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Thermodynamic aspects of vitrification ☆

Brian Wowk *

21st Century Medicine, Inc., 14960 Hilton Drive, Fontana, CA 92336, USA

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ABSTRACT

Vitrification is a process in which a liquid begins to behave as a solid during cooling without any substantial change in molecular arrangement or thermodynamic state variables. The physical phenomenon of vitrification is relevant to both cryopreservation by freezing, in which cells survive in glass between ice crystals, and cryopreservation by vitrification in which a whole sample is vitrified. The change from liquid to solid behavior is called the glass transition. It is coincident with liquid viscosity reaching 10^{13} Poise during cooling, which corresponds to a shear stress relaxation time of several minutes. The glass transition can be understood on a molecular level as a loss of rotational and translational degrees of freedom over a particular measurement timescale, leaving only bond vibration within a fixed molecular structure. Reduced freedom of molecular movement results in decreased heat capacity and thermal expansivity in glass relative to the liquid state. In cryoprotectant solutions, the change from liquid to solid properties happens over a ~10 °C temperature interval centered on a glass transition temperature, typically near −120 °C (±10 °C) for solutions used for vitrification. Loss of freedom to quickly rearrange molecular position causes liquids to depart from thermodynamic equilibrium as they turn into a glass during vitrification. Residual molecular mobility below the glass transition temperature allows glass to very slowly contract, release heat, and decrease entropy during relaxation toward equilibrium. Although diffusion is practically non-existent below the glass transition temperature, small local movements of molecules related to relaxation have consequences for cryobiology. In particular, ice nucleation in supercooled vitrification solutions occurs at remarkable speed until at least 15 °C below the glass transition temperature.

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Introduction

Matter under ordinary conditions exists in one of three states: gas, liquid, or solid. When many liquids are cooled below a characteristic temperature, the melting temperature, crystallization into a solid becomes thermodynamically favored. A process of crystal nucleation followed by crystal growth converts the disordered molecular arrangement of a liquid into the ordered molecular arrangement of a solid crystal.

Some liquids are able to avoid crystallization as they are cooled far below their melting temperature. The likelihood of this occurring is increased if the liquid is viscous and if cooling occurs rapidly. The resulting supercooled liquid retains the physical properties of a liquid until another characteristic temperature, the glass transition temperature, is reached. As the temperature of a liquid decreases below the glass transition temperature, molecules remain in the disordered pattern of a liquid. However the physical properties become more similar to those of a rigid solid. Molecules become locked in place as though the liquid were frozen in time. The resulting "solid liquid" is called a glass. The process of becoming a glass by cooling is called vitrification (from *vitri*, the Greek word for glass).

Glasses are common in nature and technology. Window glass, a mixture of silicon dioxide and other mineral oxides, is perhaps the most well-known example of a glass. Some industrial metals and most plastics are amorphous (glassy) solids [29]. Some organisms and plant seeds survive desiccation with residual water bound in a glassy state [28,90,96]. Interestingly, most of the water in the universe may exist as glass rather than ice as a result of vapor deposition on dust grains in the coldness of space [53].

Vitrification is important to cryobiology because the natural state of liquid water inside living things is disordered. Maintaining the natural disorder of water molecules and dissolved solutes inside livings things during the solidification process of cryopreservation is advantageous from the standpoint of minimally disturbing the system being preserved.

Role of vitrification in freezing

Vitrification is now known to play a central role in cryopreservation of biological materials, even in cryopreservation by slow

☆ This work was funded by 21st Century Medicine, Inc., Rancho Cucamonga, California, USA.

* Fax: +1 909 466 8618.
E-mail address: wowk@21cm.com

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freezing. Cells survive freezing by confinement in unfrozen spaces between growing ice crystals [65]. Conversion of water into ice during cooling increases the concentration of solutes in the unfrozen solution around cells, which in turn increases the concentration of solutes inside cells by osmosis. As cooling and ice growth continue, eventually the solute concentration in and around cells becomes so high that water in the solution cannot freeze. Further cooling below the glass transition temperature results in vitrification of the cells and surrounding liquid.

Survival between ice crystals is facilitated by the addition of cryoprotectants such as glycerol or dimethyl sulfoxide at several times isotonic concentration before freezing. These solutes limit the amount of ice that must form for the unfrozen solution between crystals to be able to vitrify, and prevent salts from reaching harmful concentrations during cooling.

The path of cryoprotectant concentration as a function of temperature followed by cells between ice crystals during freezing is shown in Fig. 1. Long-term stability is attained when cells pass below the glass transition temperature of the protoplasm and residual unfrozen solution. Thus even frozen cells are preserved by vitrification.

Vitrification of whole samples

In cryobiology the term vitrification is reserved for cryopreservation methods in which the entire sample volume is vitrified. Although there was at least one earlier mention [42,83], the idea of cryopreserving cells within a volume rendered glassy by rapid cooling is generally credited to Luyet [62]. Beginning in 1937, Luyet worked for years to achieve vitrification by cooling small samples of cells very quickly, attempting to outrun the processes of ice nucleation and growth in solutions without cryoprotectants. (Cryoprotectants did not become widely known until 1949 [69].) It was eventually learned that solutions without added cryoprotectant could not be vitrified in practical volumes because vitrifying water required cooling rates on the order of 10^7 °C/min [7].

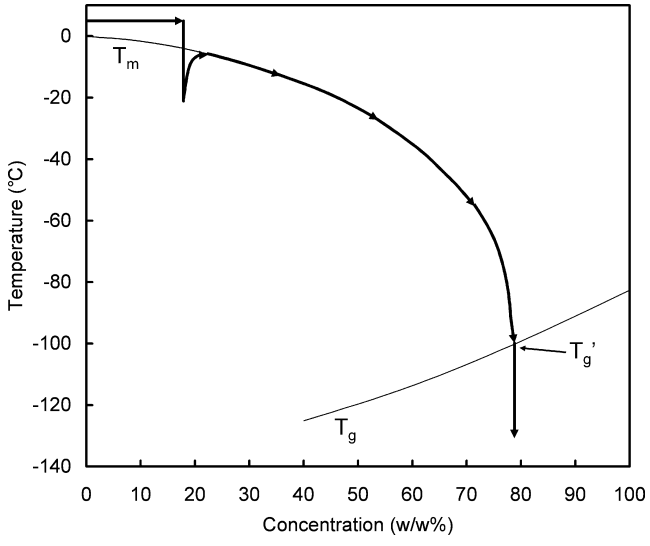

Fig. 1. Path followed on a glycerol–water phase diagram by solution between ice crystals during slow freezing, beginning with addition of cryoprotectant. T_m is the equilibrium melting temperature and T_g is the glass transition temperature. After recovery from initial supercooling, solutes concentrate during freezing so that the melting point of remaining unfrozen solution equals the solution temperature. Vitrification occurs between ice crystals when the temperature drops below T_g', the intersection of the T_m and T_g lines. (In practice the path may deviate slightly to the left of the T_m line at very low temperatures because the equilibrium melting line can only be followed exactly if the cooling rate is infinitesimal.)

In 1965 Farrant [43] and later Elford et al. [31–33] explored the idea of preserving tissue at low temperature by progressively raising cryoprotectant concentration while lowering the temperature to remain slightly above the melting point of the solution. This "Farranting" process mimicked the rise of cryoprotectant concentration that occurs between ice crystals during freezing, but without actual freezing. The main motivation for the approach was that it avoided harmful increases in salt concentration that occur when solutions freeze [86]. Experiments were performed cooling tissues down to a temperature of −79 °C in a liquid state with no ice. Farrant believed that cooling to lower temperatures would result in freezing [43]. In fact, had his samples been cooled below the glass transition temperature of the solutions used (∼−130 °C), they would have vitrified.

