Multiple Glass Transitions and Freezing Events of Aqueous Citric Acid

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ABSTRACT: Calorimetric and optical cryo-microscope measurements of 10–64 wt % citric acid (CA) solutions subjected to moderate (3 K/min) and slow (0.5 and 0.1 K/min) cooling/warming rates and also to quenching/moderate warming between 320 and 133 K are presented. Depending on solution concentration and cooling rate, the obtained thermograms show one freezing event and from one to three liquid–glass transitions upon cooling and from one to six liquid–glass and reverse glass–liquid transitions, one or two freezing events, and one melting event upon warming of frozen/glassy CA/H2O. The multiple freezing events and glass transitions pertain to the mother CA/H2O solution itself and two freeze-concentrated solution regions, FCS1 and FCS2, of different concentrations. The FCS1 and FCS2 (or FCS22) are formed during the freezing of CA/H2O upon cooling and/or during the freezing upon warming of partly glassy or entirely glassy mother CA/H2O. The formation of two FCS1 and FCS2 regions during the freezing upon warming to our best knowledge has never been reported before. Using an optical cryo-microscope, we are able to observe the formation of a continuous ice framework (IF) and its morphology and reciprocal distribution of IF/(FCS1 + FCS2). Our results provide a new look at the freezing and glass transition behavior of aqueous solutions and can be used for the optimization of lyophilization and freezing of foods and biopharmaceutical formulations, among many other applications where freezing plays a crucial role.

1. INTRODUCTION

Citric acid (CA), C6H8O7, is a weak organic acid that is naturally encountered in a variety of fruits and vegetables, especially lemons and limes, and is commercially produced in very large amount by microbial fermentation of carbohydrates. The usual form of CA is CA-monohydrate, C6H8O7·H2O, which is crystallized by the slow evaporation of water from cold saturated solutions, whereas anhydrous CA is crystallized from hot saturated solution. CA by virtue of its hydroxyl −OH and carboxyl −COOH groups is capable to form hydrogen bonding between CA molecules themselves and with solvent H2O molecules, which results in a variety of unique properties of CA/H2O solutions. Strong hydrogen bonding is believed to be a property that may prevent the crystallization of CA from cooled melt/solutions and be responsible for a liquid–glass transition that is important for freeze-drying (lyophilization) and freezing of pharmaceutical formulations. CA is widely used in foods and beverages as a flavoring and preservative additive, industry, pharmaceuticals as excipient for freeze-dried formulations, and in order to produce amorphous multicomponent excipients having high glass transition temperature, Tg, which could reduce or prevent protein denaturation in lyophilized formulations. CA increases solubility of poorly water-soluble drugs and efficiently maintains pH in the range from 3 to 6.2 that increases the stability of therapeutic proteins in frozen large scale formulations during storage. CA is also used for the solubilization and sustained delivery of anti-HIV (human immunodeficiency virus) drug and in tissue engineering (synthesis of biodegradable scaffold), biochemistry, etc.

Although CA is widely used in the different fields of science, industry, and low temperature processing, there are still gaps in understanding of freeze-induced phase separation into pure ice and freeze-concentrated solution (FCS), glass transition behavior of formed FCS, and the morphology and reciprocal distribution of ice and glassy FCS in frozen solutions. In this article, we present the differential scanning calorimetry (DSC) and optical cryo-microscope (OC-M) results of the study of 10–64 wt % CA solutions subjected to different cooling and warming rates. Our results obtained from the combined DSC and OC-M measurements give a clear picture of how ice and FCSs of different concentrations are formed during the freezing
upon cooling and subsequent warming and reveal a variety of liquid—glass and reverse glass—liquid transitions, which have not been observed before.

2. EXPERIMENTAL SECTION

We prepared 10–64 wt % CA solutions by mixing >99% anhydrous citric acid (Merck) with the corresponding amount of ultrapure water. The solutions of concentration larger than the solubility limit of ~62 wt % CA at 295 K\textsuperscript{23,24} were prepared by slow heating. We studied the phase transformations and glass transitions of CA/H\textsubscript{2}O using a Mettler Toledo DSC 822 calorimeter at the scanning cooling/warming rate of 3, 0.5, and 0.1 K/min in the temperature region between 320 and 133 K. Such cooling rates are usually applied during the lyophilization of large scale food and biopharmaceutical formulations. For the DSC measurements, we loaded and then cold sealed a half-sphere solution drop in an aluminum (Al) crucible of 40 μL by volume. The mass and diameter of drops were ~5–6 mg and ~1.5 mm, respectively. We also performed measurements at 3 K/min upon warming of quenched CA/H\textsubscript{2}O drops. To this end we placed an Al crucible with CA/H\textsubscript{2}O drop into liquid N\textsubscript{2} and then immediately inserted the crucible into the precooled calorimeter. In this procedure, the cooling rate is estimated to be 100–1000 K/s. DSC calibration and details about measurements are described elsewhere.\textsuperscript{25,26} To verify the reproducibility of results, we performed a number of repeated DSC measurements, which were of two types: (i) we sometimes measured the same drops 2–3 times, and (ii) we always performed measurements of 3–4 different drops of the same concentration.

