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Study on the Unfrozen Water Quantity of Maximally Freeze-Concentrated Solutions for Multicomponent Lyoprotectants

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A B S T R A C T

The concentration of maximally freeze-concentrated solutions W'_g and the corresponding glass transition temperature T'_g and ante-melting temperature T'_m of lyoprotectant solutions, are critical parameters for developing lyophilization process. Usually, the lyoprotectant solutions are multicomponent solutions composed of electrolytes, sugars, proteins, polymers, and other chemicals. In this article, the W'_g values of several multicomponent solutions including trehalose/NaCl, bovine serum albumin/NaCl, and hydroxyethyl starch/NaCl with water were determined by differential scanning calorimetry. A linear relationship between the unfrozen water fraction W_{un} and the initial solute concentrations W_i was found: $W_{un} = \sum(a_i \cdot W_i)$, which suggested that in the multicomponent solutions each solute could hydrate a certain amount of water a_i (g water/g solute) that could not be frozen. The hypothesis was compared with more literature data. For the same solute in different solutions, variation in the fitted coefficient a_i is noticed and discussed. If a "universal" value a_i for each solute is adopted, both W'_g and T'_g for a multicomponent solution could be predicted if Couchman-Karasz equation is adopted for calculating glass transition temperature at the same time. The prediction discrepancies for T'_g with experimental data were less than 2°C. The finding is discussed about its molecular basis and applicability.

Introduction

In the lyophilization process, several kinds of cryoprotectants and lyoprotectants including sugars, proteins, and polymers may be added to protect the pharmaceutical or biological materials from freezing and dehydration injuries. Knowledge of thermodynamic properties of these multicomponent solutions are important for the design and optimization of lyophilization procedures, among which the supplemented phase diagrams have been studied extensively (Fig. 1). For amorphous protectants, which are used mostly, the concentration of the maximally freeze-concentrated solution W'_g, the corresponding glass transition temperature T'_g, and the ante-melting temperature T'_m are critical parameters for developing the freezing and drying protocols in the lyophilization process.[1-4]

There have been numerous research studies reported in literature on the phase diagrams of solute/water binary solutions revealed through differential scanning calorimetry (DSC), the solutes include: disaccharides and other low molecular weight carbohydrates,[5-9] polymers and proteins,[10-13] and so on. Several methods were proposed to determine the T'_g or W'_g values: Roos[3] measured T'_g by DSC and fitted glass transition temperature T_g with Gordon-Tayler equation, the W'_g was calculated by extrapolating T'_g to the T_g curve; Miller et al.[14] took the intersection point of extended equilibrium melting curve T_m and glass transition curve T_g as T'_g and W'_g; Levine and Slade[15] calculated W'_g from ice melting endotherm of a single solution; Ablett et al.[16] plotted a line of freezable water content W_{ice} against solution concentration and obtained W'_g by extrapolating the line to.$W_{ice} = 0$. It has been shown that the obtained T'_g or W'_g values of the same solution by various approaches were slightly different.[17]

Up to now there have been few formula, empirical or theoretical, that copes with T'_g or W'_g. Levine et al.[15] found that for low molecular weight carbohydrates, T'_g increased linearly with the molecular weight. Matveev et al.[18,19] applied a water-clustering model to get the T_m curve theoretically, then obtained T'_g and W'_g with

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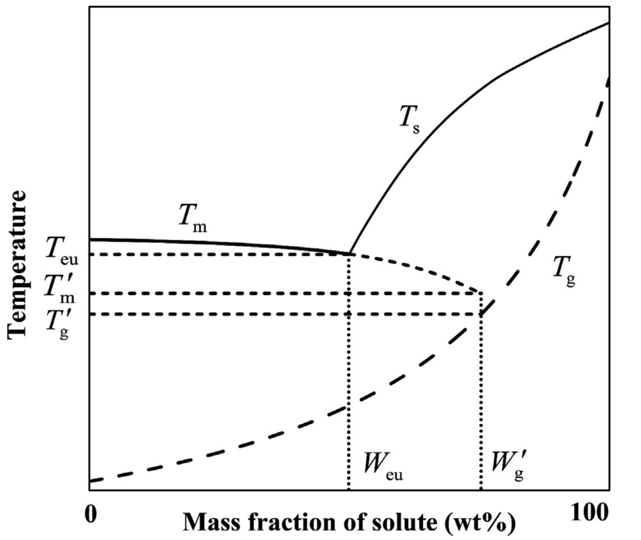

