\frac{L_p AR_g T}{v_w} \left[\ln \frac{V - V_b - V_s}{V - V_b - V_s + \phi_s n_s v_w} - \frac{\Delta h_f}{R_g} \left(\frac{1}{T_{ref}} - \frac{1}{T} \right) \right] \quad (5)$$

where v_w is the partial molar volume of water, n represents amount (in mole), ϕ_s is the dissociation constant of solutes (e.g., 2 for NaCl), V_b is the osmotically inactive volume in cells, Δh_f is the latent heat of fusion of water, T_{ref} is a reference temperature (either ice-seeding temperature or the equilibrium melting point of intracellular solution), T is thermal history, the subscripts s and w represent solute (including CPAs) and water, respectively, and L_p is the cell plasma membrane permeability to water that can be calculated as follows (Levin et al. 1976):

$$L_p = L_{pg} \exp \left[-\frac{E_{Lp}}{R_g} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right] \quad (6)$$

where L_{pg} is the permeability of the cell membrane to water at the reference temperature (T_{ref}) and E_{Lp} is the activation energy for water transport across the cell plasma membrane. In the equation, L_{pg} and E_{Lp} are two model parameters (constants) that need to be determined *a priori* using experimental data. Cell dehydration during freezing can be measured using either a specialized cryostage mounted on a light microscope (Diller 2005; Toner et al. 1991) or differential scanning calorimetry (Bischof 2000; Devireddy et al. 2001; Devireddy et al. 1998; Luo et al. 2002). Many studies have been performed to determine the two model parameters for various cells, which have been reviewed and tabulated elsewhere (Bischof 2000; Han and Bischof 2004; He and Bischof 2003; Yang et al. 2010b).

Although cell dehydration and intracellular ice formation (IIF) can be predicted using the above models, a quantitative understanding of the mechanistic link between the two biophysical events and cell injury has not been well established despite some early efforts in this respect as reviewed in (He and Bischof 2003). The incidence of significant IIF (defined as darkening of cell cytoplasm) correlates strongly with cell death in many cell types (i.e., 50% of IIF in many cell populations yields 50% of dead cells) (Toner 1993). However, the exact amount or percentage of intracellular ice that is significant enough to result in irreversible cell death is still unclear. Some studies even suggest that a small amount of intracellular ice might be beneficial to cell survival (Acker and McGann 2002; Acker and McGann 2003). Therefore, further studies to establish mechanistic models capable of accounting for the effect of all the freezing induced biophysical events including IIF and freeze concentration (i.e., the so-called solute effect), and low temperatures *per se* is important to further our understanding of low temperature biology and its biomedical applications such as cryosurgery and cryopreservation.

5. Preservation of embryonic stem (ES) cells

Currently, the two most commonly used approaches for ES cell cryopreservation are slow-freezing and conventional vitrification (Hunt and Timmons 2007; Li et al. 2010; Martin-Ibanez et al. 2008). A summary of the major advantages and drawbacks of the two approaches is given in Table 1. Although a low, non-toxic CPA concentration (usually $\leq \sim 1.5$ M) is used in slow-freezing, it is always associated with mechanical and physicochemical injury to cells due to ice formation and slow-freezing (usually ≤ 1 °C/min) induced cell dehydration (Bischof 2000; Gao and Critser 2000; Mazur 1984; Toner 1993). The conventional vitrification approach diminishes ice formation altogether to a harmless level (Fahy et al. 1987; Fahy et al. 1984; Fahy et al. 2004b; Rall and Fahy 1985; Wu et al. 2007). The unusually high (as high as 7 M) concentration of CPA required, however, can result in significant metabolic and osmotic injury to cells (Chen et al. 2000; Chen et al. 2001a; Fahy et al. 2004a; Fowler and Toner 2005; Heng et al. 2005; Hunt et al. 2006). Consequently, it is necessary to use multiple steps of CPA loading/dilution and maintain a short exposure time (within a few minutes) to high concentration CPA in each step to minimize injury (Reubinoff et al. 2001), which makes the procedure complicated, stressful, and particularly, difficult to control in that the time for the diffusion of CPAs into the cells to reach equilibrium usually takes at least 5-10 minutes (He et al. 2008b; Heng et al. 2005; Jain and Paulson 2006; Pedro et

al. 2005). In addition, a cocktail of various CPAs rather than one CPA has been commonly used to reduce the CPA toxicity.