To observe the freezing process in situ and the morphology of ice/FCS(s), we prepared ~5–10 μm thick solution films (“2-dimensional” solutions)\textsuperscript{27} and studied them with an optical cryo-microscope Olympus BX51 equipped with a Linkam cold stage and Linksys32 temperature control and video capture software. We performed optical cryo-microscope measurements at 3 and 5 K/min in the temperature region between 320 and 163 K.

3. RESULTS AND DISCUSSION

3.1. Cooling and Warming of CA/H\textsubscript{2}O at 3 K/min. In Figure 1, we present cooling and warming thermograms obtained from 10–64 wt % CA solutions. In the cooling thermograms, an exothermic \( T_f \) peak indicates the latent heat of fusion emitted during freezing.\textsuperscript{25,26} In the warming thermograms, an endothermic \( T_m \) peak is due to the absorption of latent heat during ice melting. The sharpness of the ice freezing \( T_f \) peak in the thermograms for 10–50 wt % CA indicates that the freezing process proceeds rapidly. Our numerous OC-M observations of frozen CA/H\textsubscript{2}O solutions\textsuperscript{27} show that such fast freezing process is always initiated from one ice nucleating event, as is demonstrated in Figure 2a,d. In supercooled solutions of this concentration range, the first nucleated critical ice embryo grows very rapidly and emits a large amount of latent heat of fusion. We surmise that the emitted heat raises the temperature of the surrounding liquid and prevents the nucleation of other critical ice embryos. In contrast, more concentrated solutions, namely, 55, 56, and 60 wt % CA, freeze from multiple ice nucleating events,\textsuperscript{27} as is also demonstrated in Figure 2b. The 55, 56, and 60 wt % CA cooling thermograms contain a prolonged, broad freezing \( T_f \) peak (cf. Figure 1). Such prolonged freezing is caused by the steep increase of viscosity,
which progressively slows down ice growth and, consequently, reduces the emitting of the latent heat of fusion as temperature decreases. In this case the amount of the emitted heat per unit time is less than that withdrawn by the applied cooling rate of 3 K/min. As a result, the supercooling of surrounding liquid increases with decreasing temperature, and this results in the appearance of new ice nucleating events.

In our DSC measurements, CA/H2O drops/solutions are placed on Al crucibles and, consequently, freeze heterogeneously. Figure 1 shows that the heterogeneous freezing temperature (Tf peaks) of 10–45 wt % CA is not a monotonic function of concentration. This freezing behavior differs from that of inorganic solutions, for example, (NH4)2SO4/H2O, in which a monotonous decrease of Tf is observed upon increasing solution concentration.25 We suggest that the difference can be accounted for by concentration inhomogeneities in CA/H2O, which result from the enhanced tendency of CA/H2O to form molecular CA clusters at low temperature.8,28 Supercooled CA/H2O solutions can be viewed as arbitrarily distributed concentrated regions containing a large number of CA clusters and less concentrated regions with fewer CA clusters. Heterogeneous freezing is initiated at a point where the configuration of local electrical fields of the Al substrate and H2O molecules favors the nucleation of critical ice embryo. Naturally, the onset of freezing will be governed by the warmest ice nucleating site on the Al substrate, which is in contact with a less concentrated patch within the solution. In contrast to freezing, the melting of ice is an equilibrium process, and therefore, the peak melting temperature, Tm, is a monotonic function of concentration, as is seen in Figure 1. Figure 1 also shows that in contrast to the sharp ice freezing Tf peaks, the ice melting Tm peaks are not sharp but occupy a large temperature region, which is warmer than that of Tf peaks, i.e., melting always occurs at temperature warmer than that of freezing. The melting of ice starts at the ice/FCS interface at very low temperature and gradually propagates to warmer temperatures as concentration decreases due to ice melting.

In the DSC method, a hallmark of glass transitions is a baseline step indicating a heat capacity change, ΔCp.33 An increase in heat capacity (upon heating) indicates a glass–liquid transition, whereas a decrease in heat capacity (upon cooling) indicates the reverse process, a liquid–glass transition. Figure 1 shows that the cooling thermogram of 60 wt % CA contains a prolonged ice freezing Tf peak and a liquid–glass transition with the onset at Tg1,c ≈ 185 K. Unexpectedly, in the warming scan for 60 wt % CA we find two glass–liquid transitions, Tg1,w and Tg2,w. Instead, we would expect that for each liquid–glass transition observed calorimetrically upon cooling there should be exactly one reverse glass–liquid transition upon warming at about the same temperature. In addition to the unexpected second glass–liquid transition we also observe a prolonged exothermic ice crystallization peak, Tcr, upon warming. To explain the Tcr transition we resort to simpler thermograms: In the cooling thermogram of 62 and 64 wt % CA, there is no freezing event but only a liquid–glass transition with the onset at Tg1,c ≈ 189 and 191 K, respectively. During the cooling of these solutions, their viscosity increases so steeply that it completely suppresses the freezing process. Upon subsequent warming above the reverse glass–liquid transition, Tg1,w, the glass "melts" to a highly viscous liquid (HVL).34 Upon further warming the viscosity of HVL decreases and ice starts crystallizing to produce an exothermic peak Tcr. The onset of ice crystallization is more than 40 K warmer than Tg1,w, i.e., the HVL of CA/H2O persists in a surprisingly large temperature window.

