

Chapter 12

# Vitrification: Preservation of Cellular Implants

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## Summary

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The emerging field of tissue engineering has identified product storage as a significant obstacle for commercialization of products containing living cells. Traditionally employed methods of tissue banking utilize cryopreservation by freezing. Unfortunately, cryopreservation by freezing may result in loss of tissue function and viability by several recognized mechanisms of which ice formation is the most significant. An alternative to freezing is cryopreservation by vitrification. Vitrification is the solidification of a liquid without crystallization. The basic principals of vitrification and progress in application of vitrification to tissue preservation are reviewed with emphasis on recent successes in preservation of cardiovascular tissues and cartilage. For the future, heat transfer issues during cooling and warming and minimization of devitrification and ice growth by recrystallization during rewarming appear to be the primary hurdles for scaling up from relatively small cartilage specimens and cardiovascular grafts to larger tissues and organs.

**Keywords:** Cardiovascular Implants, Cryopreservation, Freezing, Low Temperature Biology, Musculoskeletal Implants, Preservation, Tissue(s), Tissue Engineering, Transplants, Vitrification.

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## Introduction

The urgent and growing need for improved methods of cryopreservation for viable tissue engineered products has stimulated discussion and debate in the literature regarding the relative merits of traditional freezing methods versus approaches involving ice-free vitrification (Fig. 1). Prevention of freezing by vitrification means that the water in a tissue remains liquid during cooling. Vitrification is the solidification of a liquid without crystallization. As cooling proceeds, however, the molecular motions in the liquid permeating the tissue decrease. Eventually, an "arrested liquid" state known as a *glass* is achieved. It is this conversion of a liquid into a glass that is called vitrification (derived from *vitri*, the Greek word for glass). A glass is a liquid that is too cold or viscous to flow. A vitrified liquid is essentially a liquid in molecular stasis. Vitrification does not have any of the biologically damaging effects associated with freezing because no appreciable degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biological systems.

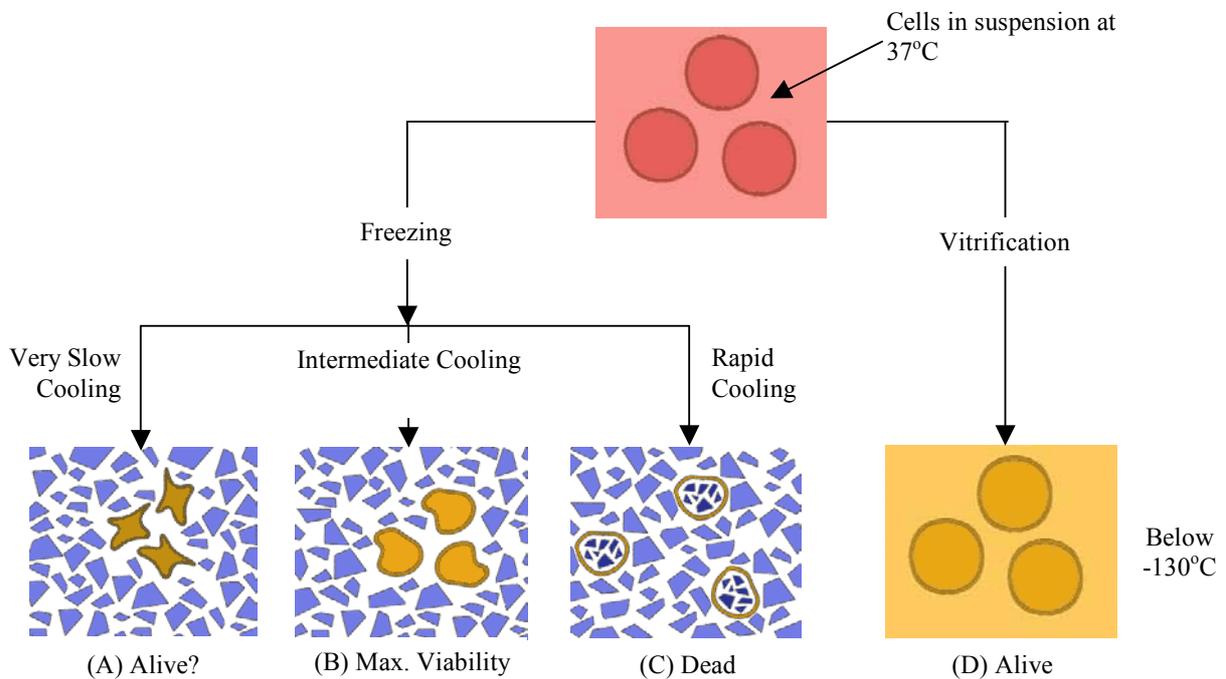


Fig. 1: Comparison of cryopreservation using vitrification or freezing strategies for cells in suspension. If the cells are cryopreserved by freezing (left side) ice forms initially in the extracellular environment and the cells undergo cooling rate dependent shrinkage due to osmotic dehydration. The slower the cooling rate the longer intracellular water has the opportunity to move out of the cell by osmosis due to the increasing osmolality of the extracellular environment as water is incorporated into ice crystals. The cells also become concentrated at slower cooling rates as they are pushed together by the forming ice (A, B). Maximum cell viability is usually achieved at an intermediate cooling rate (B) that balances osmotic dehydration and the risk of intracellular ice formation. Rapid cooling (C) permits intracellular ice formation and usually leads to cell death upon rewarming. Very slow cooling (A) may lead to excessive cell dehydration and cell death. In contrast, right side (D), cells cryopreserved by vitrification undergo neither ice formation nor shrinkage due to dehydration and most of the cells should be viable (70).

Vitrification has been shown to provide effective preservation for a number of cells, including monocytes, ova and early embryos and pancreatic islets (1-4). We review here some of the basic physico-chemical and biophysics principles of vitrification and progress on the extension of vitrification from single cells and cell aggregates to more complex structured tissues.

Cryopreservation by vitrification versus conventional freezing is illustrated using articular cartilage as a model. Despite decades of research articular cartilage has proved refractory to satisfactory cryopreservation using conventional freezing methods. Therefore, cartilage was selected as an ideal model to test the hypothesis that vitreous cryopreservation, in which the formation of extracellular ice is inhibited or prevented, will result in significantly improved preservation.