Slow-freezing	Low-CPA vitrification		Conventional vitrification
Drawbacks	Advantages	Advantages	Drawbacks
Cell injury due to ice formation and cell dehydration	Low, non-toxic CPA ($\leq \sim 1.5$ M)	Negligible ice formation and negligible cell dehydration	High CPA (4-7 M) induced metabolic and osmotic injury

Table 1. A summary of the major advantages and drawbacks of the commonly used slow-freezing and conventional vitrification approaches for cell cryopreservation today: The low-CPA vitrification approach combines all the advantages of the two commonly used approaches while avoiding their shortcomings

The inherent drawbacks associated with the two conventional approaches can result in damage that is (mild to many other types of cells though) sufficient to induce and/or accelerate apoptosis (programmed cell death or cell suicide) in dissociated ES cells considering that the ES cells are particularly susceptible to apoptosis (Heng et al. 2009; Heng et al. 2006; Martin-Ibanez et al. 2008). This may explain why adding ROCK (Rho-associated kinase) inhibitors in the cryopreservation medium to inhibit apoptosis can significantly improve the survival and function of human ES cells post cryopreservation (Baharvand et al. 2010; Claassen et al. 2009; Heng et al. 2007; Martin-Ibanez et al. 2008). Although keeping ES cells in aggregates (embryonic body or EB) can reduce apoptosis, it is even more difficult to cryopreserve the aggregates by either slow-freezing or conventional vitrification. Moreover, sub-optimal cryopreservation can induce epigenetic changes and impose a selection bias for their outgrowth (Baran and Ware 2007). Therefore, it is of great importance to achieve low-CPA vitrification of ES cells in that it combines all the advantages of the two conventional approaches while avoiding all their shortcomings, as demonstrated in Table 1. A recent study has demonstrated that an ultrafast cooling rate ($\sim 200,000$ °C/min) can be achieved by plunging a 200 μm (outer diameter), thin-walled (10 μm) quartz microcapillary (QMC, Fig. 2) into liquid nitrogen (He et al. 2008b). With this QMC ultrafast vitrification technique, R1 ES cells can be vitrified at a CPA concentration of as low as 2.5 M altogether (He et al. 2008b). Figure 9A shows the immediate (within 3 hr) and 1 day viability of the cells post cryopreservation using various CPAs. Only a small percentage of cells ($\sim 20\%$) can survive when using 2 M PROH (1,2-propanediol) alone as the CPA. When adding 0.5 M trehalose into the solution, however, the immediate cell viability increased to $\sim 80\%$, even though trehalose could not permeate the cell membrane and was present only extracellularly. The immediate cell viability for cells cryopreserved using 0.5 M trehalose alone was $\sim 65\%$.

Unlike the immediate viability, only few cells were able to survive at day 1 when using 0.5 M extracellular trehalose ($< 2\%$) as the sole CPA (Fig. 9A). This result indicates the necessity of intracellular CPA to protect cells from within during cryopreservation. Similarly, only a minimal number of cells were able to survive at day 1 when using 2 M PROH ($\sim 12\%$) as the sole CPA. The 1 day viability, however, was much higher ($\sim 72\%$) when the cells were cryopreserved using the combination of 0.5 M extracellular trehalose and 2 M cell membrane permeable CPA (PROH). Therefore, PROH and extracellular trehalose appear to have a synergistic effect on protecting the ES cells from damage during vitrification. Such

synergistic interaction between trehalose and PROH/DMSO has also been observed in other studies (Dash et al. 2008; Wusteman et al. 2003). The proliferation/growth of the attached ES cells post cryopreservation using the combination of 0.5 M trehalose and 2 M PROH was similar to that of the control (fresh) cells over a 3-day observation period (Fig. 9B).