The thermograms of 10–60 wt % CA as displayed in Figure 1 do not give sufficient information for the comprehensive understanding of the freezing and thawing phenomena. In Figures 3 and 4, we present magnified cooling and warming thermograms, which reveal transitions not visible in Figure 1. In Figure 3, a subtle exothermic event around 280 K marked by an open arrow is most likely due to the transformation of anhydrous CA to CA-monohydrate.5,35 In the 56 and 60 wt % CA thermograms, the ice freezing peak Tf is truncated to fit the figure.
ice-melting peaks were indeed observed in the thermograms obtained upon warming of frozen gelatinized-starch.\textsuperscript{37} However, in our case the warming thermograms reveal only one ice-melting peak, $T_m$, which is due to the melting of ice surrounded by FCS (Figures 2 and 4). Further, we did not observe the exothermic event around 280 K in the cooling thermograms of pure water and other aqueous solutions, for example, (NH$_4$)$_2$SO$_4$/H$_2$O.\textsuperscript{23} Thus, in Figure 3, the prolonged exothermic event around 280 K cannot be due to the condensation of water vapor on the DSC lid.

Figure 3 reveals an interesting shape of the $T_\text{f}$ peak: while it is sharp at the onset of freezing it becomes broader at the low-temperature, indicating sluggish freezing.\textsuperscript{27} In addition, the broad, inclined tail is carrying signatures for two liquid–glass transitions with the onset at $T_{g1,c} \approx 220$ and $T_{g2,c} \approx 208$ K, respectively. We observe the sluggish freezing process and the $T_{g1,c}$ and $T_{g2,c}$ transitions at the same onset temperatures in the cooling thermograms of all solutions of $\leq 56$ wt % CA. Recently we reported that the $T_{g1,c}$ and $T_{g2,c}$ transitions are indicative of two freeze-concentrated solutions of different concentrations, FCS$_1$ and FCS$_2$, which are formed during freezing.\textsuperscript{27} The FCS$_1$ is maximally freeze-concentrated and entangled with the twisted ice branches of a continuous ice framework, IF, whereas FCS$_2$ envelopes the entire IF/FCS$_1$.\textsuperscript{27} The mutual distribution of IF and (FCS$_1$ + FCS$_2$) is demonstrated in the pictures of frozen CA/H$_2$O in Figure 2. On the basis of our numerous in situ OC-M observations of freezing in supercooled CA/H$_2$O and sucrose/H$_2$O solutions, we reported that the sluggish freezing process proceeds in FCS$_2$ and it is terminated by a FCS$_2$–glass transition, $T_{g2,c}$ at low temperature.\textsuperscript{27} Correspondingly, the FCS$_1$–glass transition at higher temperature is related to $T_{g1,c}$. Thus, upon cooling of 10–56 wt % CA solutions, one freezing event, $T_\text{f}$ and two liquid–glass transitions with the onset temperatures of $T_{g1,c} \approx 220$ and $T_{g2,c} \approx 208$ K are observed.

In Figure 4, we present magnified warming thermograms from Figure 1. It is seen that in 10–56 wt % CA thermograms, a reverse glass–liquid transitions, $T_{g2,w}$ and a second transition $T_{g1,w}$ are clearly visible. In the past, these transitions have been only observed upon warming of frozen hydrocarbon solutions.\textsuperscript{35–37} Historically, they were called $T_{g1,w}$ and $T_{g2,w}$ transitions, where the former has been related to a glass–liquid transition. Recently, we reported that the $T_{g1,w}$ transition corresponds to the glass–liquid transition in the FCS$_2$ part of the sample. That is, it represents the $T_{g2,w}$ glass–liquid transition, which is the reversion of the liquid–glass transition, $T_{g2,c}$ observed upon cooling.\textsuperscript{27} However, the $T_{g1,w}$ transition does not look like a genuine glass–liquid transition because of a clearly pronounced exothermic feature. The nature of the $T_{g1,w}$ transition has remained misunderstood for decades.\textsuperscript{38–41} We demonstrated in videos, in which the freezing process was visualized in situ, that upon warming above $T_{g2,w}$ the terminated sluggish freezing of FCS$_2$ recommences and continues also in the temperature region of the $T_{g1,w}$ transition.\textsuperscript{27} We concluded that the $T_{g1,w}$ transition is a net thermal effect produced by the heat capacity change $\Delta C_p$ of a reverse glass–FCS$_2$ transition, $T_{g2,w}$ and the latent heat of fusion emitted during the recommenced sluggish freezing of FCS$_2$.$^{27}$ One can see in Figure 4 that the onset temperatures of $T_{g2,w}$ and the temperature region of the $T_{g1,w}$ transition are well reproducible and independent of the initial solution concentration. We estimated the onset temperatures to be $T_{g2,w} \approx 204$ K and $T_{g1,w} \approx 217$ K.$^{27}$ Thus, upon warming of frozen 10–56 wt % CA solutions, two glass–liquid transitions, $T_{g2,w}$ and $T_{g1,w}$, one freezing, namely, the resumed freezing of FCS$_2$, and ice melting at $T_m$ are observed.