Vitrification and freezing (water crystallization) are not mutually exclusive processes, the crystalline phase and vitreous phase often coexist within a system. In fact, during conventional cryopreservation involving controlled freezing of cells, a part of the system vitrifies. This occurs because during freezing the concentration of solutes in the unfrozen phase increases progressively until the point is reached when the residual solution is sufficiently concentrated to vitrify in the presence of ice. Conventional cryopreservation techniques are optimized by designing protocols that avoid intracellular freezing (Fig. 1). Under these cooling conditions the cell contents actually vitrify due to the combined processes of dehydration, cooling and the promotion of vitrification by intracellular macromolecules. However, the term, vitrification, is generally used to refer to a process in which the objective is to attempt to vitrify the whole system from the outset such that any ice formation (intracellular and extracellular) is avoided (5-10).

### *Physico-chemical Basis of Vitrification*

During the low temperature preservation of biological systems events rarely take place under true equilibrium conditions. Many interdependent factors determine whether an aqueous system, such as a biological system, approaches the thermodynamic state of lowest free energy during cooling. Metastability is thus often unavoidable, especially in concentrated systems. Such non-equilibrium states are, however, sufficiently reproducible and permanent to have been described as pseudo-equilibrium states and conversion of such metastable thermodynamic states to more stable forms may be subject to large kinetic barriers. The prevalence of so-called "unfreezable" or "bound" water

in the vicinity of macromolecules is a prime example, where the expected path of thermodynamic stabilization by way of crystallization is prevented by large kinetic restraints (11-13). A clear understanding of the occurrence and effects of metastable states during the cooling of compartmentalized living systems is complicated by the interaction of thermodynamic and kinetic factors. Some basic principles have been established with the aid of aqueous solutions of cryoprotective solutes and other macromolecules that interact with water by hydrogen bonding (14, 15). Phase diagrams have proved to be a useful tool in understanding the physico-chemical relationship between temperature, concentration and change of phase (Fig. 2). For detailed discussion of the role and interpretation of solid-liquid state diagrams in relation to low temperature biology please refer to the review by Taylor (13). In particular, *supplemented* phase diagrams that combine non-equilibrium data on conventional equilibrium phase diagrams serve to depict the important transitions inherent in cooling and warming aqueous solutions of cryoprotective solutes (Fig. 2).

The equilibrium melting temperature, labeled  $T_m$ , is often described as the liquidus curve and represents points at which a solution having a particular concentration will melt (or freeze) under equilibrium conditions of temperature change. Hence this curve represents the phase change boundary for the two-component solution as a function of temperature. Cooling a solution below the liquidus curve will result in ice formation if the conditions are favorable for nucleation with the result that the remaining liquid phase becomes more concentrated in the solute as defined by the curve. In practice freezing is rarely initiated at the liquidus point. Solutions tend to undercool to varying degrees before significant nucleation and ice crystal growth occur. *Heterogeneous nucleation* occurs in water at temperatures above  $-38.5^\circ\text{C}$  and it is usually catalyzed by the surfaces of particulate impurities that act as seeds for crystal growth. Pure samples of water will self-nucleate at the *homogeneous nucleation* temperature ( $T_h$ ),  $-38.5^\circ\text{C}$  (13). Both heterogeneous and homogeneous nucleation temperatures decrease with increasing dissolved solute concentration (Fig. 2).

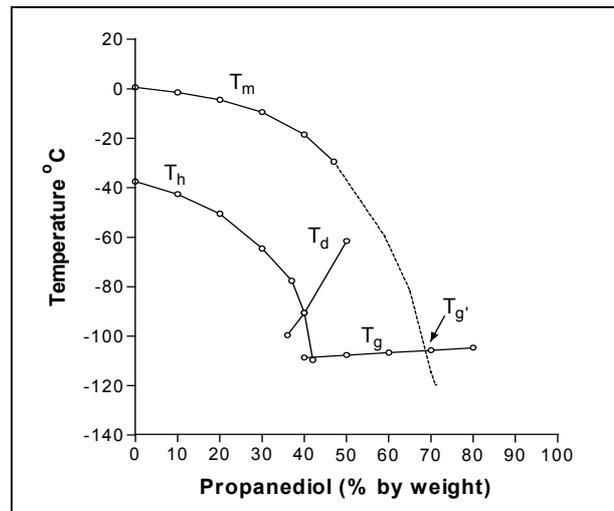


Fig. 2: Supplemented phase diagram for propanediol in water. Data derived from DSC thermograms showing the principal events and phase changes associated with cooling and heating. A supplemented phase diagram combines non-equilibrium data on a conventional equilibrium phase diagram and serves to depict the important transitions inherent in cooling and warming aqueous solutions of cryoprotective solutes. ( $T_m$  = equilibrium melting temperature,  $T_h$  = homogeneous nucleation temperature,  $T_d$  = devitrification temperature,  $T_g$  = glass transition temperature). Redrawn from the data of Macfarlane *et al.* (16, 18, 19).

The phase diagram for propanediol (Fig.2) shows that in the region of 0-35% freezing will occur at some point 5-20°C below  $T_m$ , invariably by heterogeneous nucleation. At sufficiently high concentrations and low temperatures the kinetics of the process become so slow that  $T_h$  is difficult to detect and any nucleated crystals that form in the region of  $T_h$  remain microscopic. As temperature is lowered further molecular motion is slowed to the point where translational and rotational molecular motion is essentially halted and the system is trapped in a high energy state that resembles a liquid-like configuration, or a vitreous glass (16). This glass transition is associated with a marked change in physical properties such as specific heat and refractive index and certain mechanical properties such that the glass transition temperature ( $T_g$ ) can be clearly identified. Determination of the transition temperatures that provide data for the construction of supplemented phase diagrams is usually derived from thermograms generated using differential scanning calorimetry (DSC) or the related technique differential thermal analysis (DTA) (13). A DSC derived thermogram for a complex solution of cryoprotectants in water is presented in Fig. 3. The kinetic nature of these transitions means that  $T_g$  has to be defined with reference to a particular set of experimental conditions.