Rapatz and Luyet finally showed in 1968 that red blood cells could be cooled to liquid nitrogen temperature (−196 °C) without ice formation [72]. Freeze-fracture electron micrographs showed decreasing amounts of ice in samples with increasing concentrations of glycerol until there was no visible ice. Nevertheless, interest in vitrification for cryopreservation waned until the 1980s.

Non-equilibrium vitrification

The era of practical vitrification in cryobiology began with the publications of Fahy et al. in 1982 [36] and 1984 [37], followed by the first successful recovery of living nucleated cells (mouse embryos) from liquid nitrogen temperature in a wholly vitrified medium by Rall and Fahy in 1985 [71]. Fahy recognized that aqueous solutions with suitable concentrations of cryoprotectants could be vitrified by supercooling and rewarming at practical rates through the metastable non-equilibrium zone between the ice melting temperature, T_m, and the glass transition temperature, T_g, as shown in Fig. 2. Interestingly, crossing below T_m meant that the required cryoprotectant concentrations for vitrification by supercooling were actually lower than the concentrations seen by cells at the end of freezing, or even the concentrations used by Farrant to reach −79 °C. However the concentrations were still higher, and therefore more toxic, than those used at the beginning of freezing methods. Numerous details of cryoprotectant

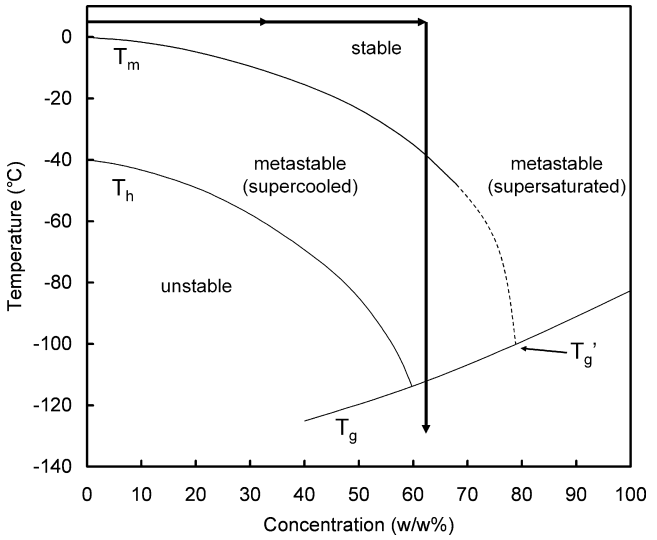

Fig. 2. Path followed on a glycerol–water phase diagram during non-equilibrium vitrification, beginning with addition of cryoprotectant. T_h is the homogeneous nucleation temperature. The T_m line is shown as a dotted line below the eutectic temperature. A sample is cooled rapidly through the metastable supercooled zone to avoid ice formation. Crystallization of solute is favored on the right side of the diagram, but is not observed in practice.

composition and introduction protocols were refined by Fahy to make it possible for living tissue to tolerate vitrification under practical conditions [42].

Fahy proposed non-equilibrium vitrification as a way to cryopreserve organized tissue, especially organs, without structural damage from freezing. The approach has since been applied to a diversity of tissues, including heart valves [23], vascular tissue [78,79,87], cartilage [77], cornea [8], and mouse ovaries [67,74]. Progress on vitrification and transplantation of the rabbit kidney as a model organ has proceeded incrementally [9,35,41,58], with one reported instance of long-term survival of an animal supported solely by a kidney that had been vitrified [34,39].

The original demonstration of vitrification for embryos has led to extensive use of vitrification in the field of animal and human reproductive cryobiology. The number of papers published about vitrification has been doubling every five years since 1990, and most of them concern reproductive applications [42].

Equilibrium vitrification

Although the connection to vitrification was not recognized by Farrant in 1965, his cryoprotectant introduction method was used four decades later to achieve vitrification of articular cartilage by Pegg et al. [68,94]. The Farrant "liquidus tracking" method followed by cooling below T_g is an example of what has come to be known as the equilibrium approach [86] to vitrification because supercooling of water is avoided. With liquidus tracking, samples remain on or slightly above the liquidus line (the ice melting temperature) of the phase diagram at all times as shown in Fig. 3. This assures that the sample will always remain free of ice regardless of cooling or warming rate. Low temperatures protect against cryoprotectant toxicity at high concentrations.

Liquidus tracking is not the only equilibrium approach to vitrification that is possible. Any cryopreservation method in which samples remain above T_m on the phase diagram prior to reaching T_g could be characterized as an equilibrium approach by virtue of avoiding supercooling. In 1978 Boutron contemplated the possibility of cryopreserving cells in solutions that would remain glassy even at very slow cooling and warming rates [18]. Such an approach, in which there is a long isothermal (or near isothermal) horizontal traversal of the phase diagram prior to cooling is shown in Fig. 3. In 2007 Brockbank et al. [22] exposed porcine heart valves to an 83% v/v cryoprotectant solution followed by cooling to −135 °C with good structural preservation. The solution was sufficiently concentrated that it could be held indefinitely at any temperature, even dry ice temperature (−79 °C) without freezing. The toxicity of such highly concentrated cryoprotectant solutions remains a challenge for the application of equilibrium vitrification methods in which cryoprotectants are introduced at high temperatures.

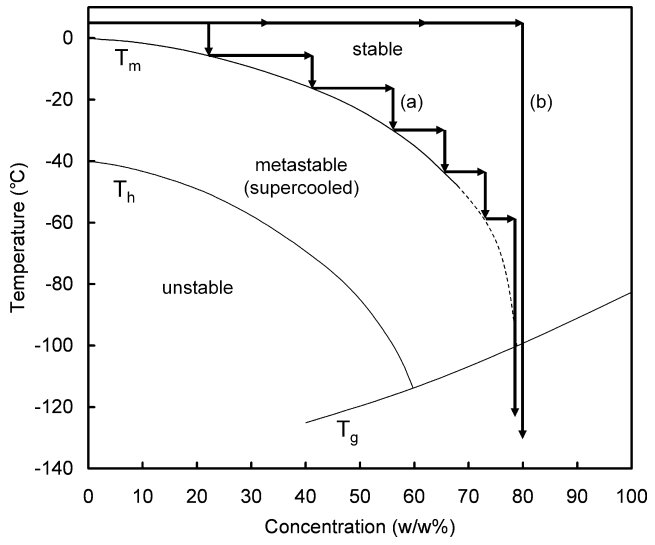

Fig. 3. Path followed on a glycerol–water phase diagram during equilibrium vitrification by (a) the liquidus tracking method and (b) warm equilibration with an unfreezable solution.

It should be noted that even though "equilibrium" approaches to vitrification avoid supercooling, samples do not remain in true thermodynamic equilibrium at all times during the process. If water supercooling is avoided by navigating above and to the right of the T_m line on the phase diagram (Fig. 3), the sample may still fall below the eutectic temperature of the solute system resulting in solute supersaturation. While supersaturated cryoprotectant solutes usually do not crystallize in solution (a requirement of good cryoprotectants), a supersaturated solution is not in equilibrium. It is only metastable. Additionally, all materials depart from thermodynamic equilibrium as they pass through the glass transition for reasons that will be discussed below.