In Figure 4, the $T_{g2,w}$ and $T_{g1,w}$ (or $T_{g1,l}$) transitions because they do not freeze upon cooling (Figure 1), and consequently, no FCS$_1$ and FCS$_2$ are formed. Therefore, only a reverse glass–liquid transition of $T_{g2,w}$ is observed upon warming. Further, Figure 3 shows that 62 wt % CA solution freezes only to a minor extent. The small amount of ice formed during freezing results in the formation of only a trace amount of FCS$_1$ and FCS$_2$, and so the $T_{g2,w}$ and $T_{g1,w}$ transitions are too subtle to be detectable with our DSC device. In Figure 5, we present OC-M pictures, which demonstrate how ice develops upon cooling and subsequent warming of 62 wt % CA solution. According to the cooling thermogram in Figure 3, the tiny exotherm indicating incipient freezing starts at $\sim 223$ K and ceases below $\sim 210$ K. Below this temperature the baseline is practically straight. Similarly, the pictures in Figure 5a,b demonstrate that between 216 and 193 K the ice indeed grows very slowly. According to the warming 62 wt % CA thermograms in Figure 4, the resumed ice growth in the HVL peaks between 220 and 230 K. Strong growth is also observed between 220 and 230 K in our OC-M measurements (cf. Figure 5c,d). By analogy to the phenomenon we observed upon cooling at $T_{\text{f}}$ phase separation into two freeze-concentrated solutions of different concentrations (FCS$_1$ and FCS$_2$) may also occur during the freezing at $T_{\text{f}}$ upon warming of the HVL. However, while FCS$_1$ and FCS$_2$ transform to glass upon continued cooling, they become more fluid upon continued warming.

The onset of liquid–glass transition at $T_{g1,c} \approx 185$ K is clearly visible in the thermogram of 60 wt % CA (Figure 3). The $\Delta C_p$ step at $T_{g1,c} \approx 208$ K is subtle but nevertheless visible on the
inclined exotherm of the slow freezing process. The FCS\textsubscript{1}—glass transition, $T_{g1,c}$ cannot be identified as it is completely concealed by the freezing $T_{f}$ peak. However, this does not mean that the $T_{g1,c}$ transition is absent. In fact, it has to exist because the FCS\textsubscript{1} forms during ice crystallization just like for the other concentrations for which $T_{g1,c} \approx 220$ K can be identified. Thus, upon cooling of 60 wt % CA, besides the freezing event of $T_{f}$ three different liquid—glass transitions with the onset at $T_{g1,c} \approx 185$ K, $T_{g2,c} \approx 208$ K, and $T_{g3,c} \approx 220$ K are produced. The three liquid—glass transitions require the existence of three liquid regions of different concentrations. How the two liquid regions of FCS\textsubscript{1} and FCS\textsubscript{2} are formed we described and demonstrated in videos in our recent work.\textsuperscript{27} In addition to FCS\textsubscript{1} and FCS\textsubscript{2}, the third region is a fraction of 60 wt % CA solution, which does not have enough time to freeze completely at the applied cooling rate of 3 K/min because of the steep increase of the viscosity of 60 wt % CA. Similar to $T_{g2,c}$ transition, which terminates the slow freezing of FCS\textsubscript{2} at ~208 K, the $T_{g3,c}$ transition terminates the freezing of remaining 60 wt % CA at ~185 K.

A natural question may arise, namely, whether the formed three (or two if concentration is less than 60 wt % CA) liquid regions of different concentrations may exist long enough in order to produce three liquid—glass transitions in a sample of only ~5 µL by volume subjected to the cooling rate of 3 K/min. It will be shown in the next subsection of 3.2, that the CA/H\textsubscript{2}O solutions subjected to the cooling rates as small as 0.5 and 0.1 K/min also produce two FCS\textsubscript{1}—glass and FCS\textsubscript{2}—glass transitions. This suggests that the main factor that determines the lifetime of the two/three FCS regions of different concentrations is viscosity, which governs the rate of molecular diffusion. Because of the limited rate of H\textsubscript{2}O diffusion between the two/three FCS regions, a concentration gradient is established between them. However, the volume of concentration—transition regions is much smaller than that of two/three FCS regions, and consequently, their liquid—glass and glass—liquid transitions are not visible in the thermograms in Figures 3 and 4.

