Vitrification of Cryoprotectant Solutions

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Abstract

Vitrification, or glass formation, is a feature of the low temperature phase behaviour of many aqueous solutions. Here the physical conditions favouring glass formation are reviewed and discussed in terms of a number of well known aqueous solution systems. The discussion will also consider the conditions under which devitrification (crystallization during warming) and recrystallization (i.e. ripening) are also important.

The role of the solute in promoting glass formation has also been the subject of study in recent years, in an effort to develop improved solutes which support glass formation at lower solute concentrations. It has been observed that one of the functions of a solute which strongly promotes glass formation is to break down the highly structured regions which tend to build up in pure water at temperatures below 0°C. This occurs via strong specific interactions between the solute and water molecules, however in the extreme of very strong interactions solute hydrate compound formation can interfere with vitrification by providing an alternate crystalline phase other than ice. The nature of the solute–water interactions depend strongly on the chemical structure of the solute and detailed trends are beginning to emerge as a result of spectroscopic studies. For example, in the family of diol compounds related to ethane 1, 2 diol it has been observed, by nuclear magnetic resonance studies, that the addition of an alkyl group to the hydroxy carbon produces a more basic hydroxy group. This results in a stronger hydrogen bond between the solute and water, and hence provides the structural disruption in the solution required to promote glass formation.

1. Introduction.

The vitrification of aqueous solutions was a subject of only rather academic interest until recently when the application of such structurally arrested, but nonetheless amorphous, solutions was recognised in Cryobiology. Prior to this, the glassy state of aqueous solutions had been investigated either as a model for the glassy state of vitreous water, which is otherwise difficult to obtain, or as an easily accessible replica of high temperature inorganic glassy systems. In 1981 Fahy prompted the recent era of interest in the vitreous state of aqueous solutions by suggesting that damage-free cryopreservation of cells might be achieved by promoting the vitrification of the solutions both inside and outside the cell. Since water and dilute aqueous solutions are extremely difficult to vitrify, the technique intrinsically involved the use of substantial concentrations of solutes which were of sufficiently small molecular size such that they were easily transported across the cell mem-
brane. To minimize the toxicity of the solutes at these relatively high concentrations, Fahy proposed that the imposition of high pressures could be used as an adjunct to solute content to achieve vitrification. These suggestions have prompted a number of groups to attempt, successfully, the vitrification of a range of cell types and also more detailed studies of the physical chemistry involved in the vitrification of aqueous solutions. Here we review the processes involved in vitrification and the role of the solute in these processes when the major component in the solution is water.

2. Vitrification

The nature of events that take place when water, or a moderately concentrated aqueous solution, is cooled below 0°C are reasonably familiar. As the temperature is lowered below the liquidus point of the solution ice will begin to freeze and the remaining liquid phase becomes more concentrated in the solute. At some lower temperature another crystalline compound involving the solute, perhaps a hydrate, crystallizes. Such behaviour is described by a typical phase diagram for the two component solution, as illustrated in Fig. 1 for the case of propane 1, 3 diol. The phase diagram represents a map of the phases that will form as the temperature and concentrations change.

It is equally well known that freezing rarely begins at the liquidus point, instead a substantial degree of "supercooling" is often required before freezing begins at a significant rate. Under certain conditions, and in the presence of certain solutes only, this supercooling phenomenon takes on a more extreme form such that crystallization of ice is difficult. This can be illustrated in a series of experiments in which a 10 wt% sample of a 35% w/w solution of dimethylsulphoxide (Me2SO, DMSO) is plunged into liquid nitrogen. Once the solution has been quenched to −196°C and removed from the liquid nitrogen, the sample appears cracked and crazed, typical of a glassy material. As the sample warms from −196°C, the cracks are observed to heal at the temperature where the sample regains liquid-like properties. Further warming produces crystallization of ice, and possibly a hydrate, via a process often termed devitrification. The ice thus formed subsequently begins to melt on further warming. These events are typical of a liquid phase substance which crystallizes only with difficulty on cooling and instead is forced into a glassy, or vitreous, state given sufficient lowering of the
temperature.