The physical process of vitrification

Solidification of liquids

Vitrification seems to be a universal phenomenon of supercooled liquids that can avoid crystallization, or liquids for which no crystalline phase is known [57]. It occurs in materials as diverse as long linear polymers, non-polar molecules, polar molecules with hydrogen bonds, or materials that form covalent network bonds. It is a process in which a liquid takes on the physical properties of a solid during cooling. It is distinct from crystallization in that no reorganization of molecules into an ordered structure occurs. The disordered molecular arrangement of a liquid is retained during the solidification process.

Consider a liquid being cooled. If the liquid becomes colder than the melting temperature, T_m, crystallization becomes thermodynamically favored. Below T_m, the entropy decrease of crystallization is exceeded by the environmental entropy increase caused by heat released by crystallization. The Gibbs free energy change associated with crystallization becomes negative; a requirement for a process to proceed spontaneously. A liquid below T_m may remain a liquid (supercool) if crystal nucleation does not occur or if crystal growth is kinetically inhibited by viscosity. Although a liquid below T_m is outside the thermodynamic equilibrium phase diagram, it can exist within local free energy minima that are stable with respect to small disturbances. Such supercooled liquids are metastable [57].

As cooling continues, the energy of molecules eventually becomes less than the height of potential energy barriers that must be overcome for molecules to move amongst each other. Although the molecules remain disordered, their physical confinement and vibration within local potential energy wells begins to resemble the confinement of molecules within a crystal. This is the glass transition. Within this regime, cooling only 10 °C can cause the viscosity to rise by a factor of one thousand [3]. Heat capacity, thermal expansivity, and compressibility suddenly fall from liquid values to near those of a crystal [6]. Vitrification has occurred. The liquid has become an amorphous solid.

One way of describing the solid formed by vitrification is to note that it is "a form of matter which maintains the structure, energy, and volume of a liquid, but for which the *changes* in energy and volume with temperature are similar in magnitude to those of a crystalline solid" [57]. Thermodynamic state variables (pressure,

volume, energy, entropy) change minimally during the glass transition. However the thermodynamic response functions that characterize how state variables change (heat capacity, thermal expansivity, compressibility) change dramatically. Thus it is the slope, not magnitude, of state variables as a function of temperature that changes abruptly (but continuously) during the glass transition. This is very different from crystallization wherein state variables such as volume, energy, and entropy change discontinuously.

Vitrification of pure glycerol

The glass transition of glycerol is useful for illustrating apparent changes in thermodynamic properties with vitrification. Measurements of the heat capacity of liquid glycerol and crystalline glycerol by Gibson et al. [48] are plotted in Fig. 4 as a function of temperature. The enthalpy and entropy of glycerol can be deduced from the heat capacity data.

The heat content, or enthalpy, of the crystal, H_{crystal}, at a temperature T can be calculated by using the crystal heat capacity $C_{\text{p,crystal}}$ to integrate heat flow starting from absolute zero

$$H_{\text{crystal}}(T) = \int_0^T C_{\text{p,crystal}}(T)\,dT. \tag{1}$$

The enthalpy of the liquid or glass, H_{liquid}, at T can be calculated by starting with the crystal enthalpy at the melting temperature from Eq. (1), adding the heat of melting (ΔH_{fusion} = 48 cal/g) to obtain $H_{\text{liquid}}(T_{\text{m}})$, and then subtracting the heat required to warm the liquid from T to T_{m} according to the liquid heat capacity $C_{\text{p,liquid}}$

$$H_{\text{liquid}}(T_{\text{m}}) = H_{\text{crystal}}(T_{\text{m}}) + \Delta H_{\text{fusion}}, \tag{2}$$

$$H_{\text{liquid}}(T) = H_{\text{liquid}}(T_{\text{m}}) - \int_T^{T_{\text{m}}} C_{\text{p,liquid}}(T)\,dT. \tag{3}$$

The resulting enthalpies of glycerol are shown in Fig. 5.

The crystal and liquid/glass entropies can be calculated similarly, and are plotted in Fig. 6

$$S_{\text{crystal}}(T) = \int_0^T \frac{C_{\text{p,crystal}}(T)}{T}\,dT, \tag{4}$$

$$S_{\text{liquid}}(T) = S_{\text{crystal}}(T_{\text{m}}) + \frac{\Delta H_{\text{fusion}}}{T_{\text{m}}} - \int_T^{T_{\text{m}}} \frac{C_{\text{p,liquid}}(T)}{T}\,dT. \tag{5}$$

The different behavior of state variables and response functions during vitrification is apparent in Figs. 4–6. In Fig. 4 the response function of heat capacity drops precipitously (but not instantaneously) as the liquid cools through the glass transition. However the state variables of enthalpy and entropy do not change at the glass transition. There is no heat gain or loss associated with the transition. There is only a smooth change in enthalpy slope over a temperature interval of about 10 degrees. Heat flows during passage through this temperature interval, but it is the usual heat of warming or cooling at finite heat capacity, not a heat of transition.

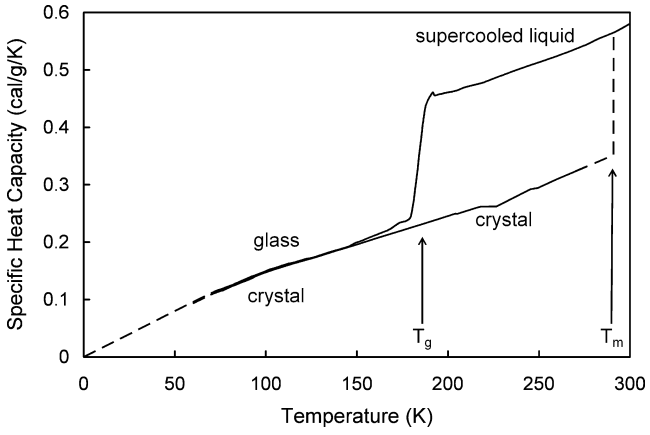

Fig. 4. Heat capacity of pure glycerol in liquid and crystal forms. The glass transition temperature and melting temperature are indicated. Dotted lines are linear extrapolations. The slope of the real heat capacity goes to zero at 0 K.

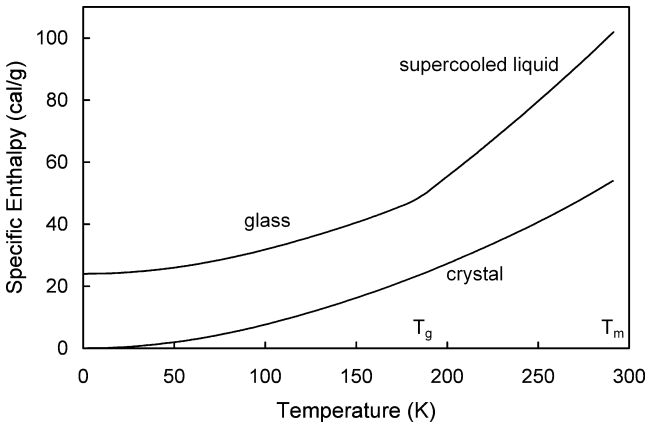

Fig. 5. Enthalpy, or heat content, of pure glycerol in liquid and crystal forms obtained by integrating the data of Fig. 4.

