

CAN CLUSTERING OF LIQUID WATER AND THERMAL ANALYSIS BE OF ASSISTANCE FOR BETTER UNDERSTANDING OF BIOLOGICAL GERMPASM EXPOSED TO ULTRA-LOW TEMPERATURES

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Plausible effect of clustering of undercooled liquid water (pentamer configuration, icosahedra formation) is discussed showing water continuous but non-periodic patterns and its impact to the either formation of ice-crystals or ice-glass, particularly when making contact with plants. These processes are viable for the cryogenic storage of biological germplasm and subject for thermoanalytical studies aimed to the determination of glass transition temperatures.

Keywords: cryogenics, glass transition temperature, icosahedron, natural freezing, non-periodic continuum models, pentamers, plants, thermal analysis, undercooling, water clusters

Introduction

The concise form of NATAS (Bowling Green, USA'06), ESTAC (Cracow, Poland'06) and JCCTA (Kyoto, Japan'06) lectures about the plant thermal behavior at lowered temperatures is presented herewith showing another example of a wider applicability of thermal studies [1]. The method can be found and conveniently used for the characterization of detrimental influence of man-affected environment assuming its injurious impact on plants growth [2–5]. In some cases, the hostile environmental conditions (such as a volcano explosion, catastrophic impact of a meteorite, global atomic war or spread out of deadly infections) may even lead to a serious depression, and in limit, extinction of the population density of certain plant specimens on our planet. On the other hand, there are also many plant species close to the edge of their disappearance under the currently deterioration effect of changing environment or even under a routine fatality of species vanishing. In the sphere of the present vast progress in genomics and the ongoing growth of genetically affected plant there is a supplementary call for the exigency to store plants with their entire genetic information preferably using the original wild-plant sources?

Fortunately, it is not necessary to store a whole plant but it is enough to have its buds or even any meristematic cell or tissue because the plant cells are totipotent; therefore it is possible to store only a small

part of plants, mostly a part of meristematic tissues of vegetatively propagated plants [4] or a seed axis of generatively propagated plants. The plant storage at low or even at ultra low temperatures (cryopreservation) is one of the appropriate ways how to keep their viability in a long term prospect.

However, the intracellular ice-crystal formation, which habitually occurs during the lower temperature exposure of plants, is in every case lethal for plant survival because of the inherent volume expansion of newly formed ice in comparison with the original volume of liquid. It grants a consequent destruction of interfacial cell membranes, sometimes happening even on reheating. One way how plants can withstand the low temperatures in the nature is their tolerance to movement of intracellular water to extra-cellular domains, which, in result, bestows a higher tolerance to cell dehydration [4, 5]. However, the undercooled states possess frequently no dehydration effects neither ice-crystal formation, but this state is noticeably unstable providing somehow not fully unpredictable processes of ice recrystallization upon reheating [4]. The exceedingly low viscosity at rapidly cooled conditions of 'freeze-in' states give often birth to the new non-crystalline state of rigid glasses and such a process of vitrification is not associated with any volume changes exhibiting only the so-called glass transformation region detectable by thermometric measurements. The freeze-in states nearly hinder any diffusion of molecules excluding

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thus biochemical transforms and, accordingly, also any genetic changes.

The formation of glassy state is yet not fully understood but expedient to help a successful storage of plants at low temperature of liquid nitrogen. From the point of view of cryopreservation, the glassy state [6, 7], which possesses the same volume as liquid, occurs as the most suitable state for a long-term biological storage of germplasm. This strategy of “being in the glassy state”, is repeatedly exploited by plants in the nature in order to withstand unfavorable freezing conditions of surrounding environment, which can be seen as a sort of miracle. For example, plant pollen flying in the air has a potential to be in glassy state keeping its viability after stigma pollination. Dormant poplar twigs can survive the low temperatures being in the glassy state in midwinter. Some sort of potatoes can be hardened to withstand low temperatures by gradual lowering their storage temperatures. Certainly, it is possible to add other examples of glassy state occurrence in plant species in the nature; however, the knowledge of conditions at which the glassy state is induced is not fully understood though the glass formation in plants can be of help to control the ‘cryogenic status’ at which the optimal undercooling achieves a suitable cryopreservation of plant germplasm.

The process of vitrification [6] is, however, closely associated with the anomalous behavior of liquid water [7], its apparent tendency to pentameric organization within the liquid state at decreasing temperatures as well as its possible interaction [8] with plant cells, which structures possess fractal and self-affinity architecture [1, 8, 9], cf. Fig. 1, therefore irreconcilable to yield focal points for the ice matching nucleation. Consequently, it seems to be necessary to reiterate some basic data about the structure of liquid water, its structural quasi-periodicity, which becomes factually important for the formation conditions of quasi-crystalline nuclei and their capability to form either state: biologically optimal glass or hazardous intracellular ice.