The course of the events which take place on cooling the solution of 35%w/w DMSO can be described as follows. The presence of the solute depresses the equilibrium melting point of ice in the solution to an extent, as indicated in Fig. 1. In samples of the solution which are free of foreign solid matter, the solution can usually be cooled to a point marked $T_h$ on the phase diagram. Samples of solution can be observed to display this behaviour when dispersed as very small droplets (~2μm diameter) in the form of an emulsion or in a cloud. Many single cells are sufficiently small to approach this limiting supercooling. $T_h$ represents the point in temperature at which the rate of homogeneous nucleation of ice becomes significant homogenous nucleation being the spontaneous generation of new crystal nuclei in the liquid without the assistance of a foreign surface. Because of the interfacial energy which is required when a new crystal nucleus is formed, homogeneous nucleation generally can only take place at a substantial supercooling, where the free energy released on transferring each molecule into the crystal has become large. At a low supercooling (i.e., close to the melting point) the free energy drive is small and hence homogeneous nucleation is an improbable event. Under these latter circumstances, nucleation of the crystalline phase can only take place on existing solid surfaces of appropriate interfacial free energy. Such surfaces are, of course, often provided via seeding, or may be present intrinsically as impurities.

Note that the effect of the added solute on the homogeneous nucleation point of ice in pure water (~38°C) is to depress $T_h$ to lower temperatures, and more markedly so than it depresses the equilibrium melting point. At sufficiently high concentrations and low temperatures, $T_h$ becomes difficult to observe, because the kinetics of the process are slow at the low temperatures involved. This region of the $T_h$ versus composition curve is dashed in Fig. 1. On continuous cooling of a solution of composition in this region of the phase diagram, nucleation begins at, or near, $T_h$ but the crystal growth rate is so low, and decreasing further as the temperature drops, that the crystals do not grow to macroscopic sizes. Instead, as the temperature drops, the rate of translational and rotational motion of the molecules becomes so low that eventually the molecules can no longer reorganize sufficiently to represent a liquid state. For example, continued cooling requires that the volume occupied by the system of molecules decreases continuously. The volume being lost in this process is the extra (often called "free") volume that the liquid has compared to the underlying close packed state. When molecular motion becomes sufficiently restricted that the molecules can no longer rearrange to take up a lower volume configuration, then the liquid can no longer contract as it should, given an infinite amount of time for the rearrangement to take place. The system of molecules is thus no longer in equilibrium; that is, its configuration is stuck in a high energy state. Such a situation describes a glassy, or vitreous, state and a material in this state displays all the mechanical properties of a solid even though it is amorphous on a molecular level.

It is interesting to note from the above description of the transition into the glassy state that the process is a kinetic one. Given a different cooling rate the events would happen over a different range of temperatures. For this reason, the glass transition temperature, $T_g$, which is often used to characterize the process, can only be defined with reference to a set of experimental conditions. Typically, glass transition temperatures of aqueous solutions have been measured by differential scanning calorimetry (or by a related technique, differential thermal analysis) on warming at a rate of 10K/min. In such an experiment the glass is heated continuously, begins to exhibit the motional and other properties of the liquid at $T_g$ and is properly liquid-like (albeit a viscous liquid) by the time the temperature has risen 20°C or so above $T_g$. Heating at a different rate will cause the glass transition to appear at a different temperature.

The glass obtained by the continuous cooling of the 35%w/w DMSO solution has been found to also contain an array of crystal nuclei which began to grow during cooling. However, because the sample
was cooled into glassy state, the growth of these nuclei is arrested along with all other motional processes in the sample. The most common manifestation of the existence of these nuclei is the fact that the liquid obtained on heating to temperatures above $T_g$ rapidly crystallizes, because the growth rate of the crystalline phase has returned to a substantial value. Crystallization on warming a glassy substance to temperatures in the vicinity of $T_g$ is often referred to as devitrification.