Figure 1. Phase diagram of a model binary solution. (T_m: equilibrium melting curve, T_s: solubility curve, T_{eu} and W_{eu}: eutectic temperature and concentration, for amorphous solute, T_g: glass transition curve, W'_g: maximally freeze-concentrated concentration, T'_g and T'_m: glass transition temperature and ante-melting temperature at maximally freeze-concentrated solution.)

incorporation of Couchman-Karasz (C-K) equation, the predicted values were in good agreement with experimental results for some binary solutions. The formula developed by Levine et al. or Matveev et al. are applicable for single solute solutions and difficult to be extended for describing the behavior of practical lyophilization solutions which usually consist of at least 2 solutes. There were also many experiments to measure the T'_g or W'_g values for ternary or quaternary solutions, some qualitative conclusions had been drawn, for example, the addition of electrolytes decreases T'_g and W'_g; increasing the mass contents of protein, starch, or other macromolecules decreases the T'_g of disaccharide solutions.[10,20-24] However, a quantitative relationship for multicomponent solutions is still urgently needed if it exists. Fonseca et al.[25] made such an attempt; they proposed an empirical equation to calculate the T'_g of a culture medium composed of lactose, galactose, lactic acid, and glucose based on experimental data, but they did not show the applicability of the equation for other solutions.

In this article, an equation was put forward to estimate W'_g for multicomponent solutions based on the concept of water hydration. Experimental W'_g data for ternary solutions of trehalose/NaCl/water, bovine serum albumin (BSA)/NaCl/water, hydroxyethyl starch (HES) (200/0.5)/NaCl/water at different mass ratios were measured and used to check the equation. Because T'_g can be further predicted along with C-K equation, more literature data involving W'_g and T'_g values for multicomponent solutions were also used to verify this relationship. Similar water hydration capabilities for the same solute in different systems were noted, so "universal" values for frequently used solutes were adopted, the prediction of W'_g and T'_g thus becomes possible. A comparison was made between our proposed method and that of Fonseca et al.[25]

Theory and Experiment

Theory for Estimation of W'_g for Multicomponent Solutions

It is well known that W'_g for a binary aqueous solutions is independent of initial solution concentration, this implicates that the unfrozen water content hydrated by or associated with a certain amorphous solute may be constant. For multicomponent solutions composed of several solutes, assuming that the unfrozen water content hydrated by each solute is not affected by the interaction forces between solute molecules or ions, the unfrozen water fraction W_{un} can be expressed as the sum of hydrated water:

$$W_{un} = \sum (a_i \cdot W_i) \tag{1}$$

where parameter a_i (g water/g solute) indicates the hydration ratio of solute i, W_i is the initial weight fraction of solute i. The concentration of maximally freeze-concentrated solution, W'_g, can be calculated as follows:

$$W'_g = \sum W_i \Big/ \sum [(1 + a_i) \cdot W_i] \times 100\% \tag{2}$$

Prediction of T'_g for Multicomponent Solutions

For a multicomponent solution without electrolytes, its glass transition temperature may be calculated by "modified" C-K equation,[26] treating all the solutes as one component, the glass transition temperature is:

$$T_{gmix} = \sum (\Delta C_{pi} \cdot W_i \cdot T_{gi}) \Big/ \sum (\Delta C_{pi} \cdot W_i) \tag{3}$$

$$\Delta C_{pmix} = \sum (\Delta C_{pi} \cdot W_i) \Big/ \sum W_i \tag{4}$$

where, ΔC_{pmix} is the heat increment for solute mixture, ΔC_{pi}, W_i, and T_{gi} are heat increment, weight fraction, and glass transition temperature for solute i, respectively. Thus, the glass transition temperature for the solute mixture and water binary solution is:

$$T_g = \frac{\Delta C_{pmix} \cdot T_{gmix} \cdot \sum W_i + \Delta C_{pw} \cdot T_{gw} \cdot (1 - \sum W_i)}{\Delta C_{pmix} \cdot \sum W_i + \Delta C_{pw} \cdot (1 - \sum W_i)} \tag{5}$$

where ΔC_{pw} and T_{gw} are heat increment and glass transition temperature for water. With the definition of C-K coefficient: $k_i = \Delta C_{pw} / \Delta C_{pi}$, Equation 5 can be re-expressed as:

$$T_g = \frac{\sum (W_i \cdot T_{gi} / k_i) + T_{gw} \cdot (1 - \sum W_i)}{\sum (W_i / k_i) + (1 - \sum W_i)} \tag{6}$$