Structuring in the undercooled water and its impact to the glass-formation tendency

It is clear that life on Earth depends on the unusual structure and as all organisms consist mostly of liquid water. This water performs many functions and it can never be considered simply as a kind of inert diluents or filler, because it carries the transports, makes lubrication, helps reactivity, evaporative cooling, stabilizes makeup, structures and partitions arrangement and may exhibit different character on the contact with biological surfaces or within

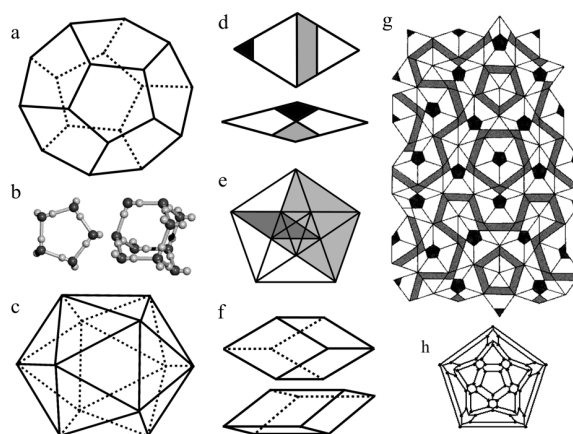


Fig. 1 Some constructions (associable with the cluster structure of liquid water): a – Dodecahedron, which Plato associated with the firmament, b – the models of cyclic pentamer and tricyclo-decamer, which are a relatively stable small clusters of water easy interconnecting with the larger clusters of yet below c – icosahedron (which geometry Plato associated with water). d – Diamond (so called Penrose) basic tiles with a specific shape called rhombus can be expanded to cover greater areas, g – forming an unbroken structure where we can localized both the chains (like polymeric water) and h – pentagons (like water clusters, where the below connectivity map of the water molecules shows the molecules orderliness within an icosahedron). Such a structuring can also be applied to a spatial distribution if the two kinds of f – rhombohedra are assembled to form icosahedron matching thus the larger clusters of water discussed in the text in more details

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membranes. The living world should be thought of as an equal partnership between the structure of biological molecules and the architecture of water clusters. Recently the water was also shown to be the best vehicle medium when assuming proton-issued quantum diffusion [10]. In spite of numerous studies, many of the properties of water are yet puzzled not mentioning yet unidentified relations to living organisms [11]. Enlightenment comes from a better understanding that water molecules form an infinite hydrogen-bonded network with confined and structured clustering which become more intensive and better localized under decreasing temperatures. Recent studies done via molecular dynamics [12] identified experimentally water clusters, which grow in size and become more compact as temperature decreases. Their size can be characterized by a fractal dimension consistent with that of lattice animals having the correlation length proportional to the configurational entropy. Upon cooling, ice-like crystals were more easily transformed from the smaller clusters of $(H_2O)_n$ with n up to 20, more likely

existing at temperatures $>5^{\circ}\text{C}$, while the larger clusters ($n>20$) were readily altered to glassy structures at temperatures $\ll 0^{\circ}\text{C}$ [13]. The apparent decrease of the glass transition temperature with the increasing cluster size was experimentally authorized to be fewer effective than the corresponding change of melting temperatures. Consequently, the mutual succession of the melting and glass-transition temperatures was found even inverted when compared with that observed for bulk water.

In order to get a better understanding of processes of water crystallization and/or vitrification it is necessary to look at the cluster size development [14]. Small standard assemblies of the four-bonded molecules of liquid water may join at lowered temperatures together to form cyclic pentamer, tricyclo-decamer (cf. Fig. 1b) and large bicyclo-octamers, which arises from the competition between maximizing Van-der-Waals interactions (higher orientation entropy and thus density) and maximizing hydrogen bonding. Though cohesive in nature the hydrogen bonding is curiously holding the water molecules apart, which endows water with many of its yet unusual properties. The tricyclo-decamer and by themselves forming a well symmetric but spatially quasi-periodic $[(\text{H}_2\text{O})_{280}]_x$ icosahedral macromolecules may contain a variety of substructures being able to interlink and tessellate throughout the volume. A mixture of water containing cyclic pentamers and tricyclo-decamers can bring about similar ensuing clusters, which provide aperiodic tiling (i.e., a continuous pattern that never repeats exactly).