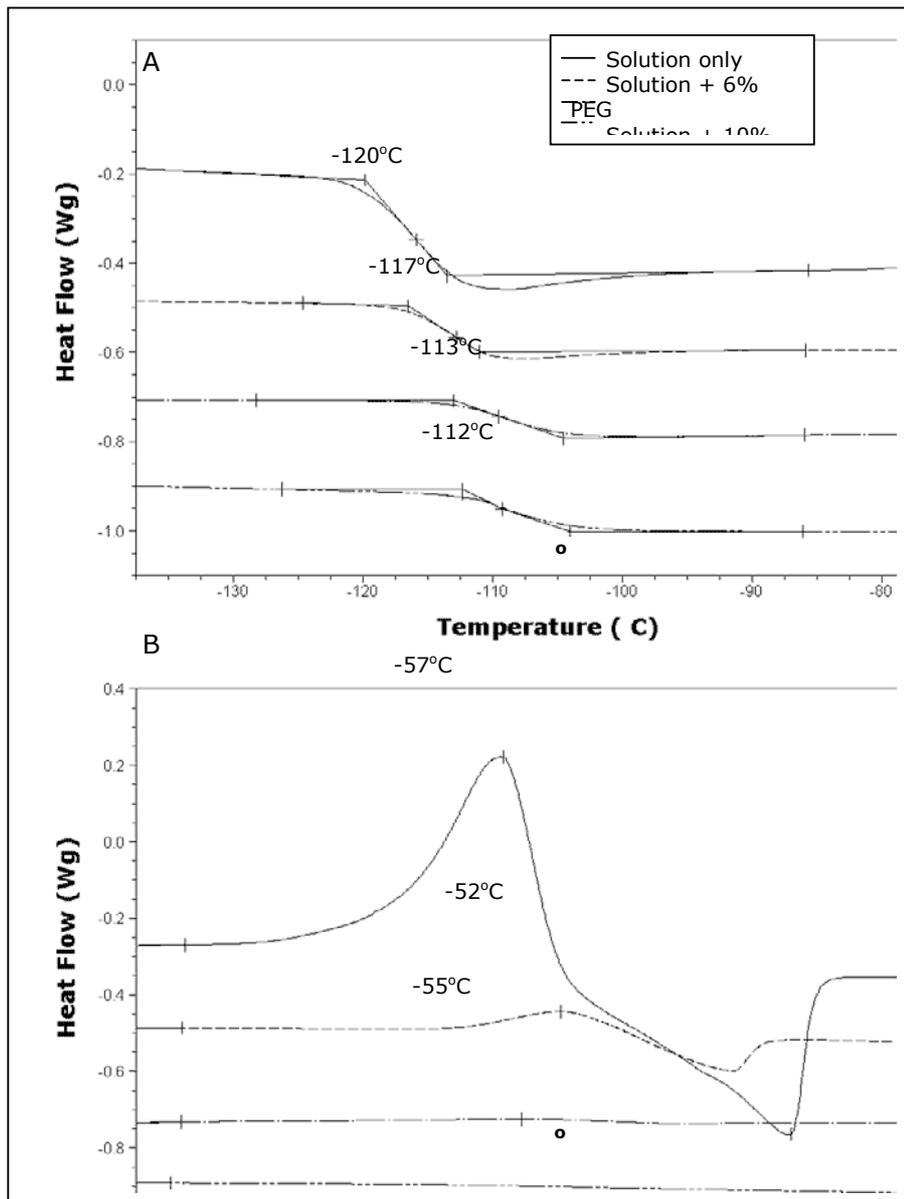


Fig. 3: Representative thermograms illustrating the influence of polyethylene glycol 400 concentration on the glass transition (A) and peak devitrification (B) temperatures of EuroCollin's solution containing of 3.0 M DMSO and 3.0 M Propanediol. PEG400 elevated the glass transition temperature (A), reduced the energy associated with the transition (A) and reduced the risk of devitrification (B) in a concentration dependent manner.

Reference to Fig 2 shows that in the region of 35-40% PG it is possible to cool samples through the  $T_h$  curve without apparent freezing and form what have been referred to as *doubly unstable glasses* (17). This term reflects the high probability that the vitreous system contains ice nuclei and if warming is not sufficiently rapid further nucleation and crystallization will occur. This event is known as devitrification and is depicted on the supplemented phase diagram as  $T_d$ . Hence, during cooling the sample attains the glassy state but it invariably contains ice nuclei the growth of which is arrested along with all other molecular motions in the sample. However, upon rewarming crystallization can be detected, either visibly or by an exothermic event in a thermogram (Fig. 3), reflecting the growth of ice by devitrification (transition of glassy to crystalline state) and recrystallization (growth of existing ice crystals) (13). The phenomenon of crystallization on warming a glassy sample to temperatures in the vicinity of  $T_g$  is often referred to as devitrification of a doubly unstable glass since it is unstable with respect to both the liquid and solid states (17, 18). Hence, the process by which a metastable glass, or supercooled liquid obtained by heating the glass above its glass transition temperature, forms the stable crystalline phase is generally referred to as devitrification (18).

In the higher concentration range of 41-50% for propanediol (Fig. 2) the  $T_h$  curve meets  $T_g$  and in this region it is possible to slowly cool even bulk liquids directly to  $T_g$  without experiencing any detectable freezing events and devitrification can be avoided by using moderate warming rates. Devitrification ceases to be detectable at concentrations above 50% even at low warming rates and the system can be regarded as stable (Fig. 2). The intersection of the melting curve and the glass transformation curve at  $T_g'$  indicates the minimum concentration of propanediol in aqueous solution that will vitrify irrespective of cooling rate. The concentration at which a glass transition occurs varies according to the nature of the solute. It appears that those systems with the strongest solute-solvent hydrogen bonding provide the best suppression of ice nucleation and promote vitrification (16, 19).