It has been shown that devitrification can even take place in aqueous solutions at temperatures below $T_g$. For this reason the glasses which exhibit devitrification behaviour have been described as "doubly unstable" - since the glass is simultaneously unstable with respect to both the liquid and solid states.

Carrying out the same continuous cooling experiment on a DMSO solution of concentration 50% w/w, produces a situation where $T_g$ for the solution has dropped well below (as indicated by extrapolation) $T_\phi$. The liquid should, therefore, exhibit no tendency to generate nuclei of the crystalline phase during cooling. The glass that is obtained is stable with respect to homogeneous nucleation, and should not exhibit a devitrification phenomenon during warming. Experimentally, however, there are inevitably foreign surfaces of various types present in, or around, the sample, on which the crystallization can begin. Hence the appearance, or lack of appearance, of a devitrification event cannot be used to indicate the underlying stability of the glass with respect to homogeneous nucleation.

The above description of the events that take place during glass formation intentionally overlooks a number of other effects. Firstly, the solute itself, or one of its hydrate compounds, may crystallize during cooling. Obviously this is the principal event that takes place on cooling most reasonably concentrated solutions. However, under the conditions (described above) used to remove the influence of heterogeneous effects, hydrate compounds crystallize only slowly and often only as a result of the crystallization of some of the ice from the solution. Secondly, the applied pressure has an effect on all of the thermodynamic and kinetic aspects of the processes involved, and third, the solute may influence, in a specific way, the tendency to form ice. These latter two aspects are addressed further in the next sections.

3. High Pressures Promote Vitrification

Fahy pointed out some time ago that high pressures could be a useful adjunct to added solute in the cryopreservation of cells by vitrification, since most cell lines showed at least some tolerance to high pressures. As Fig. 2 illustrates, the homogeneous nucleation, glass transition and melting temperatures are all a function of the applied pressure. To an extent, the apparent effect of increasing pressure is very similar to the effect of increasing solute concentration. Thus, as Fig. 2 indicates for the mixture of solutes known as VS1 (see reference 9 for composition of this solution), increasing the pressure causes a steady drop in the equilibrium melting point and a rather more rapid drop in the homogeneous nucleation temperature of ice in the solution. At the same time, $T_g$ tends to rise at the rate of about 4 K/kbar. Both of these effects tend to promote glass formation, such that, in the case in point in Fig. 2, some of the solutions becomes glass forming at high pressures, even though they were not at ordinary pressures.

The only additional feature that arises at high pressures (not shown in Fig. 2) is the appearance of melting of ice III, a high pressure polymorph of ice. The formation of this type of ice represents an upper limit to the glass forming region as a function of pressure. Thus a window of glass formation appears, often in the region between 1-5kbar. Whether cell lines of interest can survive such pressures is a question that remains to be investigated, however initial results seem encouraging.

4. The Role of the Solute in Promoting Vitrification

Pure water was not thought to be vitrifiable until recently. Work in the 1960s and 1970s showed that, treated as a normal liquid, water might require cooling rates in excess of $10^7$K/s to bypass
crystallization\textsuperscript{[10]}. Other workers focussed on the curious behaviour of the properties of supercooled water and it was suggested on fundamental grounds that water faced mechanical decomposition on rapid cooling\textsuperscript{[11]}. The implication of this was that the vitreous state could never be achieved except from the vapour phase. These propositions have been the subject of considerable and on-going debate in the literature. In recent years Mayer and coworkers\textsuperscript{[13]} have reported the successful vitrification of pure liquid water by a jet quenching method capable of producing cooling rates in excess of $10^6$ K/s. This suggests that bulk liquid water is in fact vitrifiable, but confirms the estimations which indicated that the cooling rates required were enormous and certainly beyond everyday applications of the process.

The fundamental role played by the solute in aiding vitrification of an aqueous solution has been a vexing question for some time and in a number of areas. The addition of the solute lowers the equilibrium freezing point of the solution, but not sufficiently such that the solution is capable of forming a stable glass, except in the case of a limited number of solutes. The solute also causes a related decrease in the homogeneous nucleation temperature of the solution, however, the magnitude of this effect is not independent of the solute. Some solutes also have a substantial effect on $T_d$ of the solution, such that $T_d$ rises as the concentration increases. The solutes which are most effective in promoting vitrification of a solution at low concen-
trations are thus those which have the greatest depressant effect on \( T_n \), as well as raising \( T_n \). A number of groups have investigated these relationships during the last decade\(^{15,16} \).