Some constructions, which are associable with the cluster structure of liquid water can be found tiling in a 5-fold symmetry that were thought, for long run, as impossible to fill areas completely and regularly [8], Fig. 1. Upper thick rhomb (d) has longer diagonal equal to the famous 'golden ratio' phi ($\phi=1.618034\dots$), which is related to the number 5 by formulae $(1-\sqrt{5})/2$. Fascinatingly, it is playing a crucial role in various aspects of natural creations and art constructions. The thinner rhomb has his shorter diagonal equal to $1/\phi$ and both rhombs can be derived from a shaded area of pentagon (e), which all five diagonals match ϕ and which 5-side structure leaves gaps when used to be continuously repeated in space. On the other hand, the derived rhombs can fill the surface in an asymmetrical and non-replicating manner (Fig. 1g), which is known as a continuous but non-repeating structure [8] (sometimes called 'quasi-crystals' when recently discovered in quenched glassy alloys). On expanded tiling to cover greater areas, the ratio of the quantity of thick rhombs to thin ones approaches ϕ again and if the rhombs are

marked by shadow strips they form the unbroken structure (g) where we can localize both the chains (like polymeric water) and pentagons (like water clusters), where the below connectivity map of the water molecules shows the molecules orderliness within an icosahedron (h). Such a structuring can also be applied to a spatial distribution if the two kinds of rhombohedra are assembled (Fig. 1f) to form icosahedron matching thus the larger clusters of water discussed in the text in more details.

All of three basic clusters are comparatively stable and it is expectable that their interaction can produce larger embryos of icosahedra (IS), which geometry was already foretold as a water geometrical representation by the Greek philosopher Plato (427-347 BC) [9, 15], Fig. 1c). Dynamically it forms a continuous but quasiperiodic network of open, low-density and condensed structures. Such a fluctuating and rather self-replicating network of water molecules [16], with localized and overlapping of clusters, can interconvert between lower and higher density forms by bending some of the hydrogen bonds but not by their interruption.

Less common water dodecahedra (cf. Fig. 1a) have been found in various aqueous solutions (curiously in the gas phase, too) and such clusters were observed at hydrophobic and protein surfaces, where low-density water with stronger hydrogen bonds and lower entropy has been noticed.

Water clustering and biological systems

There exists a certain dynamical equilibrium with its collapsed, but more frequent form of more symmetrical dodecahedra (CS). The position of the $\text{IS} \leftrightarrow \text{CS}$ equilibrium around biological molecules was revealed to be important for their proper activity, e.g., with the enzyme balanced between flexibility (IS-environment) and rigidity (ES-environment). Character of water is critical not only for the correct folding of proteins [17] but also for the maintenance of this structure due to the impact of heat, i.e., denaturation and loss of its biological activity, which is likely linked to the breakup of the 2-dimensional spanning water network around the protein (due to increasing hydrogen bond breakage with temperature) restrictive on the protein vibrational dynamics. As the temperature increases the average cluster size, their integrity and the proportionality in low-density forms decrease and warmer water ($\sim 15^{\circ}\text{C}$) thus develops a predisposition to solidify to ice-crystals more promptly upon quick cooling than colder water does. Therefore the highly clustered water near freezing becomes incommensurable with hexagonal ice, which effect is the factual source for

easy undercooling of the liquid water but, on the other hand, does structurally correlate with the non-crystalline state of glass. This is not only important fact for a quick preparation of ice in a refrigerator but explains the higher sensitivity of plants to freeze down under big temperature drops when temperature starts to decline from higher temperatures.

The water clusters can tessellate in three dimensions as each cluster has twelve potential sites at its icosahedral vertices to be exploitable as centers for the neighboring overlapping clusters. However, when the network grows the structure, only locally symmetrical, becomes more distorted in longer order, and expectably, the structure then becomes quasi-periodic while true tessellation can be possibly achieved by the clusters pulsating between expanded and collapsed structures. Though such an icosahedral cluster is capable of continuous but non-periodic tiling it is incapable of direct tessellation to such a crystalline structure that is needed for the congruous nucleation of hexagonally ordered ice due to its residual (and resistant) five-fold symmetry. Collapse of all four dodecahedral structures would be expected to increase the density about a further three-fold form that between the (ES) and (CS) structures to gives a density of about 1.18 g cm^{-3} close to the 1.17 g cm^{-3} [18]. Such a collapse also gives an increase in the closely approaching the so-called 'interstitial' water, when some hydrogen-bonds are removed from given water molecules. Therefore it would not be surprising that the neighboring organic molecules can drastically change the conditions for water makeover, particularly if scanning the fractal surface image of their biological interfaces, which tends to exhibit the pentagonal-like symmetry, too, yet supporting above mentioned incommensurability equally applicable to an interfacially induced heterogeneous nucleation of ice [19, 20]. The icosahedral structure contains large that may allow occupancy by suitable solute (e.g., sucrose) or gases (CH_4) and the vicinal water on solid surfaces uses to exhibit further, rather curious 'extra-transitions' to occur roughly at temperatures 15, 30 and 45°C , possibly caused by the gradual breakage of hydrogen bonds due to the differences in surface potentials.