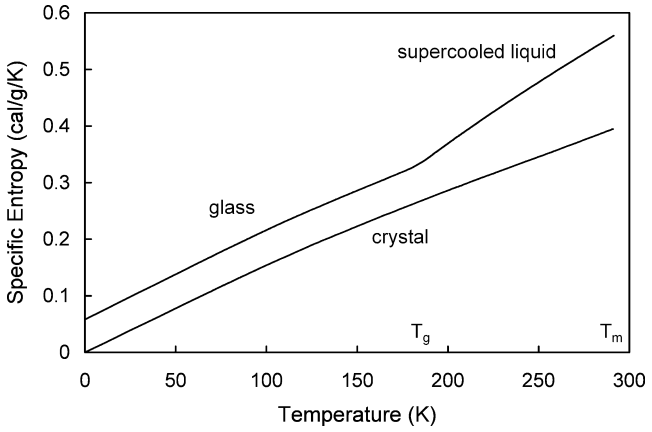

Fig. 6. Entropy of pure glycerol in liquid and crystal forms obtained by integrating the data of Fig. 4.

The surplus enthalpy and entropy of the glass over the crystal in Figs. 5 and 6 is real. This extra heat and entropy content is an important property of glasses, and extends all the way to absolute zero [25].

Onset of the glass transition

The approximate temperature at which vitrification occurs is the glass transition temperature, T_{g}. For pure substances, T_{g} is typically found near 2/3 T_{m} on the absolute temperature scale [29]. Angell has pointed out that this empiricism may be a tautology based on the viscosity constraints on liquids for vitrification to be observable in the laboratory [3]. For cryobiologists, it is important to note that T_{m} of ice in aqueous solutions can be reduced and moved closer to T_{g} by addition of solutes, and even reduced below T_{g} as is the case for equilibrium vitrification methods discussed above. Not all liquids that can be vitrified have an observable crystallization process.

A remarkable aspect of the glass transition is that so many apparently independent physical properties undergo large changes

within the same narrow temperature range. For example, the T_g values determined by noting the temperature of rapid change of heat capacity (calorimetric glass transition), thermal expansivity, or viscosity are nearly identical [57]. Evidently all these properties are influenced in parallel by the changes in molecular mobility that occur during the glass transition.

Liquid viscosity is typically observed to be near 10^{13} Poise [3,29] (10^{15} times the viscosity of water) at the calorimetric glass transition, independent of the nature or initial viscosity of the liquid being vitrified. Viscosity is related to the shear stress relaxation time by the Maxwell relation

$$\eta = G_\infty \tau_s, \tag{6}$$

where η is the shear viscosity, G_∞ is the infinite frequency shear modulus for liquids (typically $0.5–4 \times 10^{11}$ dyn cm^{-2}), and τ_s is the shear stress relaxation time [3,5]. The shear relaxation time corresponding to a viscosity of 10^{13} Poise is about 10^2 s [5]. Thus the similar viscosity observed for disparate liquids at the calorimetric glass transition is the result of a similar shear relaxation time of 100 s at the glass transition.

A relaxation time on the order of minutes is also observed for enthalpy relaxation and dielectric relaxation at the glass transition [3,4,57]. Similar relaxation times are indicative of common dependence on molecular mobility for different types of relaxation.

Relaxation times at the glass transition exhibit another seemingly remarkable coincidence. According to viscosity data for glycerol [6], the relaxation time changes from 10^{-10} s at 290 K to 10^8 s at 150 K (extrapolated), a variation of 18 orders of magnitude between room temperature and cryogenic temperatures. Why over such an enormous range of different relaxation times is the glass transition calorimetrically observed to occur at a temperature with a relaxation time of minutes, a very human timescale? The answer is that it is not a coincidence that the glass transition is observed to occur when the relaxation time approaches laboratory measurement timescales. There is a connection between the timescale over which thermodynamic response functions are measured, the liquid relaxation time, and whether a glass transition is detected. The glass transition has an "observer dependence".

The non-equilibrium nature of glass

At temperatures far above T_g, molecules in a liquid have energies much greater than potential barriers that impede motion past adjacent molecules. Liquids quickly explore the configuration space of all possible distinct molecular positions and orientations consistent with the total energy of the system. The value of a physical property measured over a laboratory measurement time interval Δt will be based on a complete sampling of configuration space. The system is said to be ergodic [73]. The system is also in thermodynamic equilibrium because the rapid cycling through configuration space during the time of measurement assures that the measured value of a property will remain the same regardless of when a measurement is made.

At lower temperatures molecules in a liquid begin to get trapped in potential energy wells between other molecules. As temperature decreases, a diminishing fraction of molecules have the energy needed to change position in the liquid. A particular molecule must wait longer and longer for random energy exchanges to give it the energy necessary to escape its potential well and change position. The time required for the system to explore its space of possible molecular configurations expands. When the time required to sample configuration space grows to become longer than the laboratory measurement time Δt, the system is no longer ergodic. The phenomenology of the glass transition becomes evident [29].

Consider the measurement of heat capacity. If heat is removed from a system faster than a system can relax to new lower energy arrangements of its molecules, heat removal will be limited to vibrational degrees of freedom. The heat capacity will appear lower than if measurements were done slowly enough for energy to be released by positional rearrangements. For this reason, the observed temperature of the glass transition is lower at slower cooling rates [24], typically lowering by 3–5 °C for a factor of 10 decrease in the cooling rate [30].

Thus, within a certain temperature range, whether a material is seen to be a liquid or a glass depends on the measurement timescale. For a given measurement time, there is a temperature below which ergodicity is broken. The thermodynamic response functions are altered because of insufficient time to sample configuration space during the measurement interval. Translational and rotational degrees of freedom appear to be absent. The material is seen to be a glass. Furthermore, because sampling of configuration space to find the state of lowest free energy is incomplete during the measurement time, the properties of the glass will be seen to change with time. Glasses formed at non-zero cooling rates are therefore intrinsically out of thermodynamic equilibrium.

Strong vs. fragile glass formers

The dependence of molecular relaxation on escape from local potential wells might be expected to give relaxation time in liquids an Arrhenius dependence, such as

$$\tau = \tau_0 \exp(E_a / k_B T), \tag{7}$$

where τ is the relaxation time, τ_0 is a constant, k_B is Boltzmann's constant, and E_a is some activation energy. What is usually found instead is that the viscosity of liquids (proportional to the relaxation time via Eq. (6)) increases supra-exponentially with decreasing temperature. This non-Arrhenius behavior of viscosity is termed "fragility", and is believed to be related to increasingly large cooperative motions being necessary for molecular rearrangements as temperature decreases, with associated increasing activation energies [60].

Fig. 7 shows the logarithm of viscosity as a function of inverse temperature for three different liquids at temperatures above the glass transition temperature. The viscosity of silicon dioxide has an Arrhenius temperature dependence, giving a straight line on

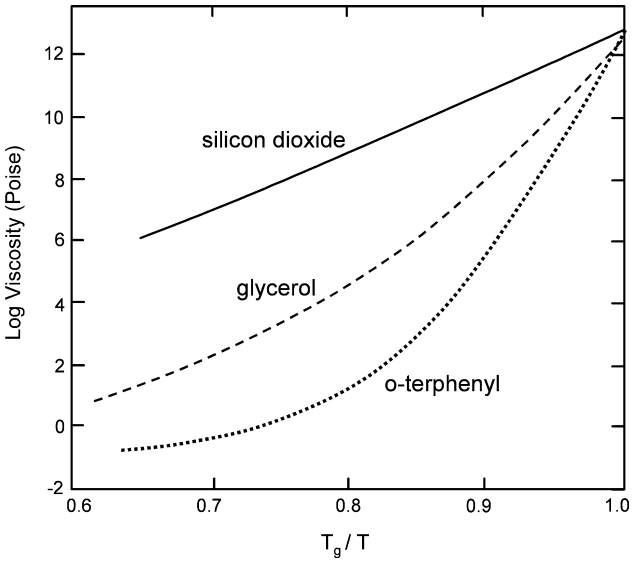

Fig. 7. Viscosity as a function of glass transition temperature divided by temperature for three liquids of different fragilities. The figure is adapted from reference [6].

this plot. Silicon dioxide is a classic "strong" glass former. In contrast, *o*-terphenyl deviates markedly from Arrhenius behavior. *O*-Terphenyl is a "fragile" glass former. Glycerol behaves in between these two extremes. Liquids made of atoms that form directional covalent bonds make the strongest glass formers, while molecular liquids with weak non-directional van der Waals interactions tend to be the most fragile [29]. Polar liquids that form hydrogen bonds are intermediate. The correlation between intermolecular bond weakness and viscous fragility means that there is a correlation between viscous fragility of liquids and the mechanical strength of glasses that they form [66].