The existence of three liquid—glass transitions upon cooling of 60 wt % CA requires the existence of three reversible glass—liquid transitions upon warming. We mentioned above that the warming thermogram of 60 wt % CA in Figure 1 contains only two glass—liquid transitions, $T_{f2,w}$ and $T_{f3,w}$ and a prolonged ice crystallization peak of $T_{cr}$. It is reasonable to assume that the third glass—liquid transition, namely, the reverse glass—FCS\textsubscript{1} transition, $T_{g1,w}$, is concealed by the prolonged $T_{cr}$ peak. Most likely, the prolonged $T_{cr}$ peak consists of two exothermic peaks, which are produced by the resumed slow freezing of FCS\textsubscript{2} and the resumed freezing of the unfrozen fraction of 60 wt % CA. To verify this assumption, we performed several additional DSC measurements of 60 wt % CA. The thermograms displayed in Figure 6 demonstrate that different amounts of 60 wt % CA remain unfrozen upon cooling of samples of similar mass (5.05, 5.74, and 5.64 mg). In the DSC method, peak areas are proportional to the amount of material undergoing a phase transition. It is seen that the area of the $T_{cr}$ peak and, consequently, the amount of unfrozen 60 wt % CA decreases from top to bottom in Figure 6. At the same time, the $\Delta C_{p}$ step of $T_{f2,w}$ transition decreases, whereas the $T_{f2,w}$ transition becomes more pronounced in the thermograms from top to bottom. Thus, the thermograms in Figure 6 demonstrate that the prolonged $T_{cr}$ peak indeed indicates resumed freezing of FCS\textsubscript{2} and freezing of unfrozen 60 wt % CA. The former transition is at the origin of the exothermic feature of the $T_{f2}$ transition, whereas the latter is at the origin of the exothermic $T_{f1}$ transition. Taking into account that the $T_{f2}$ transition is a net thermal effect produced by the resumed slow freezing of FCS\textsubscript{2} and reverse glass—FCS\textsubscript{1} transition, $T_{g1,w}$ (see discussion above and ref 27), we conclude that three reverse glass—liquid transitions with the onset at $T_{g1,w} \approx 180$ K, $T_{g2,w} \approx 204$ K, and $T_{g3,w} \approx 217$ K are experienced upon warming of frozen 60 wt % CA. Note that there is a difference of about 3–4 K between the onset temperatures of liquid—glass transition observed upon cooling and reverse glass—liquid transition observed upon warming as illustrated in Figures 1, 3, 4, and 6. Thus, the total number of freezing events, which are observed upon cooling and warming of 60 wt % CA is three, namely, the freezing upon cooling, $T_{f}$ and the resumed freezing of FCS\textsubscript{2}, and resumed freezing of the unfrozen 60 wt % CA upon warming.

3.2. Cooling and Warming of CA/H\textsubscript{2}O at 0.1 and 0.5 K/min. To verify whether the applied cooling rate impacts the appearance and onsets temperature of $T_{g1,w}$ and $T_{g2,w}$ transitions and, consequently, the concentration of FCS\textsubscript{1} and FCS\textsubscript{2} regions and mutual distribution of IF/(FCS\textsubscript{1} + FCS\textsubscript{2}), we scanned 40 and 50 wt % CA solutions at cooling/warming rates as small as 0.5 and 0.1 K/min. Since the glass transition is a kinetic phenomenon, glass transition temperatures, $T_{g}$ shift upon changing rates, by contrast to transitions of thermodynamic origin such as melting. Here, it should be noticed that DSC measurements performed at small scanning rates are not only time-consuming but also produce a small signal-to-noise ratio that may disguise the $\Delta C_{p}$ step.\textsuperscript{33} The rate dependence is summarized in Figures 7 and 8 for 50 and 40 wt % CA solutions, respectively. The thermograms obtained at 3 and 0.5 K/min are practically identical, i.e., the onset temperatures of $T_{f}$ freezing event, liquid—glass $T_{f1,w}$, $T_{f2,w}$ and reverse glass—liquid $T_{f1,w}$, $T_{f2,w}$ transitions are practically the same (cf. Figure 7). Also the thermograms obtained at 0.5 and 0.1 K/min (cf. Figure 8) are practically identical. Although the $\Delta C_{p}$ step of $T_{g1,c}$ and $T_{g2,c}$ transitions disappears in the noise, the $\Delta C_{p}$ steps