### ***Vitreous Stability***

Stability of the vitreous state is critical for the retention of vitrified tissue integrity and viability. Comprehensive studies of vitreous stability for a variety of potentially important cryoprotective mixtures have been made (20). Glass stability of vitrified blood vessel samples stored in vapor phase liquid nitrogen storage with retention of smooth muscle function has been demonstrated up to 4

months of storage (21). The stability of the amorphous state has been defined empirically in terms of the critical heating rate,  $V_{cr}$ , above which there is insufficient time for a vitreous sample to crystallize before  $T_m$  is reached. The smaller the value of  $V_{cr}$  the more stable the amorphous state. The dependence of  $T_d$  on the rate of warming can be measured and  $T_m - T_d$  has been used to define the stability of the vitreous state (20, 22). The warming rate for which  $T_m - T_d$  is zero is defined as the  $V_{cr}$  for which the supercooled mixture neither devitrifies nor recrystallizes. Such studies have shown that stability of glasses formed from aqueous solutions of 1,2-propanediol are much greater, for the same water contents, than for all other solutions of commonly used cryoprotectants including, glycerol, dimethyl sulfoxide, and ethylene glycol. Unfortunately solutions of polyalcholic CPAs such as propanediol and butanediol that show the most promise in terms of cooling rates and concentrations necessary for vitrification, also required unrealistically high heating rates to avoid devitrification. Moreover, due principally to isomeric impurities that form a hydrate at reduced temperatures, 2,3-butanediol has proved to have an unanticipated biological toxicity at concentrations below that necessary for vitrification (23-26). Despite developments to devise solutions that would vitrify at practically attainable cooling rates for sizeable biological tissues, the corresponding critical warming rate necessary to avoid devitrification remains a critical challenge. Conceptually, elevated pressures (27), electromagnetic heating (28-30, 30) the use of naturally occurring antifreeze molecules (31), and synthetic ice blockers (32) have been proposed as means to tackle the problem.

## ***Importance of Vitrification for Tissue Preservation***

Advances in biostabilization and low temperature biology have produced high viability preservation technologies for cells and tissues in the areas of hypothermic storage, cryopreservation by freezing and vitrification, as well as anhydrobiotic preservation (33). However, the development of preservation methods is not straightforward. Process development requires the optimization of chemical and thermal treatments to achieve maximal survival and stability. In a recent editorial (34) the need for ice-free cryopreservation methods was emphasized. The consensus opinion was that viable tissues such as blood vessels, corneas and cartilage that have proven refractory to

cryopreservation by conventional freezing methods, despite decades of intense research by many investigators, can only be successfully preserved if steps are taken to prevent or control the ice that forms during cooling and warming. Mathematical modeling may ultimately improve our ability to optimize freezing procedures for tissues (35), but has not yet contributed to significant advances. Our laboratory has developed a cryopreservation approach using vitrification, which thus far has demonstrated >80% preservation of smooth muscle cell viability and function in cardiovascular grafts (36, 37).

<b>Tissue Type</b>	<b>Survival Outcome</b>	
	Frozen (%)	Vitrified (%)
Jugular vein (50, 51)	6-22	84-87
Carotid artery (52)	< 30	> 80
Tissue Engineered Blood Vessels (53)	10.7	82.7

Table 1: Vascular tissue functions after either frozen or vitrified cryopreservation and storage.

Avoidance of ice by vitrification can be generally achieved with one of two approaches or a combination of both. The first approach employs cooling highly concentrated solutions (typically >50% w/w) that become sufficiently viscous at low temperatures to suppress crystallization rates. Typically, a vitrified material is considered solid when the viscosity reaches  $10^{15}$  poise (11). Vitrification can also be achieved by selecting sufficiently high cooling rates to prevent ice crystallization in relatively dilute solutions (<50% w/w). This second approach generally produces a metastable state that is at risk of devitrification (recrystallization) during warming. Ice formation during warming is just as potentially injurious as during cooling.

The tissue vitrification technology described here employs >50% w/w cryoprotective agents. The formulation and method was licensed from the American Red Cross, where it was intended for organ preservation (38, 39). However, even though rabbit kidneys were vitrified they could not be

rewarmed. Viability was lost due to ice formation upon rewarming by the process outlined above. The rewarming of vitrified materials requires careful selection of heating rates sufficient to prevent significant thermal cracking, devitrification and recrystallization during heating. The use of carefully designed warming protocols is necessary to maximize product viability and structural integrity. Vitrified materials, which may contain appreciable thermal stresses developed during cooling, may require an initial slow warming step to relieve residual thermal stresses. Dwell times in heating profiles above the glass transition should be brief to minimize the potential for devitrification and recrystallization phenomena. Rapid warming through these temperature regimes generally minimizes prominent effects of any ice crystal damage. It is presently not possible to rewarm organs rapidly enough due to their high volume relative to the volume of tissues. Development of optimum vitrification solutions requires selecting compounds with glass-forming tendencies and tolerable levels of toxicity at the levels required to achieve vitrification. Due to the high total solute concentration within the solution, stepwise protocols should be used at low temperatures for the addition and removal of cryoprotectants to limit excessive cell volume excursions and lower the risk of cytotoxicity. For a current comprehensive review of vitrification see Taylor *et al.* (32).

In addition to *in vitro* studies of cardiovascular tissues, transplant studies have been performed that demonstrate normal *in vivo* behaviour of vitrified tissues (36, 37, 40). More recently we have discovered that vitrification decreases the rate of calcification observed in subcutaneously implanted frozen rat heart valves (41), suggesting that vitrification may have other benefits for tissues in addition to increasing cell viability and tissue functions.

More recently we have extended our vitrification studies to musculoskeletal tissues. Although, fresh osteochondral allografts have proven to be effective and functional for transplantation, the limited availability of fresh allograft tissues necessitates the use of osteoarticular allograft banking for long-term storage (42-45). Conventional cryopreservation by means of freezing is currently a preferred method for storing tissue in general until needed, however, such protocols result in death of 80-100% of the chondrocytes in articular cartilage plus extracellular matrix damage due to ice formation. These detrimental effects are major obstacles preventing successful clinical utilization of osteochondral allografts (44, 46, 47) and commercial success of tissue-engineered cartilage constructs. Consequently, the search has continued for a better preservation method. The method we have employed for vitrification of articular cartilage is outlined below as an example of a

vitrification protocol. This method is contrasted with the method (48) that we have employed for cryopreservation by freezing of cartilage in Fig. 1 and Table 2.