Instead of the Arrhenius equation above, the temperature dependence of viscosity for fragile liquids is found to more closely follow the Vogel–Fulcher–Tammann (VFT) equation [45,84,89]

$$\eta = \eta_0 \exp\left(B/(T - T_0)\right), \tag{8}$$

where η is the viscosity, η_0 is the viscosity in the high temperature limit, and B and T_0 (the VFT temperature) are constants chosen to fit the data. This equation is also variously known as the Vogel–Tammann–Fulcher (VTF) equation, the Vogel–Fulcher–Tammann–Hesse (VFTH) equation, or just the Vogel–Fulcher (VF)) equation [4]. If the equation is rewritten in the form

$$\eta = \eta_0 \exp\left(DT_0/(T - T_0)\right), \tag{9}$$

then D is a dimensionless parameter that characterizes the fragility of the liquid [2,3], with D being 100 for silicon dioxide (strong) and 6.8 for *o*-terphenyl (fragile) [11]. T_0 approaches 0 K for strong liquids, and becomes closer to T_g for fragile liquids.

Although the VFT equation predicts that viscosity diverges to infinity at T_0, the equation does not provide a good fit to data at temperatures below T_g. Near the glass transition temperature, it is frequently found that the viscosity and relaxation times of even fragile liquids are better modeled by Arrhenius dependence on temperature (linear behavior on the far right side in Fig. 7) [6,52]. Therefore near T_g the principal difference between strong and fragile liquids is that fragile liquids have a steep viscosity/relaxation time slope as function as temperature (high Arrhenius activation energy). The glass transition of fragile liquids is sharp, occurring over a brief temperature interval.

The failure of the VFT equation below T_g can be understood by recognizing that it applies to a liquid in equilibrium [57], and glasses depart from equilibrium at the time of their formation. If a liquid were cooled so slowly that it remained in equilibrium to lower temperatures, the viscosity would indeed diverge as the temperature approached T_0.

Relaxation and aging of glass

The practical definition of the glass transition temperature is the temperature at which relaxation times in a liquid begin to exceed measurement timescales, causing loss of ergodicity. Glasses therefore drop out of equilibrium at the time of their formation, and have un-relaxed stress almost by definition. The relaxation of glass toward equilibrium is called aging, or annealing if taking place relatively quickly at temperatures just below T_g.

Let $\sigma(t)$ be the value of a physical property at time t. A relaxation function $F(t)$ can be defined as

$$F(t) = \frac{\sigma(t) - \sigma(\infty)}{\sigma(0) - \sigma(\infty)} \tag{10}$$

such that the movement of $F(t)$ from 1 to 0 with increasing time reflects the relaxation of a property toward equilibrium. Most systems relax exponentially according to

$$F(t) = \exp(-t/\tau), \tag{11}$$

where τ is the relaxation time. However glasses are found to relax according to the Kohlrausch–Williams–Watts (KWW) function [59,95]

$$F(t) = \exp\left(-(t/\tau)^{\beta}\right), \tag{12}$$

where $0 < \beta < 1$. This stretched exponential behavior is believed to result from the existence of distinct relaxing domains with the glass [29]. The value of β depends on microheterogeneities locked into the glass at time of formation, so it is independent of temperature [4].

The continued relaxation of glass at temperatures below T_g has physical consequences. The calorimetric onset of the glass transition during cooling is caused by liquid being unable to rearrange structure fast enough to maintain enthalpy equilibrium. Therefore heat will continue to be slowly lost below T_g as the glass seeks enthalpy equilibrium, even if temperature is held constant. This is enthalpy relaxation. Entropy reduction and volume relaxation (shrinkage to equilibrium volume) accompanies enthalpy relaxation.

At any given temperature, a glass seeks to reach the equilibrium state of a liquid at the same temperature. It does so according to Eq. (12) at a rate that depends on the value of the relaxation time constant τ at that temperature. For glasses formed under typical laboratory conditions, τ will be on the order of 10^2 s at T_g, but increases by a factor of 10 for every 3–5 °C further temperature reduction.

One consequence of relaxation below T_g is hysteresis. As shown in Fig. 8A, a glass has a higher enthalpy and volume during cooling than it does later during warming through the same temperature range. During cooling below T_g, the glass leaves the equilibrium liquid line because some degrees of freedom cannot lose heat fast enough to maintain equilibrium. However heat loss still continues below T_g by relaxation, slowly pulling the enthalpy, entropy, and volume of the glass down toward equilibrium liquid values (the dotted line in Fig. 8A). This downward pull continues during rewarming back toward T_g, causing the glass to follow a lower enthalpy/volume path, and meet the equilibrium liquid line at lower temperature than it left the line during cooling. Now the glass has the opposite problem it had during cooling; it cannot gain heat

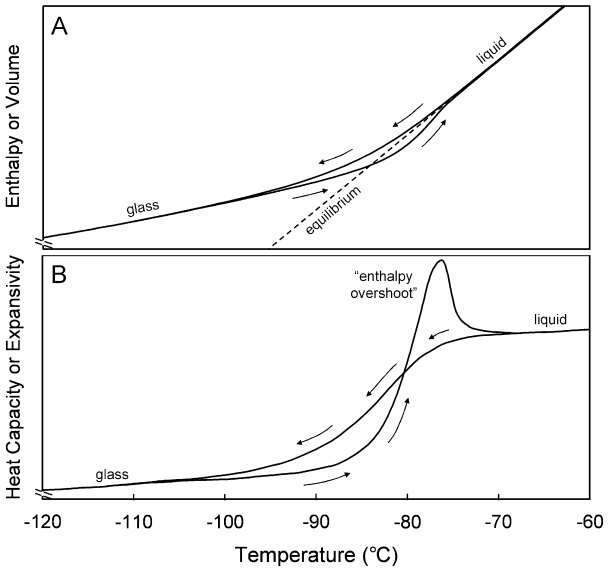

Fig. 8. Hysteresis of pure glycerol during cooling at 10 °C/min followed by warming at 10 °C/min according to data of Angell [3]. Graph (B) corresponds to the thermogram that would be seen on a differential scanning calorimeter (DSC) as the glass transition is traversed in both directions.

fast enough to follow the liquid equilibrium line back up. The glass crosses below the liquid equilibrium line, and is said to become superheated [25]. At T_g the relaxation time finally shortens enough for the superheated glass to race toward liquid equilibrium. The rapid absorption of heat appears as an "enthalpy overshoot" in calorimetry measurements, as shown in Fig. 8B. The magnitude of the enthalpy overshoot during warming through the glass transition has been found to increase with increasing annealing or aging of cryoprotectant solution glasses, as expected [26,46,93].