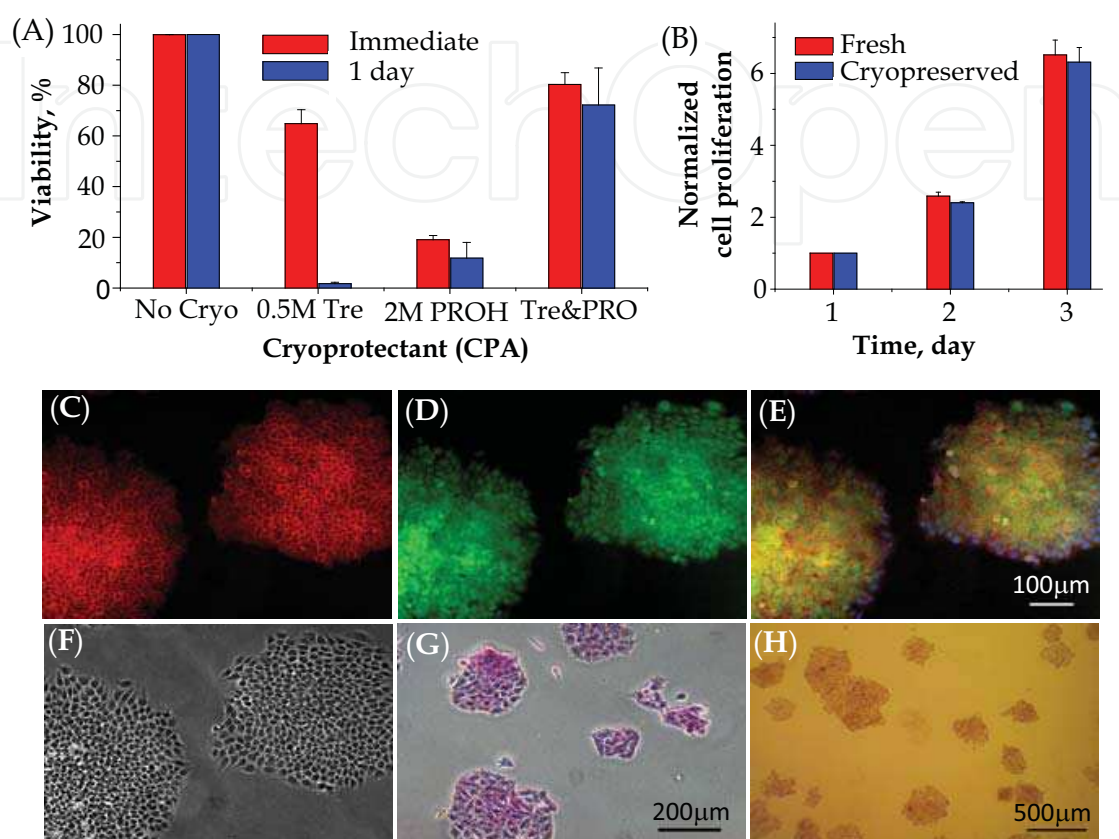


Fig. 9. (Adapted from (He et al. 2008b)) (A) Immediate and 1 day viability of fresh (No Cryo) cells and cells cryopreserved using 0.5 M trehalose (0.5 M Tre), 2 M PROH (1,2-propanediol), and the combination of 0.5 M trehalose and 2 M PROH (Tre&PROH); (B) normalized (to the data at day 1) proliferation of fresh and cryopreserved (using 0.5 M trehalose and 2 M PROH) cells over three days in culture; and micrographs showing undifferentiated properties of the ES cells post vitrification: (C) staining for the surface glycoprotein SSEA-1, (D) green fluorescence protein (GFP) expression denoting transcriptional activity, (E) merged view of SSEA-1, GFP and nuclei staining (in blue using DAPI), (F) phase contrast image of two ES cell colonies, and alkaline phosphatase expression viewed at both high (G) and low (H) magnifications

Typical Micrographs showing the undifferentiated properties of the ES cells post vitrification are given in Fig. 9C-H. Preservation of the undifferentiated properties were verified by the high level staining of the membrane surface glycoprotein SSEA-1 (C) and expression of GFP (green fluorescence protein) under the control of the transcription factor OCT-4 (D). The merged view (E) of the red (SSEA-1), green (GFP), and blue (DAPI to stain cell nuclei) channels indicates extensive co-expression of the two markers overlapping with the cell nuclei. The phase image (F) shows cells with high nuclei/cytoplasm ratios and compact colony formation typical of pluripotent mouse ES cells. The histochemical staining

shows strong expression for alkaline phosphatase at high magnification (G) which was well distributed within each colony as observed at a lower magnification (H). These results suggest that the ES cells retained their undifferentiated properties post cryopreservation by ultrafast vitrification at the reduced CPA concentration (2.5 M altogether). Further studies to test the capability of the cryopreserved cells in differentiating into different types of cells *in vitro* and forming specific tissue *in vivo* are necessary to ultimately confirm preservation of the pluripotent properties of the cells after vitrification using the reduced CPA.

6. Outlook

Although the use of QMC can significantly reduce the required CPA concentration for ES cell vitrification from 4-7 M to 2.5 M, it is desired to further decrease the CPA concentration to ≤ 1.5 M which is usually used for slow-freezing. Therefore, it is of great interest and importance to further test the efficacy of the low-CPA vitrification technique in preserving the more stress sensitive ES cells by encapsulating the ES cells in small alginate microcapsules. The other advantage of microencapsulating the ES cells for vitrification is that the microencapsulated ES cells can be used directly for transplantation *in vivo*, provided that the wall permeability of the microcapsules is low enough to exclude immunoglobulin and other immunological factors from getting into the microcapsules to kill the encapsulated cells. The latter allows the use of non-autologous cells for the treatment of diseases, which significantly expands the capability of the ES cell-based medicine. Ultimately, it is important to achieve lyopreservation of ES cells at ambient temperature to allow convenient and wide distribution of the ES cell-based medicine to end users (just like what we are doing with lyophilized pharmaceutical drugs today), particularly those in remote areas. However, no successful and consistent lyopreservation of mammalian cells (not to mention ES cells) has been reported in the literature. Hopefully, with the advances of modern nanotechnology for the intracellular delivery of small hydrophilic molecules (disaccharide such as trehalose) and our understanding on anhydrobiotism in nature and the biophysics of freeze-drying and evaporative drying, lyopreservation of ES cells can be realized in the near future.

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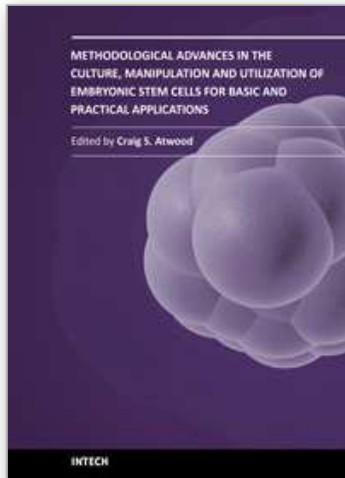
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