Let us mention that all of the natural ice on the earth is hexagonal ice, which is well manifested in the existence of six-cornered snowflakes. However, at lower temperatures and at higher pressures (above 2 kbar) many other ice phases with different crystalline structures exist, including various forms of glasses (phase diagram reveals it at about -130°C). No other known substance exhibits such a high variety of forms. If water vapor is forced to condense on a cold substrate below -80°C , a cubic modification of ice is formed,

which is related to the classical ice in the same way as a cubic diamond is related to a hexagonal diamond having also almost the same density. However, below -110°C a non-crystalline amorphous solid known as low-density glassy ice appears.

For biological purposes there is important electrical conduction in ice, which is carried by the protons, whose motion is closely tied to the motion of ionic defects. Since the small proton jumps occur collectively along a favorably oriented chain of H-bonds, the ionic defects and the effective charge carried by them have a very high mobility in the ice structure, ten times greater than the mobility of ions in normal ionic conductors. Protonic conduction [10] plays a major role in biological charge transfer processes across membranes or along nerves and the proton conduction in ice and in other H-bonded materials is periodical in nature [9] and analogous to electron conduction in semiconductors. Besides the protonic quantum behavior the self-organization can commence with the soluble cations of sodium and calcium. These cations exhibit most excellent decoupling from the classical noise (of thermally agitated mechanical impacts) so that they can move more straightforward in aqueous solutions being thus 'quantum' sensitive [1, 9, 10, 21].

Also the surface of ice shows unique properties as near the melting point, the surface contains many dangling broken bonds that promote the existence of a liquid-like layer (needed for skating) also of importance to biological contacts. In a whole class of solids, such as clathrates and all biological molecules, where the ice forms a H-bonded host lattice, that can encage a great variety of small guest atoms or molecules like argon or methane (large resources of methane hydrates deposited at higher static pressure of water on the sea bottom). Many forms of ice can be viewed as self-clathrates, where two equal ice lattices are interpenetrating each other but are not H-bonded to each other. Ice-like structures or structurally adjusted water molecules are encountered in hydrates, and hydrated compounds including all macromolecules that would not be biologically active without the presence of their structured water or ice-like shells, which can be viewed as a partially broken structure of distorted ice [22, 23]. Surely, there are many other phenomena, which can strongly effect the character of liquid water and its clustering behavior as that all is falling, however, beyond the scope of this brief article.

Natural plant behavior while exposed to freezing

Woody plants tolerating ice crystals in their tissues have two major strategies to survive temperatures

below zero. The first living strategy is aimed to avoid freezing by either undercooling or lowering of melting point of their tissues and organs [24–26]. Undercooling is a known effect in many plants and plant tissues, such as parenchymatic cells [27], whole organs as generative buds of [28, 29], or vegetative buds of [30]. The level of supercooling goes down to -41°C [31] as the solid hexagonal ice have the structure that is uncanonical with respect to the pentagonal-like symmetry of a solidifying liquid water that contains a high number of incommensurable nuclei of icosahedra and dodecahedra.

The second living strategy of plants is tolerating of extracellular freezing [32, 33]. Survival of such plant cells/tissues is based on their tolerance to excessive dehydration of the protoplast. Some plant tissues survive temperatures even below -40°C in the nature and in the techniques used for preservation of plant genetic material they survive cryo-temperatures as low as -196°C . The third strategy of plants to outlast the sudden frost is the process called extraorgan freezing [32]. This type of strategy protects the whole plant organ, such as bud, against ice nucleation and ice spreading and the external ice formation exposed the bud to frost dehydration [33]. The above mentioned survival strategies of plant species while tolerating ice in their tissues can become combined, for example, the woody plants can exhibit extraorgan freezing in their buds, extracellular freezing in their bark tissues and supercooling in their parenchymatic cells of xylem rays [34]. The question is what the state of water or, better to say, the state of protoplasm is in the plant cells subjected to subzero and low temperatures. Physical rules states that undercooled liquid can change into the form of glass during the process of glass transition at the narrow temperature range (T_g). The glassy state found in dormant twig of a poplar tree [33] offers the explanation of what states of solid water can be found in frozen tissues supposing that this state occurred thanks to freezing dehydration of tissues.