**Example: Protocol for cryopreservation of cartilage by vitrification**

Full thickness cartilage specimens (0.6 mm deep) from New Zealand White rabbits femur-heads were gradually infiltrated with an 8.4M vitrification solution consisting of 3.10M DMSO, 3.10M formamide and 2.21M 1,2-propanediol in EuroCollins solution at 4°C (36, 37). Precooled vitrification solution (4°C) were added in six sequential, 15-minute steps. After addition of the final vitrification solution, the specimens were placed in glass scintillation vials (Dia. x H, 25X60 mm) containing 2 ml of the pre-cooled vitrification solution. The top of the vitrification solution was then covered with 0.7 ml of 2-methylbutane (isopentane, freezing point: -160°C, density: 0.62) at 4°C to prevent direct air contact. A thermocouple was inserted into a separate dummy sample of the same vitrification solution and its output monitored via a digital thermometer throughout the cooling process. Samples were cooled rapidly (43°C/min) to -100°C, followed by slow cooling (3°C/min) to -135°C, and finally storage in a freezer at -135°C for a minimum of 24 hours. Vitrified specimens were rewarmed in two stages, first, slow warming to -100°C (30°C/min) and then rapid warmed to melting (225°C/min). After rewarming, the vitrification solution was removed in a stepwise manner.

	<b>Freezing</b>	<b>Vitrification</b>
Cryoprotectants	1M DMSO 2.5% Chondroitin Sulphate	3.1M DMSO 2.2M 1,2-propanediol 3.1M Formamide
Cooling Rate	-1°C/min to -80°C	-40°C/min to -100°C -3°C/min to -135°C
Storage	Vapor phase nitrogen	
Warming	Rapid	
Cryoprotectant Removal	3 steps	6 steps

Table 2: Cryopreservation Methods

### Cartilage Vitrification

Cryosubstitution studies of frozen and vitrified articular cartilage revealed negligible ice in the vitrified specimens (Fig. 4), and extensive ice formation, both in the extracellular matrix and deeper lacunae, in frozen specimens (Fig. 4). Some cell shrinkage was observed in the lacunae of vitrified specimens (Fig. 4), which is most likely related to high cryoprotectant concentrations. The presence and absence of ice are better appreciated in representative electron micrographs of frozen (Fig. 5) and vitrified, cryosubstituted (Fig. 5) cartilage samples.

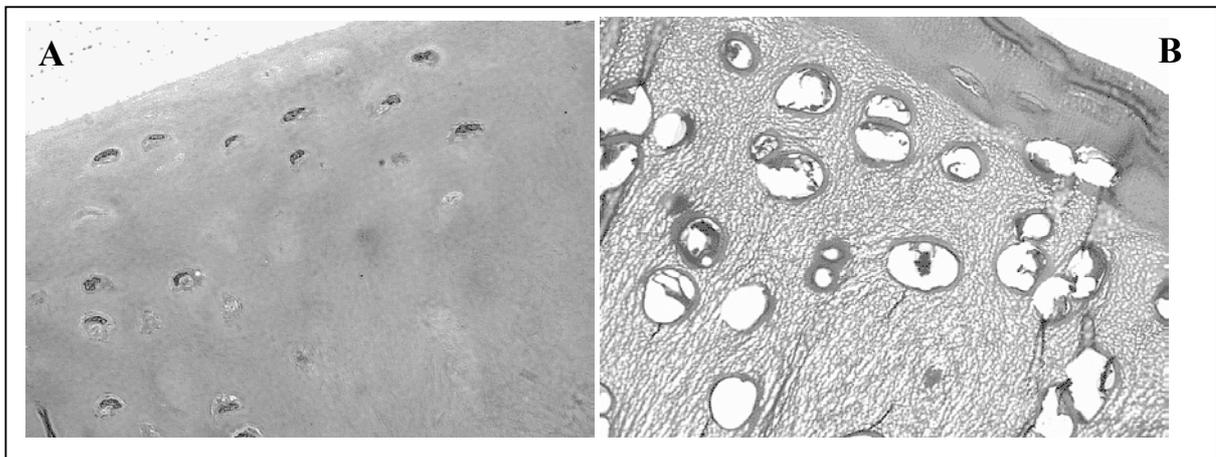


Fig. 4: Light microscopic comparison of frozen and vitrified cartilage specimens that were cryosubstituted during low temperature storage. The vitrified specimen (A) is free of ice. In contrast the frozen specimen (B) demonstrates extensive ice in both the lacunae and in the extracellular matrix. Cells on the outermost edge (top, right side) of the frozen cartilage specimen are free of ice. Cryosubstitution is a method for demonstrating the presence or absence of ice during cryopreserved storage (71). Cryosubstitution was performed using chilled (-90°C) 1% osmium tetroxide in 100% methanol in high density polyethylene scintillation vials containing cryopreserved specimens -90°C. The tissues were dehydrated by replacing the frozen or vitrified water with cryosubstitution medium over a period of several days at -90°C. The heat-sink and vials were then placed in a -20°C freezer overnight, followed by 4°C for one hour, and then finally brought to room temperature. This gradual warming of the tissue and cryosubstitution media assures complete osmium tetroxide tissue fixation. Finally, these tissues were transferred to 100% acetone, infiltrated with araldite resin and polymerized, sectioned, stained and viewed by light microscopy.