Is the glass transition a phase transition?

The glass transition is sometimes characterized as a second-order phase transition [61], which is a phase transition with no latent heat. While the glass transition has no associated latent heat, the glass transition is not a phase transition [52,57]. Phase transitions are changes from one thermodynamic equilibrium state to another equilibrium state with different properties. However glasses formed at non-zero cooling rates are not in thermodynamic equilibrium. Whether a glass transition has even occurred is ambiguous because the transition happens over a temperature interval, with the location of the interval dependent upon the timescale of observations. The glass transition is a kinetic phenomenon in which certain degrees of freedom are lost from liquids depending on observation timescales [6]. While the glass transition mimics a second-order phase transition, it is not a phase transition in a strict thermodynamic sense.

Although the glass transition is not a phase transition when observed on practical timescales, it has been speculated that a true phase transition to an equilibrium "ideal glass" may occur at temperatures tens of degrees below the laboratory T_g [6,29,47,52]. However avoiding departure from equilibrium during cooling requires cooling so slowly that reaching an ideal glass phase transition would take eons [52].

The possibility of an ideal glass can be viewed in terms of the Adam–Gibbs theory for kinetic processes in cooperative systems [1]. The Adam–Gibbs model predicts that relaxation time τ is given by

$$\tau = A\exp(B/TS_C), \tag{13}$$

where A and B are constants, T is the temperature, and S_C is the configurational entropy. A modern approach is to calculate the configurational entropy by computer exploration of a system's multidimensional configuration space of molecule positions [4,29]. S_C is proportional to the logarithm of the number of system configurations with the same total potential energy. This "energy landscape" paradigm is emerging as a valuable approach for understanding glass properties such as fragility [29]. In the Adam–Gibbs energy landscape interpretation, an ideal glass would be the very bottom of a large potential energy basin in which, like a crystal, there is one unique molecular configuration and $S_C = 0$ and $\tau = \infty$ (equilibrium).

The Kauzmann paradox

Fig. 9 plots the entropy excess of liquid glycerol over crystal glycerol from the data of Fig. 6. The entropy excess plummets with temperature until a glass transition causes the liquid to depart from equilibrium and lock in extra entropy that persists to 0 K. The glass transition shifts to lower temperatures as the cooling rate decreases, underscoring the kinetic nature of the transition. In 1948 Kauzmann observed that this suggests a paradox [57]. If a liquid were cooled slowly enough to follow the extrapolated liquid equilibrium line to a low enough temperature, the entropy of the liquid would become less than that of the crystal, causing an "entropy crisis" [97]. The temperature at which this event is extrapolated to occur is now called the Kauzmann temperature, T_K. It is a paradox because the configurational entropy of such a material would be negative, which is unphysical.

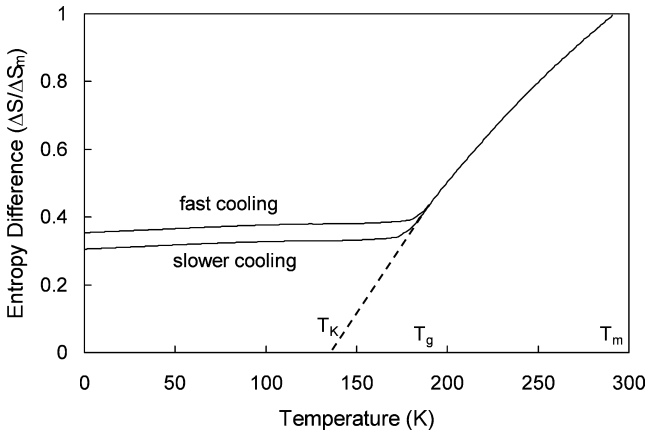

Fig. 9. The entropy difference ΔS between liquid/glass glycerol and crystalline glycerol as a fraction of the entropy of melting, ΔS_m. The "fast cooling" curve is computed from the data of Fig. 6. The "slow cooling" curve is a more relaxed glass that would be obtained at a slower cooling rate. T_K is the Kauzmann temperature, the temperature at which the liquid equilibrium entropy is extrapolated to become equal to that of the crystal.

There are several possible resolutions to the Kauzmann paradox. It is generally assumed that an equilibrium glass with the same heat capacity as the crystal must exist at some point before the equilibrium liquid entropy line dives below the crystal entropy. This glass may be the product of a phase transition as discussed above, or a smooth transition to a state in which a lower configurational entropy is dynamically impossible [60]. Kauzmann himself speculated that crystal nucleation and growth would occur in a glass over eons of time so that the only way for a glass to reach the entropy of a crystal would be to become a crystal itself [57].

Temperatures related to the glass transition

Several different temperatures are encountered in discussions of vitrification. It is useful to summarize them.

T_g is the glass transition temperature. The glass transition temperature is usually defined calorimetrically as the temperature at which heat capacity is halfway between liquid and glass values. T_g depends on cooling and warming rate. It coincides closely with the temperature of intersection of extrapolated entropies of the glass and liquid, and also with the inflection point of heat capacity measured through the transition region [103]. In cryobiology there is a tradition established by Boutron [18–21] and others [12,14,15,98] of reporting T_g of vitrification solutions as the inflection point of heat capacity measured during warming in a differential scanning calorimeter (DSC), preferably at rates less 10 °C/min to minimize instrument lag effects.

T'_g is the glass transition temperature of maximally freeze-concentrated aqueous solutions [44]. This is the glass transition temperature of residual unfrozen solution between ice crystals when a solution containing a freezable concentration of water is frozen very slowly. It is the temperature of the intersection of the T_m and T_g curves in Fig. 1. T'_g is important to cryopreservation by freezing because it is near the glass transition temperature of the medium that cells are stored in at the end of freezing. Frozen biomaterials need to be stored at temperatures below T'_g for long-term stability.

T_0 is the Vogel–Fulcher temperature, the temperature at which the VFT equation, Eq. (8), predicts liquid viscosity goes to infinity. It is typically tens of degrees below T_g. Although the VFT equation is

not valid below T_g, T_0 computed by parameter fitting to viscosity data at higher temperatures is often found to be near T_K [85].

T_K is the Kauzmann temperature, the temperature at which the entropy of a liquid that remains in thermodynamic equilibrium during slow cooling is extrapolated to fall to the same entropy as a crystal. Per Fig 9, it is computed by linear extrapolation of liquid entropy on the ergodic (pre-T_g) portion of the phase diagram down to the crystal entropy.

T_f is the fictive temperature. The fictive temperature is a property of a particular glass sample that measures how far the glass is from thermodynamic equilibrium. T_f is the temperature at which the equilibrium liquid would have the same structure and configurational entropy as the glass [49,88]. A glass with a fictive temperature T_f is a glass that could be formed by instantaneously cooling the liquid starting from equilibrium at temperature T_f. The fictive temperature of a glass decreases with aging.

Vitrification of cryoprotectant solutions

Ice nucleation and growth

Non-equilibrium vitrification of aqueous solutions requires cooling through the metastable part of the phase diagram shown in Fig. 2 down through T_g, the glass transition temperature, and then warming back up again without significant crystallization of water or solutes. Cryoprotectant solutes are generally chosen to have the desirable property of not crystallizing or forming hydrates at low temperature in aqueous solution. Phase separation (spinodal decomposition) may occur with some hydrophobic solutes [63,66], but this is usually not a concern. The primary physics challenge of cryopreservation by vitrification is avoidance of water ice.