The aim of our ongoing project is aimed to the characterization of the state of water in some selected species specified for the germplasm storage in ultra low temperatures (liquid nitrogen) such as apple dormant bud or garlic shoot tips [3].

Peculiarities of thermal analysis applied to cryogenic samples

Thermal analysis methods [1, 6] are of a favorite use in the study of thermal behavior of both phenomena observed under the process of rapid cooling, ultra low temperature storage and re-heating. We are particularly curious about the determination of concen-

tration-temperature dependences of glass transition as well as the examination of phase relations in the simulated systems such as aqueous carbohydrate mixture [35, 36]. The shoot tips of garlic (*Allium sativum* L. landrace Djambul 2) dissected from bulblets were dehydrated over various saturated salt solutions of known water activity until the steady state of water was reached. The temperature of glass transition was measured in a heat flow type calorimeter, during cooling and warming with rate of $10^{\circ}\text{C min}^{-1}$ rate. For higher survival of garlic shoot tips after dehydration and cooling/warming we often used alginate bead with encapsulated shoot tip during dehydration. A typical

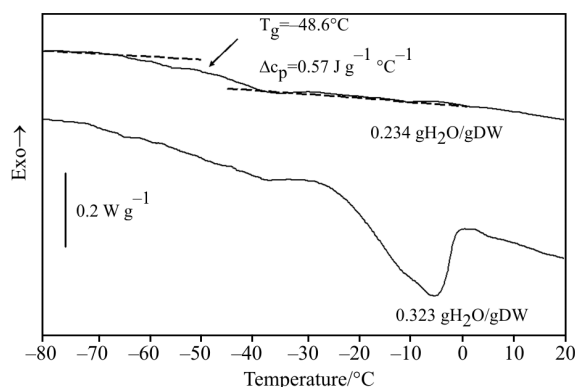


Fig. 2 Thermal characteristics of shoot tips dissected from bulblets of *Allium sativum* L. landrace Djambul 2 measured by DSC. The endothermic peak was measurable at water content $0.323 \text{ gH}_2\text{O/g dry mass}$. After dehydration to the lower water content ($0.234 \text{ gH}_2\text{O/g dry mass}$) the glass transition (T_g) with typical difference in a heat capacity occurred. The measurement were made during heating $10^{\circ}\text{C min}^{-1}$ (after previous cooling at $10^{\circ}\text{C min}^{-1}$)

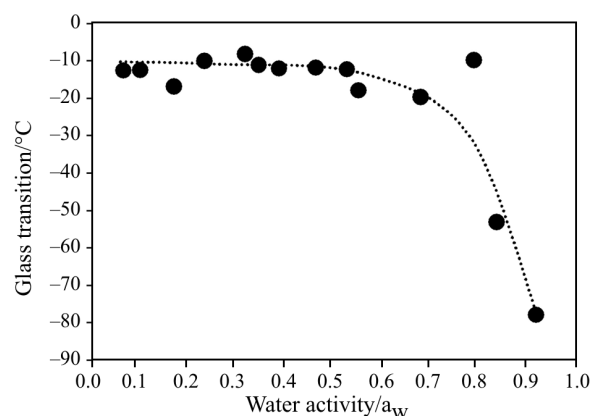


Fig. 3 Typical result of DSC study providing the plot of the degree of dehydration vs. the glass transition temperatures (T_g 's). The shoot tips of garlic (*Allium sativum* L. landrace Djambul 2) dissected from bulblets were dehydrated till the equilibrium with water activity of vapor above saturated salt solutions. The glass transition temperature was measured, during warming

output from our DSC measurements is depicted in Fig. 2. The development of temperature of the glass transition, shows (Fig. 3, shows relationship with the water activity of the plant shoot tips).

Conclusions

In the outlook of present vast progress in genomics, the deterioration effect of changing environment and the ongoing disappearance the biodiversity of plant species there is an important appeal for the exigency to store plants with their complete genetic information typically inherent in the original wild-plant samples. The convenient cryogenic methods need, however, allocation of addition data of the thermal behavior of species subjected to the process of thermal quenching and consequent reheating. The measurement of glass transition temperature by DSC/DTA is a good tool for estimation of optimal level dehydration to the optimal level for cryopreservation. The information about glass transition temperature also serves for checking of cryopreserved plant material during storage in liquid nitrogen.

Portrayal of nonperiodic but continuous patterns of water on basis of variously shaped tiles [8] may be of help in other geometrical models used, e.g., in the phenomenological description of reaction mechanism in chemical kinetics [1, 9, 37].

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