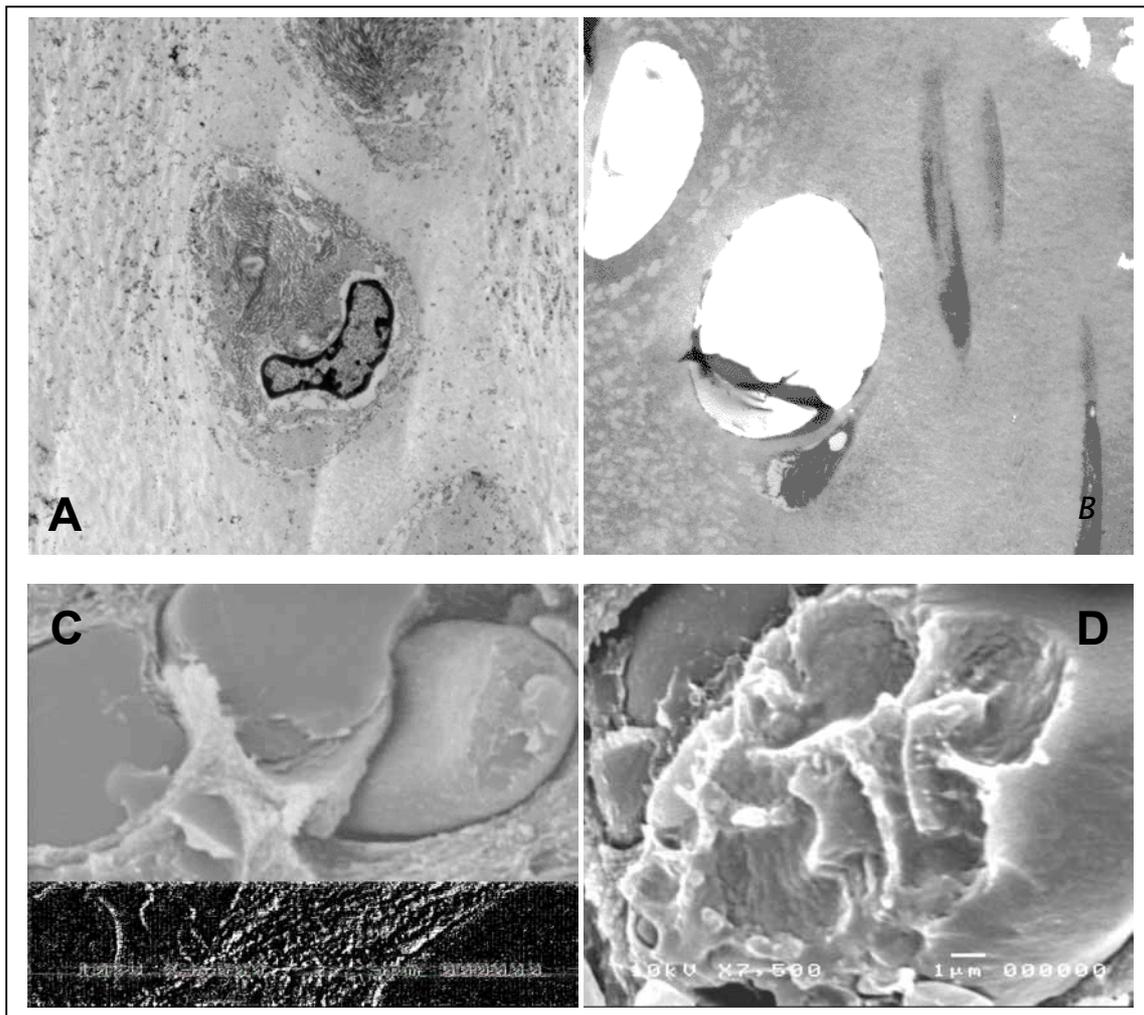


Fig. 5: Transmission and scanning microscopic comparison of frozen and vitrified cartilage specimens that were cryosubstituted during low temperature storage. Both the transmission electron and scanning electron micrographs (A & C), illustrate the absence of cellular material in the articular cartilage lacunae. In contrast, the cells are still present in the lacunae of vitrified articular cartilage specimens (B & D). Specimens were cryosubstituted as indicated in the legend for fig. 4. Tissue for transmission electron microscopy was dehydrated in acetone and gradually infiltrated with an araldite epoxy resin and embedded, and polymerized for 18 hours at 60° C. Sections (75nm) were cut using a diamond knife with a Reichert OMU2 ultramicrotome. The sections were then double stained using uranyl acetate followed by lead citrate. The sections were viewed in a JEM-1210 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 80kV accelerating voltage. For scanning electron microscopy three changes of 10ml Hexamethyldisilizane were used in the drying process. The tissue was then oriented on a specimen stub and coated with 20nm of gold/palladium using an ion-sputter coater. The samples were viewed using a JSM 5410 scanning electron microscope (JEOL USA Inc., Peabody, MA) operated at 10kV accelerating voltage.

Viability assessment of fresh and cryopreserved rabbit articular cartilage specimens demonstrated that oxidation-reduction in vitrified samples was approximately 85% of fresh samples (Fig. 6). Similar values have been obtained using live/ dead stains for assessment of cell viability (49). Transplantation studies have also been performed in rabbits to compare the performance of fresh, frozen and vitrified specimens. These studies demonstrated that vitrified cartilage performance was not significantly different to fresh untreated cartilage. In contrast, frozen cartilage performance was significantly different when compared to either fresh or vitrified cartilage (49). These studies combine to demonstrate that the vitrification process results in ice-free preservation of rabbit articular cartilage and that about 85% of cellular metabolic activity is retained following rewarming. Frozen tissues contained ice within the cells and the matrix, with the exception of the articular surface, where some viable cells were observed (Fig. 4).