For a multicomponent solution containing electrolytes, there has been no equation proposed to calculate its glass transition temperature so far. Some experiments showed that the addition of NaCl or $MgCl_2$ had no effect on T_g value of sugar/water binary systems.[20,22] If this conclusion holds true for other solutions containing electrolytes, Equation 6 can be modified slightly to calculate their T_g values:

$$T_g = \frac{\sum (W_i \cdot T_{gi} / k_i) \cdot (\sum W_e / \sum W_i + 1) + T_{gw} \cdot (1 - \sum W_i - \sum W_e)}{\sum (W_i / k_i) \cdot (\sum W_e / \sum W_i + 1) + (1 - \sum W_i - \sum W_e)} \tag{7}$$

where W_e is the initial weight fraction of electrolytes.

Combining Equation 7 with Equation 2, the T'_g for a multicomponent solution can be deduced:

$$T'_g = \frac{\sum(W_i \cdot T_{gi}/k_i) \cdot (\sum W_e/\sum W_i + 1) + T_{gw} \cdot [\sum(a_i \cdot W_i) + \sum(a_e \cdot W_e)]}{\sum(W_i/k_i) \cdot (\sum W_e/\sum W_i + 1) + \sum(a_i \cdot W_i) + \sum(a_e \cdot W_e)} \quad (8)$$

For a single solute i, the above equation reduces to:

$$T'_{gi} = (T_{gi}/k_i + T_{gw} \cdot a_i)/(1/k_i + a_i) \quad (9)$$

After defining the denominator: $\beta_i = 1/k_i + a_i$, Equation 8 could be rewritten as:

$$T'_g = \frac{\sum(\beta_i \cdot W_i \cdot T'_{gi}) + [\sum(W_i \cdot T_{gi}/k_i) \cdot \sum W_e/\sum W_i + T_{gw} \cdot \sum(a_e \cdot W_e)]}{\sum(\beta_i \cdot W_i) + [\sum(W_i/k_i) \cdot \sum W_e/\sum W_i + \sum(a_e \cdot W_e)]} \quad (10)$$

For the case where there is no electrolyte, $\sum W_e = 0$, Equation 10 reduces to:

$$T'_g = \sum(\beta_i \cdot W_i \cdot T'_{gi}) \Big/ \sum(\beta_i \cdot W_i) \quad (11)$$

It should be noticed that the “modified” C-K equation has the same form as the Gordon-Taylor (G-T) equation, though the physical meaning of G-T coefficient and C-K coefficient was totally different, but the glass transition temperatures for trehalose/water system calculated by “modified” C-K equation, with C-K coefficient calculated by heat increment ratio, was obviously larger than the experiment values,[26] then in this article, the C-K coefficient was determined by fitting “modified” C-K equation to experimental glass transition temperatures, which could be equal to the G-T coefficient.

Materials and Solution Preparation

Chemicals include: trehalose dihydrate (Wako Pure Chem. Ind. Ltd., HPLC API grade ≥98.0%), BSA (fraction V, Sangon Biotech Co., Ltd., Shanghai, Molecular Biology Grade, purity ≥98.0%), HES (200/0.5, Wuhan HUST Life Science & Technology Co., Ltd., active pharmaceutical ingredient grade), NaCl (Aladdin, purity >99.99%). The contents of Na^+ and K^+ in trehalose, BSA, and HES reagents were measured by atomic absorption spectrophotometry, it was found that impurities were very little and would have no significant effect on experimental results (analysis process were not shown), therefore all the reagents were used without purification.