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure6.png}
\caption{Cooling and warming thermograms of three 60 wt % CA drops. The upper thermograms are those from Figure 1. The middle cooling thermogram is that from Figure 3. The mass of drops, from which the upper, middle, and bottom thermograms were obtained, are 5.05, 5.74, and 5.64 mg, respectively. $T_{g}$ and $T_{cr}$ mark the liquid—glass and reversible glass—liquid transitions of a fraction of 60 wt % CA, which does not freeze upon cooling. The remaining symbols have the same meaning as in Figure 4.}
\end{figure}
The onsets of these glass transitions are practically unshifted. The constant onset temperatures of \( T_{g1,c}, T_{g2,c} \) and \( T_{g1,w}, T_{g2,w} \) transitions indicate that the applied cooling rate does not impact on the concentration of FCS\(_1\) and FCS\(_2\). Thus, the negligible differences between the thermograms obtained at different cooling rates suggest that the freezing process in supercooled CA/H\(_2\)O proceeds in the same way at 3 K/min and at 0.1 K/min. In other words, the concentration of FCS\(_1\) and FCS\(_2\) regions and mutual distribution of IF/(FCS\(_1\) + FCS\(_2\)) is independent of the applied cooling rate. Our OC-M observations of IF formed during freezing (not shown) suggest that the applied cooling rate also does not impact the morphology of IF, i.e., on the thickness and spatial distribution of dendritic ice branches/protuberances as long as the degree of solution supercooling before freezing is similar.

### 3.3. Quenching of CA/H\(_2\)O and Subsequent Warming at 3 K/min.
We also investigated the impact of very large cooling rates on the freezing/thawing phenomenology. In particular, it is of interest to check whether vitrification or freezing takes place upon quenching. Furthermore, if freezing is still observed, it is of interest to check whether the appearance of \( T_{g2,w} \) and \( T_{tr2} \) transitions and, consequently, the concentration of FCS\(_1\) and FCS\(_2\) regions and the mutual distribution of IF/(FCS\(_1\) + FCS\(_2\)) is affected. In Figure 9, we present thermograms obtained upon warming of quenched 10–55 wt % CA solutions quenched in liquid N\(_2\). \( T_{g2,w} \) and \( T_{tr2} \) mark glass–FCS\(_2\) and glass–FCS\(_1\) transitions (see text for details). The circle shows a magnification of the \( T_{g2,w} \) transition in 50 wt % CA. Other symbols have the same meaning as those in previous Figures.

![Figure 7](image1.png)

**Figure 7.** Comparison of cooling and warming thermograms obtained from two different drops of 50 wt % CA cooled/warmed at the scanning rate of 3 K/min (upper thermograms) and 0.5 K/min. The mass of drops are 6.22 mg (upper thermograms) and 6.35 mg. The upper thermograms are from Figure 1. All symbols have the same meaning as those in the Figures above.

![Figure 8](image2.png)

**Figure 8.** Comparison of 40 wt % CA thermograms obtained at the scanning rate of 3, 0.5, and 0.1 K/min. The upper thermograms are those from Figure 1. The middle thermograms are obtained at the cooling and warming rate of 0.5 K/min. The bottom cooling thermogram is obtained at 0.1 K/min and warming thermogram at 0.5 K/min. A sharp exothermic peak at ~305 K is due to the transition from anhydrous CA to CA-monohydrate.\(^{35,36}\) All symbols have the same meaning as those in the Figures above.

![Figure 9](image3.png)

**Figure 9.** Warming thermograms of 10–55 wt % CA solutions quenched in liquid N\(_2\). \( T_{g2,w} \) and \( T_{tr2} \) mark glass–FCS\(_2\) and glass–FCS\(_1\) transitions (see text for details). The circle shows a magnification of the \( T_{g2,w} \) transition in 50 wt % CA. Other symbols have the same meaning as those in previous Figures.
quenched solutions the concentration of FCS\textsubscript{1} is slightly smaller than that of FCS\textsubscript{2} formed at 3, 0.5, and 0.1 K/min cooling rates, i.e., the FCS\textsubscript{1} formed during quenching is not maximally freeze-concentrated.

Figure 9 shows that 50 and 55 wt % CA thermograms drastically differ from those of 10–45 wt % CA. In contrast to 10–45 wt % CA solutions, which freeze during quenching, the 50 and 55 wt % CA solutions partly and completely transform to glass, respectively. This vitrification upon quenching is inferred from the appearance of the \( T\textsubscript{g,w} \) transition, which is first followed by a short temperature window, in which the HVL exists, and finally followed by ice crystallization (\( T\textsubscript{cr} \)). The \( \Delta C\textsubscript{p} \) step of the \( T\textsubscript{g,w} \) transition, the temperature window of HVL, and the area of the \( T\textsubscript{cr} \) peak are smaller for the 50 wt % CA solution than for 55 wt % CA, in spite of similar drop masses, i.e., 5.61 and 5.78 mg, respectively. This implies that the former solution partly freezes and partly vitrifies. Further evidence that ice and, consequently, FCS\textsubscript{1} and FCS\textsubscript{2} are formed during the quenching of 50 wt % CA is the existence of the \( T\textsubscript{g,w} \) and \( T\textsubscript{fi,22} \) transitions. The fact that the onset of the \( T\textsubscript{g,w} \) transition is \( \sim 4 \) K colder than that in Figure 4 indicates that the concentration of FCS\textsubscript{1} formed during quenching is less than that of FCS\textsubscript{2} formed at 0.1–3 K/min cooling rate. The smaller FCS\textsubscript{2} concentration results in lower viscosity and, consequently, colder onset temperature of the resumed freezing of FCS\textsubscript{1} (compare Figure 4). To verify whether there is a shift of the \( T\textsubscript{g,w} \) transition, in Figure 10, we compare the 50 wt % CA