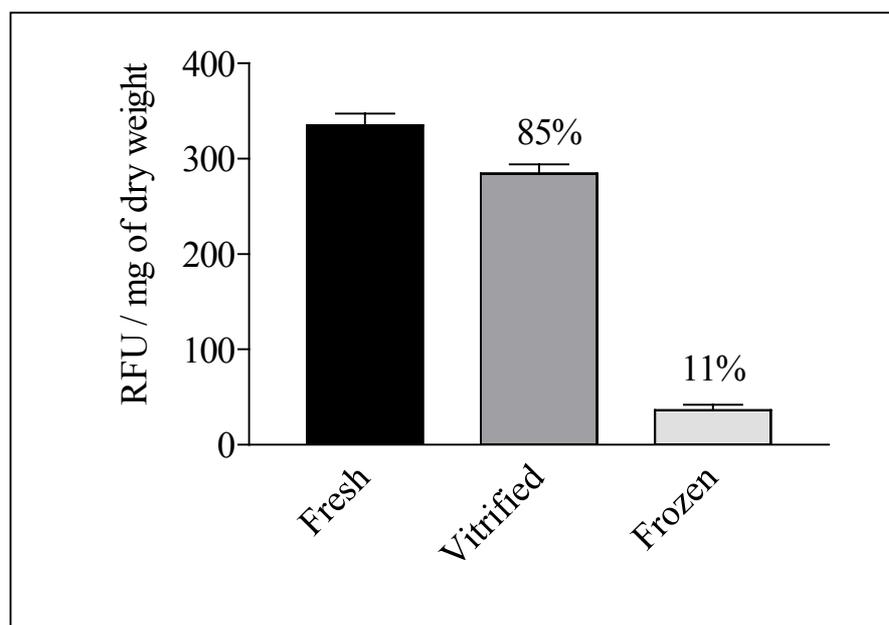


Fig. 6: Metabolic comparison of frozen and vitrified articular cartilage specimens after rewarming under tissue culture conditions. The alamarBlue™ assay was employed as a non-cell specific viability assay. This assay utilizes a water soluble fluorometric viability indicator based on the detection of metabolic activity, specifically, an oxidation-reduction (REDOX) indicator which both fluoresces and changes color in response to chemical reduction of the cell culture medium caused by cell metabolism. Aliquots of medium from tissue samples were incubated with alamarBlue™ working solution in microtiter plate wells and read on a microtiter plate spectrofluorometer at 590 nm. The data is expressed as the mean ( $\pm$ sem) of ten samples of articular cartilage assayed using alamarBlue™. Viability is expressed as relative fluorescence units (RFU) relative to the dry weight of each articular cartilage sample. Reproduced with permission from Taylor *et al.* (32).

We believe that the experimental data presented here makes a strong case for cryopreservation of cartilage by vitrification for osteochondral grafts. The baseline vitrification process, that has yet to be optimized, protected the cartilage from ice formation, demonstrated retention of cell metabolism and viability (49). These observations support the hypothesis that vitrification may be applicable to all biological systems.

Simple freezing of cells or tissues results in nonviable, nonfunctional materials. Little advance was made in the field of cryopreservation until Polge *et al.* (50) discovered the cryoprotective properties of glycerol. Subsequently, Lovelock and Bishop (51) discovered that dimethyl sulfoxide (DMSO) could also be used as a cryoprotectant. Since the discovery of these cryoprotective agents, cryoprotection during freezing and thawing of biological materials has become established and many other cryoprotectants have been identified that may play a role in cryopreservation by either freezing or vitrification methods (Table 3). Isolated chondrocytes in suspension, in common with many other cell types, can be preserved using conventional cryopreservation methods involving freezing. In such methods the cells may be concentrated and vitrified in channels between regions of extracellular ice (Fig. 1) however, chondrocytes embedded in their natural matrix are extremely difficult to preserve by similar freezing methods, presumably because the cells can not move away from forming extracellular ice.

Small molecular compounds	Permeable (e.g. DMSO, glycerol, 1,2-propanediol)
	Non-permeable (e.g. Sucrose, PVP)
Used in slow rate freezing and vitrification	
Protect cells via several mechanisms	Displacement of freezable water
	Suppression of high electrolyte concentration
	Used to control frozen fraction of water

Table 3: Cryoprotective Agents

Studies using a variety of animal articular cartilage models (43, 44, 52, 53) and human cartilage biopsies (46) have revealed no more than 20% chondrocyte viability following conventional cryopreservation procedures employing either DMSO or glycerol as cryoprotectants. Ohlendorf *et al.* (44) used a bovine articular cartilage, osteochondral plug model to develop a clinical cryopreservation protocol. This protocol employed slow rate cooling and 8% DMSO as the cryoprotectant. They observed loss of viability in all chondrocytes except those in the most superficial layer at the articular surface. Muldrew *et al.* (52) previously investigated chondrocyte survival in a similar sheep model. These researchers observed cells surviving post-cryopreservation close to the articular surface and deep at the bone/cartilage interface. The middle layer was devoid of viable cells. More recently, Muldrew *et al.* demonstrated improved results using a step-cooling cryopreservation protocol, but cell survival post-transplantation was poor and again there was significant loss of cells in the mid-portion of the graft (54). The reason for lack of cell survival deeper than the superficial layers of articular cartilage is most likely multifactorial and related principally to heat and mass transfer considerations (55). Surface cells freeze and thaw more rapidly than cells located deep within the matrix. This phenomenon could result in a greater opportunity for ice to form, both within cells and in the extracellular matrix, deeper within the articular cartilage. Furthermore, typically employed concentrations of DMSO (8-20%) may not penetrate adequately to limit intracellular ice formation. Recent data from Jomha *et al.* (56) demonstrated that increasing DMSO concentrations to 6M can result in higher overall cell survival (40%) after cryopreservation. These observations suggest that use of higher DMSO concentrations results in better penetration of the DMSO in to the cartilage.

We are aware that other factors, in addition to ice formation, may have biological consequences during freezing procedures. Two of these factors are the inhibitory effects of low temperatures on chemical and physical processes, and, perhaps more importantly, the physiochemical effects of rising solute concentrations as the volume of liquid water decreases during crystallization. This latter process results in a decrease in cell volume and the risk of solute precipitation. Several hypotheses have been published on mechanisms of freezing-induced injury based upon such factors (55, 57), but our own experiences with mammalian tissues concur with others that the principal disadvantage of conventional cryopreservation revolve primarily around ice formation (36, 37, 39, 58-60).