Unlike vitrification, freezing of liquid water into ice is a thermodynamic phase transition. It is a first-order phase transition because the transition occurs with the release of latent heat. Ice formation consists of nucleation followed by growth. Very small ice crystals have a larger Gibbs free energy per molecule than large ice crystals because of interfacial surface energy. This requires supercooling to a temperature lower than T_m, the large ice crystal melting temperature, for formation of small ice crystals to be thermodynamically favored. In the limiting case of the first few water molecules gathering together to form the nucleus of a new ice crystal (nucleation), the temperature at which crystallization is favored is approximately −40 °C in pure water. This is the homogeneous nucleation temperature, T_h. As indicated in Fig. 2, T_h decreases by approximately 2 °C for every 1 °C decrease in T_m as solute concentration is increased [66]. Below T_h the solution is unstable against ice formation because homogeneous nucleation occurs quickly and extensively. Between T_m and T_h the solution is metastable against ice formation. Ice can form, but only if nucleation is assisted by particles or surfaces that lower free energy barriers to nucleation. This is heterogeneous nucleation. Heterogeneous nucleation is the predominant mechanism of ice formation in cryobiology.

The solute concentration at which T_h crosses T_g in Fig. 2 is the lowest concentration at which it is possible to avoid homogeneous nucleation during vitrification. This concentration has been deemed the "concentration-needed-to-vitrify" (CNV), and correlates well with the minimum concentration needed to avoid visible ice formation during vitrification of 6–8 mL solution samples at moderate cooling rates of 5–30 °C/min [34]. Since solutions of low molecular weight cryoprotectants typically have a T_g near −120 ± 10 °C, CNV usually correlates with a solution melting point near −40 °C (80 °C depression of T_h). Solutes with a low CNV are termed "good" glass formers (not to be confused with strong vs. fragile glass formers). There is considerable variation of CNV between solutes [64]. Glycerol is a poor glass former (CNV ~60% w/w) compared to meso-depleted 2,3-butanediol (CNV ~35% w/w), a very good glass former [17]. Vitrification using poor glass formers may actually be advantageous for reducing toxicity of vitrification solutions [40]. The empirical concentration needed to vitrify can be reduced by adding small quantities of "ice blockers" that directly inhibit ice nucleation and growth [56,86,99,102].

Whether below or above CNV, non-equilibrium vitrification is a race to avoid the kinetic processes of ice nucleation and growth. The lower the concentration of cryoprotectant, the faster cooling must proceed to avoid ice formation [42]. The minimum cooling rate necessary to avoid significant ice formation during cooling is the "critical cooling rate" of a cryoprotectant solution. The minimum warming rate to avoid significant ice formation during warming from a vitrified state is the "critical warming rate". Critical warming rates are typically two or more orders of magnitude greater than critical cooling rates. Ice formation during warming, termed "devitrification", happens faster than during cooling because ice nucleation occurs at lower temperatures than ice growth. Nucleation at very low temperatures primes the solution for extensive ice growth at warmer temperatures.

Fig. 10 shows ice nucleation and growth rates measured by DSC [101] in a solution used for vitrification of organs called M22 [41]. The ice growth rate peaks about 10 degrees below T_m. Ice growth requires diffusion to bring new water molecules to the crystal surface, so it is inhibited by increasing viscosity at lower temperatures. Nucleation requires only local reorientation of molecules, so it continues even below T_g. M22 is exceptionally stable against ice formation, with critical cooling and warming rates of 0.15 °C/min and 0.4 °C/min, respectively [100]. More dilute solutions show broader and overlapping nucleation and growth regimes [51], but in which the nucleation rate declines at temperatures below T_g in the same manner as Fig. 10.

There is a substantial body of literature on physical aspects of ice nucleation and growth relevant to cryopreservation by vitrification. For further information, including discussion of pressure, which is neglected here, readers are referred to the detailed reviews by Mehl [66] and MacFarlane et al. [64].

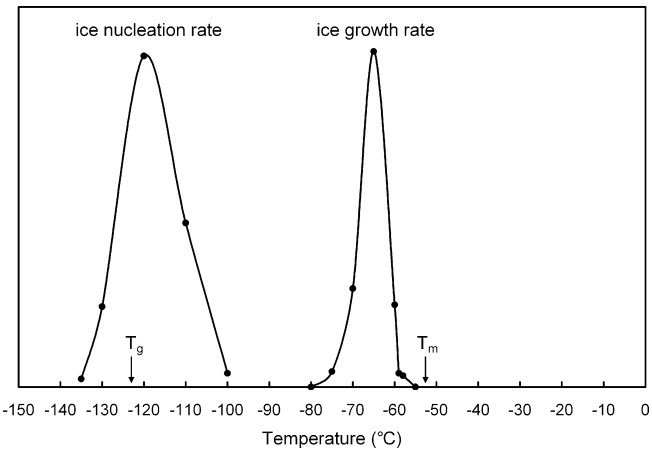

Fig. 10. Ice nucleation and growth rates for M22 vitrification solution obtained by DSC study [101]. The vertical axis is proportional to the amount of ice formed when holding a constant growth temperature after holding different nucleation temperatures (nucleation rate measurement), or after holding a constant nucleation temperature followed by holding different growth temperatures (growth rate measurement). Ice growth occurs in a narrow zone below T_m, while nucleation peaks near T_g and persists until well below T_g. This nucleation is heterogeneous nucleation because T_h is depressed far below T_g in this concentrated solution.

Thermomechanical aspects of vitrification

Cooling cryoprotectant solutions below the glass transition temperature causes mechanical stress inside the resulting glass. As temperature decreases, a liquid or a solid will seek to occupy a smaller volume in accordance with the equation

$$\alpha = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_P, \tag{14}$$

where T is the temperature, V is the volume (measured at constant pressure), and α is the thermal expansion coefficient, a constant that is characteristic of a particular material. Representative values of α for cryoprotectant solutions are 85×10^{-6} per °C (liquid) and 40×10^{-6} per °C (glass) [54]. These are an order of magnitude larger than thermal expansion coefficients for typical solution container materials, such as borosilicate glass. Cryoprotectant solutions will therefore undergo larger volume contraction with cooling than the containers they occupy. A liquid solution in an open container can easily contract without stress to reduce volume. However a solid (vitrified) solution may be prevented from contracting if it adheres to container walls. This causes stress to accumulate inside the cryoprotectant glass with cooling. Non-adherent hydrophobic materials, such as polyethylene, can minimize stress by allowing vitrified solutions to retract away from container surfaces as they contract.

Stress can develop inside vitrified cryoprotectant solutions even without contact with container walls. Volume change is proportional to temperature change, so if temperature changes non-uniformly within a sample, volume will seek to change non-uniformly as well. The sample will seek to change shape. If a sample is stiff (high elastic modulus), internal stress will develop instead of shape change. The most common manifestation of such stress in vitrified systems occurs as a consequence of samples developing a non-uniform temperature distribution during cooling to achieve vitrification. Different parts of a sample may then pass through the glass transition at different times, and possibly even different temperatures due to different cooling rates. It is possible to "lock in" a specific volume, or density, distribution within the sample that is associated with the non-uniform temperature distribution. Temperature must later change by different amounts at different locations within the solid sample to achieve temperature uniformity. This results in stress. The faster the cooling rate through T_g, the larger the temperature non-uniformity that must eventually resolve, and the larger the stress that results.