The extent to which such effects are related to the chemical structure of the solutes rather than simple mole concentration has been the subject of a number of studies in recent years\(^{15,16} \). These have focused on the nature of the interaction between the solute and water molecules and relationship between these solute–water interactions and the molecular structure of the solute. Below we consider the mode of interaction of various solute types with water and the effect these interactions have on the ability of the solutions to vitrify.

The thermodynamic effect of salt concentration on the properties of an electrolyte solution is, as indicated by the lowering of the melting point of course, independent of the nature of the ions. Slight variations in the liquidus lines for various ionic solutes result from differing interactions between solute and solvent, which produce different enthalpies of solution. However, as Fig. 3 indicates, even at fixed \( T_n \) different solutes produce quite different degrees of effect on \( T_n \). Looking beyond the thermodynamic effects, MacFarlane and Forsyth\(^{16} \) recently suggested that the extent to which different salts suppressed ice nucleation was related to the ability of the ions to disrupt the structure of the solvent. One of the well known anomalous properties of water is the relatively small degree of supercooling it exhibits, ie, \( T_n \) is much closer to \( T_n \) than expected from comparison with similar liquids\(^{11} \). It has been postulated that this is related to the fact that water becomes highly structured at low temperatures and that this hydrogen bonded structure has certain elements in common with ice. The ability of a solute to suppress the nucleation of ice is thus predicted by this model to be related to its ability to disrupt the structuring of water as the temperature is lowered.

This hypothesis has been tested by examining the temperature dependence of the proton magnetic resonance (pmr) response of the \( H_2O \) protons in pure water and a number of electrolyte solutions\(^{16} \).

It is well known that the effect of hydrogen bonding on the pmr chemical shift of a proton in water is to increase the magnitude of the chemical shift (chemical shift is a measure of the local electronic, and hence bonding, environment of the proton). Thus as the temperature is lowered below 0°C and the hydrogen bonded structure increases, the chemical shift begins to increase quite rapidly, as shown in Fig. 4. This figure also indicates the effect of solute on this behaviour; that is, the chemical shift increases much less rapidly in the electrolyte solutions. Fig. 4 shows how the ability of the solute to depress \( T_n \) correlates with its ability to decrease the temperature dependence of the water chemical shift.

Hence, it has been concluded that at least one of the important effects that electrolytes have on water, which promotes glass formation, is their ability to destroy the structure of the water. Further evidence for this mode of action has been derived recently from a study of the properties of several solutions of solutes which are known to
Fig. 4. Temperature and solute dependence of the chemical shift of water protons in a number of electrolyte solutions. The chemical shifts have been normalized with respect to their values at 20°C. The strong temperature dependence of water and the dilute solutions is indicative of developing hydrogen bonded structure at lower temperatures. (Reproduced from reference 16).

enhance the structure of water\textsuperscript{17}. As predicted from this model, \( T_b \) was seen to increase on addition of the solute, (although only when observed at high pressures where the natural structure of water is suppressed). This broad class of molecular solutes includes all those able to dissolve as uncharged molecules in water. Examples of solute compounds typical of this class include a number of alcohols, ethers, ketones, aldehydes, amines, amides, various sulphur containing compounds and many organic acids. The common feature of all of these types of compound is some ability to enter into a hydrogen bonding interaction with water. In the absence of this interaction, or where the hydrocarbon part of the molecule is large, the molecule will generally not be soluble in water.