Furthermore, cryopreservation by vitrification of tissues offers several important advantages compared with procedures that allow or require ice formation. First, complete vitrification eliminates concerns for the known damaging effects of intra- and extracellular ice crystallization. Secondly, tissues cryopreserved by vitrification are exposed to less concentrated solutions of cryoprotectants for shorter time periods. For example, Rall (8) has calculated for embryos that during a typical cryopreservation protocol involving slow freezing to  $-40^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  the cells are exposed to cryoprotectant concentrations of 21.5 and 37.6 osmolal respectively. In contrast, cells dehydrated in vitrification solutions are exposed for much shorter periods to  $<18$  osmolal solution, although the temperature of exposure is higher. Finally, unlike conventional cryopreservation procedures that employ freezing, vitrification does not require controlled cooling and warming at optimum rates. A principle benefit of vitrification is the elimination of requisite studies to determine optimal cooling rates for tissues with multiple cell types. Successful vitrification, requires that the thermal processing be rapid enough to transition regions of maximal ice crystal nucleation and growth that occur above the glass transition temperature of the solution. Thus, it is only necessary to cool solutions at rates in which a negligible fraction of the solution forms ice (typically  $< 0.2\%$ ) (61). Vitrified materials have a similar rate requirement during heating, when samples are rewarmed for subsequent use, to limit ice formation to negligible levels (typically  $<0.5\%$ ) (62). A major objective of our current research is the development of non-toxic vitrification formulations that have a minimal risk of ice formation during cooling and warming of bulk samples.

## ***Future Perspectives in The Application of Vitrification to Tissues***

There are a number of significant challenges for deployment of vitrification methods for tissue engineered medical products. Vitrification approaches to preservation has some of the limitations associated with conventional freezing approaches. First, both approaches require low temperature storage and transportation conditions. Neither can be stored above their glass transition temperature for long without significant risk of product damage due to inherent instabilities leading to ice formation and growth. Both approaches employ cryoprotectants with their attendant problems and require competent technical support during rewarming and cryoprotectant elution phases. The high

concentrations of cryoprotectants necessary to facilitate vitrification are potentially toxic because the cells may be exposed to these high concentrations at higher temperatures than in freezing methods of cryopreservation. Cryoprotectants can kill cells by direct chemical toxicity, or indirectly by osmotically-induced stresses during suboptimal addition or removal. Upon completion of warming, the cells should not be exposed to temperatures above 0°C for more than a few minutes before the glass-forming cryoprotectants are removed. It is possible to employ vitrified products in highly controlled environments, such as a commercial manufacturing facility or an operating theater, but not in a doctor's outpatient office, or in third world environments. Formamide, one of the components of VS55, is a known mutagen. Alternatives to formamide with less safety risks and potentially easier clinical acceptance are being sought. However, the cytotoxicity of complex cryoprotectant formulations containing formamide is surprisingly much less than the cytotoxicity of single component formulations at the same concentrations.

Heat transfer issues are the primary hurdle for scaling up the successes in relatively small tissue specimens to larger tissues and organs. The limits of heat and mass transfer in bulky systems result in non-uniform cooling and contribute to stresses that may initiate cracking. In fact, the higher cooling rates that facilitate vitrification will typically lead to higher mechanical stresses. Macroscopic scale instability manifested as cracking or fractures was first reported during the vitrification of glycerol solutions (63). Fractures may also provide an interface for nucleation that can initiate devitrification (64). Very little information on the material properties of vitreous aqueous solutions exists. Material properties such as thermal conductivity and fracture strength of vitreous aqueous solutions have many similarities with their inorganic analogues that exist at normal temperatures e.g. window glass and ceramics. Any material that is unrestrained will undergo a change in size (thermal strain) when subjected to a change in temperature. Materials in general, and tissues as they are cryopreserved in particular, shrink when they are brought from physiological temperature down to lower temperatures. Extensive testing is necessary to determine how the thermal strain (shrinkage) of a material depends on the temperature and possibly on the rate at which the temperature is changed. Under these unrestrained conditions when thermal expansion or contraction is free to occur, the stress remains zero; that is, no forces act on the material. As tissues are cryopreserved, they are externally free to shrink. However, in practice it is impossible to cool a tissue uniformly. The outer surface decreases in temperature more rapidly than the inside. The outside of the tissue is forced to shrink less and the inside to shrink more. The stresses that arise to

accommodate the differential shrinkage are dependent on the stiffness and relaxation of the material. If these stresses are too severe fractures may occur.

Calculations of stress in frozen biological tissues have shown that thermal stress can easily reach the yield strength of the frozen tissue resulting in plastic deformations or fractures (65-67). The driving mechanism of thermal stress is the constrained contraction of the frozen or vitrified tissue. It is commonly assumed that thermal expansion of frozen biological tissues is similar to that of pure water ice crystals (65, 68), and Rabin *et al.* (69) have confirmed this experimentally. Moreover, their studies provided some preliminary insight with regard to the effect of the presence of cryoprotectants on the thermal expansion. Results of pilot expansion tests of rabbit muscle permeated by the cryoprotectants DMSO and glycerol solutions, and pig liver perfused with DMSO solution indicated that the cryoprotectants dramatically reduced the thermal expansion at higher temperatures and created a maximum value of thermal expansion within the temperature range of -70 to -100°C. Rabin (personal communication) has preliminary data indicating that tissue thermal expansion decreases with increasing DMSO concentration that may be explained by a change in physical properties associated with a glass transition. The competing needs of vitrification and minimization of mechanical damage demand a greater understanding of both vitrification and stress development. Although mechanical stress has long been recognized as an important mechanism of tissue destruction, it has received very little attention in the context of cryobiology.

Another major hurdle for deployment of vitrification methods is the development of effective rapid warming techniques for larger specimens (greater than 10 ml volume) to prevent devitrification and ice growth by recrystallization. Microwave warming has been attempted but has never been successful due to the uneven warming of specimens and problems with thermal runaway which results in heat-denatured tissues. In 1990, Ruggera and Fahy (30) reported success in warming test solutions at rates of up to about 200°C/min using a novel technology based on electromagnetic techniques (essentially microwave heating). Unfortunately, unpublished results indicate that this method is also problematic due to the uneven warming of specimens and problems associated with thermal runaway. Others have taken a systematic approach to develop a dielectric heating device to achieve uniform and high rates of temperature change (28, 29). This has been achieved in some early model systems but application of this technology to the survival of cells and tissues has yet to be reported.

## **Acknowledgements**

Supported by U.S. Public Health Grants #HL66688, #HL59731 and AR47273. The authors would like to thank Elizabeth Greene and Kelsey K. Brockbank for their assistance in manuscript preparation and acknowledge Fred Lightfoot for preparation of the light and electron micrographs.

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