![Figure 10. Comparison of the glass—liquid transition onsets in the warming thermograms of quenched (upper line, from Figure 9) and slowly cooled (3 K/min, bottom red line, from Figure 4) 50 wt % CA. All symbols have the same meaning as those in previous Figures.](image-url)

thermograms from Figures 4 and 9. It is seen that there is no visible shift of the warmer part of the \( T\textsubscript{fi,2} \) transition, which, as we already know, contains the \( T\textsubscript{g,w} \) transition. This fact suggests that there is no shift of the onset of the \( T\textsubscript{g,w} \) transition and, consequently, the FCS\textsubscript{2} solution expelled from ice during the quenching of 50 wt % CA is maximally freeze-concentrated, i.e., of the same concentration as FCS\textsubscript{1} solution expelled from ice during freezing at slow cooling rates.

A unique feature in the freezing/thawing of CA solutions can be seen in the 55 wt % CA thermogram in Figure 9. It does not contain the exothermic feature of the \( T\textsubscript{fi,2} \) transition, but a massive \( T\textsubscript{cr} \) peak. This indicates that no ice crystallizes upon quenching, and hence, no freeze-concentrated solutions form. Ice crystallizes, though, upon subsequent slow warming above \( T\textsubscript{g,w} \) when the formed HVL freezes (\( T\textsubscript{cr} \) peak). This phenomenon of cold-crystallization itself is not unique, but known for many quenched substances, including the single-component system amorphous ice.\textsuperscript{53} However, what is unique is the observation of two glass—liquid transitions above the cold-crystallization. The existence of two glass—liquid transitions upon warming at \( T > T\textsubscript{g} \) requires the expulsion of two glassy solutions of different concentrations during cold-crystallization of ice at \( T\textsubscript{cr} \). That is, we put forward that freezing upon warming also produces two freeze-concentrated solutions. Judging from the onset of the \( T\textsubscript{g1,w} \), transition at \( \sim 217 \) K, one of them is maximally freeze-concentrated, FCS\textsubscript{1} (cf. Figure 4), whereas the other (FCS\textsubscript{22}) is less concentrated and transforms from glass to liquid at \( T\textsubscript{g22,w} < T\textsubscript{g1,w} \). This phenomenon has the paradoxical implication that two distinct immobile, glassy solutions seem to be expelled upon cold-crystallization of ice. How can a glass be mobile enough to suddenly phase-segregate during freezing on the scale of micro- and millimeters? This mystery can easily be unraveled if we recall that the large amount of the latent heat of fusion associated with cold-crystallization at \( T\textsubscript{cr} \) intermittently heats the freezing solution so that the freeze-concentrated solutions, FCS\textsubscript{22} and FCS\textsubscript{1}, are in the mobile, liquid state for a moment. As the freezing process decays between \( \sim 194 \) and 198 K, the amount of emitted latent heat abruptly reduces and the sample temperature, and consequently, the temperature of FCS\textsubscript{22} and FCS\textsubscript{1}, start rapidly equilibrating with the environmental temperature due dissipation of heat to the environment. Since the environmental temperature at the \( T\textsubscript{cr} \) cold-crystallization is much colder than the onset glass—liquid transition temperatures \( T\textsubscript{g22,w} \approx 211 \) and \( T\textsubscript{g1,w} \approx 220 \) K, the FCS\textsubscript{22} and FCS\textsubscript{1} regions immediately transform to the glassy state after segregation. (We estimated the onset temperature of the FCS\textsubscript{22}—glass transition as \( T\textsubscript{g22,w} \approx 211 \) K because it should be \( \sim 3–4 \) K warmer than the onset temperature of the reverse glass—FCS\textsubscript{22} transition, which is \( T\textsubscript{g22,w} \approx 208 \) K as is seen in Figure 9.) Signatures for the double FCS\textsubscript{22}/FCS\textsubscript{1}—glass transition at \( \sim 194–198 \) K cannot be seen in Figure 9 because they are masked by the latent heat evolution. However, its effects are clearly visible in the aftermath of the cold-crystallization event in the form of the anomalously elevated descending warm-side shoulder of the \( T\textsubscript{cr} \) peak. In fact, the warm-side shoulder of the \( T\textsubscript{cr} \) peak should have been lower than the baseline of HVL because the heat capacity of ice is less than that of water, and therefore, the height of the baseline lowers during freezing and increases during melting, as has been shown in Figure 7 in ref 26. The elevation of the warm-side shoulder of the \( T\textsubscript{cr} \) peak is brought about by the large \( \Delta C\textsubscript{p} \) step of the double FCS\textsubscript{22}/FCS\textsubscript{1}—glass transition. That is, we suggest that also hidden liquid—glass transitions contribute to the complex phenomenology of CA solutions upon freezing and thawing. The formation of two FCSs (FCS\textsubscript{22} and FCS\textsubscript{1}) during the freezing upon warming to our best knowledge has never been reported before. (Note, the formation of one FCS during the freezing upon warming of quenched aqueous glycerol, ethylene glycol, sucrose, and glucose was observed for the first time by Luyet and Rasmussen\textsuperscript{34} (see Figure 1 in ref 54); however, then this freeze-induced phase separation upon warming was not understood.) Thus, the total number of liquid—glass and glass—liquid transitions that occur during the warming of quenched-glassy 55 wt % CA solution is five, namely, \( T\textsubscript{g} \approx 177 \) K, double FCS\textsubscript{22}/FCS\textsubscript{1}—glass transition in the temperature region of \( \sim 194–198 \) K, \( T\textsubscript{g22,w} \approx 208 \) K and \( T\textsubscript{g1,w} \approx 217 \) K. Following this line of thought, the total number of liquid—glass and glass—liquid transitions is even six in the case of quenched 50 wt % CA solution, namely, \( T\textsubscript{g} \approx 172 \) K, and double FCS\textsubscript{22}/FCS\textsubscript{1}—glass transition between \( \sim 182 \) and 186 K (in the tail of the cold-crystallization peak). \( T\textsubscript{g22,w} \approx 200 \) K, \( T\textsubscript{g22,w} \approx 208 \) K, and \( T\textsubscript{g1,w} \approx 217 \) K (cf. Figure 9).
similarity of concentrations of freeze-concentrated solutions in quenched and slowly cooled CA solutions as well as the very similar morphology observed in OC-M experiments suggests that the degree of supercooling prior to the heterogeneous freezing event is very similar at all rates studied here.