A range of solutes of this type have been investigated for cryobiological applications, but attention has focussed in recent years on (i) DMSO and (ii) a variety of alcohols, including diols and triols. Luyet and colleagues\textsuperscript{19} and Fahy and colleagues\textsuperscript{18} have carried out extensive investigations of the glass forming ability of a large range of solutions containing solutes such as glycerol, dimethylsulfoxide, propylene glycol and the family of butane diol isomers. Typically, solutions of these relatively low molecular weight solutes require between 30 and 50% w/w solute to suppress ice nucleation to achieve vitrification in small samples. A comparison of the concentration required to achieve vitrification from the work of Fahy\textsuperscript{18} is presented in Table 1, where it can be seen that butane 2, 3 diol is one of the most effective solutes from the point of view of achieving vitrification.

Also obvious from this table are a number of comparisons between isomers, for example, propane 1, 2 diol and propane 1, 3 diol, which behave quite differently. The cause of these differences were investigated recently by Forsyth and

Table 1. Physical properties of some pure isomeric diols. (After reference 16).

<table>
<thead>
<tr>
<th>Solute</th>
<th>B. P./°C</th>
<th>Density/g cm(^{-3})</th>
<th>( T_b )/°C</th>
<th>( C_v ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>propane 1, 2 diol</td>
<td>189</td>
<td>1.036</td>
<td>-100</td>
<td>44</td>
</tr>
<tr>
<td>propane 1, 3 diol</td>
<td>213.5</td>
<td>1.0597</td>
<td>-120</td>
<td>57</td>
</tr>
<tr>
<td>ethylene glycol</td>
<td>198</td>
<td>1.1088</td>
<td>-120</td>
<td>53</td>
</tr>
<tr>
<td>2,3 butanediol</td>
<td>178,\text{-}181</td>
<td>.9869</td>
<td>-81.5</td>
<td>34</td>
</tr>
<tr>
<td>1,2 butanediol</td>
<td>190.5</td>
<td>1.0024</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>1,3 butanediol</td>
<td>204</td>
<td>1.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,4 butanediol</td>
<td>235</td>
<td>1.0171</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
MacFarlane\textsuperscript{19}, again by pmr. It was found that when water was examined in the role of solute in the almost pure diol, the pmr chemical shift indicated quite different behaviour for a number of groups of related compounds. Fig. 5 illustrates the data obtained, and the contrasts between, similar solute molecules. By analysing the behaviour of the various solutes and comparing with the limited amount of infra-red spectroscopy information that exists in the literature, it was deduced that the better ice suppressants among this group of compounds were the stronger bases. The hydroxy groups in these diol compounds are rather weakly basic, but their basicity is stronger than that of water. Where the molecular structure tended to enhance this basicity, the molecule was found to be a better ice suppressant. Presumably, this results via the stronger covalent type hydrogen-bond which is formed by the stronger bases. The strong interaction influences the motional freedom of the water molecules involved and hence also decreases their ability to participate in ice crystal growth. The strength of the interaction was thus predicted to correlate with both $T_g$ and $T_s$ in the solution, as was found to be the case. Thus in the case of a solute exhibiting strong interactions with water, eg, 2, 3 butanediol, $T_g$ of the solution is considerably higher, and $T_s$ is considerably depressed, compared with other related solutions.

5. Summary and Conclusions

The vitrification of aqueous solutions has become a subject of importance as a result of the possible application of the process in cryobiological techniques. Extensive studies have taken place in recent years of the conditions of concentration, solute type and cooling rate under which vitrification is readily achievable. An understanding of the curious differences in effectiveness in promoting vitrification between apparently similar solutes is only just beginning to develop. However, on the basis of such studies and others in the same vein, it seems likely that further improvements in the concentrations required to induce vitrification can be expected in the future.

6. References

6) The term stable is used here in an experimental sense. In most cases the glass remains unstable.
with respect to the crystalline phase, however the kinetics of the transition to the crystalline state are prohibitively slow.


Körber: Ternary systems with 1, 2 propanediol - A new gain in the stability of the amorphous state in the system water-1,2 propanediol-1-propanol. Cryobiology, 19, 550-564 (1982).


(g) Boutron, P.: Levo- and dextro-2, 3 butanediol and their racemic mixture: Very efficient solutes for vitrification. Cryobiology, in press.