If stress exceeds the mechanical strength of the glass, fracture will occur. Likelihood of fracture during cooling increases with cooling rate and sample size. Fracture at temperatures tens of degrees Celsius below T_g is typical for cryoprotectant solutions with volumes on the order milliliters, but can occur as little as a couple of degrees below T_g for solutions with volumes on the order of a liter according to a study by Fahy et al. [38]. It was noted in this study that fracturing depends strongly on cooling rate and thermal uniformity, and that chances of fracturing could be reduced by cooling samples at a declining cooling rate to just above T_g and then raising the environmental temperature to T_g for a period of annealing before continuing descent to lower temperatures. Bourne et al. [16] also found that fracturing of vitrified corneas could be avoided by slowing the cooling rate near T_g. Baudot et al. [13] found that 3 mL vitrified samples could be cooled all the way to liquid nitrogen temperature without fracturing if they were first cooled just below T_g, slowly rewarmed to just above T_g, and then held for a period of time before final temperature descent. The efficacy of all these maneuvers for avoiding fracturing can be understood in terms of minimizing temperature non-uniformity within samples at T_g before further cooling.

In recent years the understanding of the thermomechanics of vitrification has been advanced greatly by the quantitative measurements and mathematical models of Steif and Rabin et al. [10,54,55,70,80–82]. For storage temperatures far below T_g, they found stress to be proportional to the cooling rate, the elastic modulus, the thermal expansion coefficient, the inverse thermal conductivity, and the square of the sample size [80]. Notable quantitative findings include the aforementioned thermal expansion coefficients of cryoprotectant solutions, fracture strain of approximately 0.3%, and fracture stress of approximately 3 MPa [70].

The foregoing discussion assumes that stress results from glasses seeking an equilibrium volume dictated by integration of Eq. (14). It should be noted that the volume given by the value of the thermal expansion coefficient below the glass transition temperature is not a true thermodynamic equilibrium volume, but a kinetic equilibrium volume over moderate timescales based on loss of non-vibrational degrees of freedom over such timescales. Over long timescales, structural relaxation can carry equilibrium volumes to even smaller values. Stress and strain in cryopreserved materials can continue evolving over periods of years.

Long-term stability of cryopreserved materials

Since the goal of cryopreservation is to preserve cells and tissue in a solid state for long time periods, the stability of vitrified biological materials over time is an important issue. Stability near the glass transition temperature is of particular interest because storage near T_g is advantageous for reducing thermomechanical stresses that can cause fracturing, and because the considerable molecular mobility that persists near T_g makes the stability of such storage temperatures non-obvious.

The thermodynamic state variables of enthalpy, entropy, and volume (density) change on timescales of minutes to years between T_g and T_K (tens of degrees below T_g), respectively, as structural relaxation proceeds according to Eq. (12). Relaxation is caused by molecular mobility below T_g, and relaxation data is used as a conservative measure of degradation risk for vitrified biological materials [75,90]. However, the molecular movements underlying relaxation are localized and limited in possible chemical effects. A weak correlation between glass relaxation times and chemical degradation of amorphous pharmaceutical preparations was found by Shamblin et al. [76]. In some cases, increasing the structural relaxation rate actually decreased the rate of chemical change. The coupling between chemical change and glass relaxation depends on the specific chemical reaction, and whether the reaction can be driven by the small movements of adjacent molecules that are associated with structural relaxation.

The localized nature of glass relaxation on a molecular scale can be appreciated by examining how strongly translational diffusion is inhibited at T_g. The diffusion coefficient, D, can be computed from the Stokes–Einstein relation

$$D = \frac{k_B T}{6\pi\eta r}, \tag{15}$$

where k_B is the Boltzmann's constant, T is the temperature, η is the dynamic viscosity, and r is the radius of the diffusing object. The position variance as a function of time, t, is given by

$$\sigma^2 = 6Dt. \tag{16}$$

The time to diffuse a given distance therefore increases almost linearly with viscosity. These relations were used by Fahy in conjunction with the VFT equation for viscosity of a typical cryoprotectant solution to compute the time at various temperatures required for the equivalent of one minute of diffusion at −20 °C [42]. This time

expands from one day at −80 °C, to one year at −100 °C, to millennia as the glass transition is approached near −120 °C. The Stokes–Einstein relation is known to understate translational mobility of host solvent molecules by up to two orders magnitude in highly fragile liquids near T_g [4,27,29]. Even with this adjustment, the diffusion equivalent of one minute at −20 °C (η = 1 Poise) is 10^5 years at T_g ($\eta = 10^{13}$ Poise). It appears that any chemical changes dependent upon diffusion will be slowed to negligible rates in vitrified cryoprotectant solutions.

A caveat to these calculations is that molecules smaller than the dominant molecules determining viscosity will diffuse faster than predicted by Stokes–Einstein [4]. This is a particular issue for aqueous solutions of large molecules with high a glass transition temperature, in which water and other small molecules can remain relatively mobile at T_g. However for cryoprotectant solutions made with low molecular weight solutes, and with a T_g near that of pure water, diffusional mobility of all molecules is expected to be very restricted near or below T_g.

Recent studies by Walters et al. [92] found apparent time-dependent deterioration of seeds stored at cryogenic temperatures, even liquid nitrogen temperature, over a period of years. Several possible explanations have been suggested [91]. One possibility is a degradation mechanism driven purely by vibrations of adjacent molecules.

For samples vitrified by non-equilibrium methods, the most active process of change below T_g that impacts subsequent recovery of biological materials seems to be ice nucleation. The temperature dependence of nucleation below T_g suggests that nucleation is related to the same types of small molecular movements that allow structural relaxation. Nucleation could be a limiting factor for storage of vitrified tissue at temperatures near T_g. An important question is whether heterogeneous nucleation ever stops at a tolerable amount. Further study is required.

Discussion

Vitrification can be understood as a process in which a liquid takes on the properties of a solid by its molecules losing translational and rotational freedom at low temperatures. For liquids of intermediate fragility, such as cryoprotectant solutions, the transition takes place over a temperature interval of approximately 10 °C. At temperatures below this glass transition interval, material behavior over short measurement timescales is governed by molecule vibrations only. Heat content and density change with temperature as though the material were a solid. This change in thermodynamic response functions is a result of the removal of degrees of freedom from the system on the laboratory timescales over which measurements are made [6]. Performing measurements over longer timescales causes liquid degrees of freedom and liquid physical properties to be retained at lower temperatures.

The timescale dependence of the glass transition, and departure from liquid equilibrium that occurs at the glass transition, means that the state of cryopreserved cells in cryobiology is intrinsically a non-equilibrium state. The practical significance of this depends on how close the storage temperature is to the glass transition temperature, and the effect of residual mobility and relaxation processes on samples being stored. Possible sample changes over time include volume, mechanical stress, chemical change, and ice nucleation for samples vitrified by non-equilibrium methods (supercooling). A necessary requirement for any change in the glassy state is that it results from small local movements of molecules because diffusion below the glass transition temperature is strongly inhibited. This restriction, plus activation energy considerations, would seem to limit possibilities for chemical change in vitrified cells stored at cryogenic temperatures.

Starting from Fahy's idea of "CNV", practical vitrification has now expanded to include dilute solutions cooled at very high rates similar to the vitrification sought by Luyet [50], and very concentrated solutions able to vitrify at very slow cooling rates [100]. These developments notwithstanding, the non-equilibrium supercooled realm between the melting temperature and glass transition temperature remains a rich and complex field of study for improvement of cryopreservation by vitrification.

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