4. CONCLUSIONS
In this work, we present the results of DSC and OC-M measurements of 10–64 wt % CA solutions subjected to different cooling/warming rates, including the quenching of DSC crucibles with a drop into liquid N$_2$. We observe in the cooling thermograms of 10–56 wt % CA one freezing event, $T_g$ and two FCS$_g$–glass and FCS$_c$–glass transitions at $T_{g1,c} \approx 220$ K and $T_{g2,c} \approx 208$ K, respectively. In the corresponding warming thermograms, we observe the reverse glass–FCS$_g$ transition at $T_{g1,w}$ $\approx 204$ K, $T_{g2,w}$ transition, and prolonged ice melting event, $T_m$. The $T_{g1,w}$ transition is a net thermal effect produced by the reverse glass–FCS$_g$ transition at $T_{g1,w} \approx 217$ K and heat of fusion emitted during the resumed slow freezing of FCS$_c$. In our OC-M measurements of 10–56 wt % CA, we are able to observe in situ how IF (FCS$_g$ + FCS$_c$) is formed during freezing and how ice (IF) melts during subsequent heating. We also observe the slow freezing of FCS$_c$ both upon cooling and subsequent warming of frozen solutions.

In addition to the thermal events of 10–56 wt % CA, the cooling/warming thermograms of 60 wt % CA reveal a third liquid–glass transition, $T_{g3,c}$, reverse glass–liquid transition, $T_{g3,w}$, and cold-crystallization of ice, $T_c$. These additional thermal events are due to the fraction of 60 wt % CA, which does not freeze but transforms to glass upon cooling. Upon cooling of 62 wt % CA, the cooling thermogram reveals only a subtle freezing, which indicates that practically all 62 wt % CA transforms to glass. Upon cooling of 64 wt % CA, there is no freezing but only a liquid–glass transition. Upon warming, glassy 62 and 64 wt % CA “melts” to the HVL at different $T_{g3,c}$. Upon further warming, ice cold-crystallizes in the HVL at $T_{c}$ and then melts at $T_{m}$.

Upon warming of quenched 50 wt % CA, which partly freezes and partly transforms to glass during the quenching, we observe six liquid–glass and glass–liquid transitions and two freezing and one ice melting events. Upon warming of quenched 55 wt % CA, which completely transforms to glass during the quenching, we observe five liquid–glass and glass–liquid transitions and one freezing and one melting events. In these solutions, two FCS$_{g2}$ and FCS$_1$ are formed during the freezing upon warming, the finding that, to our best knowledge, was not reported before. We can only rationalize our observations when assuming a hidden double FCS$_{g2}$/FCS$_1$ transition behavior of aqueous solutions. Our results can be used for the optimization of time- and energy-consuming lyophilization and freezing of foods and biopharmaceutical formulations and, consequently, for improving quality attributed to lyophilized products, among many other fields of science and applications where freezing plays a crucial role.

### REFERENCES


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