The trehalose/NaCl/water, BSA/NaCl/water, HES/NaCl/water ternary solutions, and trehalose/BSA/NaCl/water, trehalose/HES/NaCl/water quaternary solutions were prepared by dissolving solutes in distilled water, using electronic balance (Mettler, AL104, 0.0001g) for weighing. To promote dissolution water bath was used. The solutions were used immediately after preparation. The solution concentrations measured were in the range of 0-77 wt%.

For ternary solutions, mass ratio (MR) was defined as the MR of nonelectrolyte to electrolyte, and MR = ∞ indicated the binary solution. For trehalose/NaCl/water solutions, MR was controlled to be no less than 2. The reason is that when MR <2, the eutectic formation of $NaCl \cdot 2H_2O$ during freezing cannot be fully inhibited,[23,27] whereas for BSA/NaCl/water and HES/NaCl/water solutions, MR was controlled to be no less than 4.[21,28]

Differential Scanning Calorimetry

Low temperature DSC (NETZSCH, Germany, DSC 200 F3) was used to measure the thermograms of sample solutions. The temperature and heat sensitivity of DSC were calibrated by 5 standards: C_6H_{12}, Hg, H_2O, KNO_3, and indium. The thermograms were analyzed by NETZSCH Proteus Analysis software. The temperature scanning protocol were set as follows: cooling at −10°C/min from room temperature to −120°C and holding for 3 min, then heating at 5°C/min to the annealing temperature and holing for 30 min, recooling at −10°C/min to −120°C and holding for another 3 min, finally heating at 5°C/min to room temperature. The annealing temperature was chosen between the T'_g and T'_m to create maximally freeze-concentrated solution.[29-31] All the measured values were determined at the last heating stage.

The glass transition temperature: T_g and T'_g, were reported as the midpoint of baseline shift transition.[11,29] The ice melting latent heat ΔH_m was obtained by integrating the ice melting endothermic peak area above the thermogram baseline.[30] Each kind of solution was measured 3 times and average values were reported.

Experimental Determination of W'_g

The concentration of maximally freeze-concentrated solution W'_g was determined by 2 methods: “T'_g extrapolating” method and “freezable water extrapolating” method. The former was adopted for trehalose/NaCl/water solutions, for which measured glass transition temperatures were fitted to “modified” C-K equation, and W'_g was obtained by extrapolating T'_g to the glass transition curve.[21,32] The latter was adopted for BSA/NaCl/water and HES/NaCl/water solutions. A linear regression was made between freezable water content W_{ice} and solute concentration, and W'_g was determined by assuming $W_{ice} = 0$.[5,16,33] The freezable water content was calculated by:

$$W_{ice} = \Delta H_m / L_e \quad (12)$$

where ΔH_m (J/g) is the ice melting heat per gram of solution, and L_e (J/g) is the effective latent heat of ice melting, a function of melting temperature.[34]

Results and Discussion

In this part, first the applicability of Equations 1 and 2 was tested against our experimental data, then more data from literature were taken for validation. For ternary solutions, Equation 1 became $W_{un} = a_1 \cdot W_1 + a_2 \cdot W_2$, it could be transformed into:

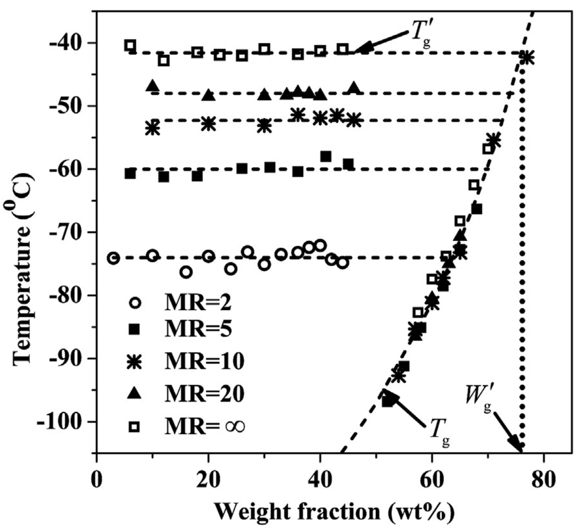

Figure 2. Phase diagram for trehalose/NaCl/water solutions at MR = 2, 5, 10, 20m and ∞, glass transition temperatures were fitted by C-K equation with $T_g = 115^{\circ}$C, $k = 5.18$.

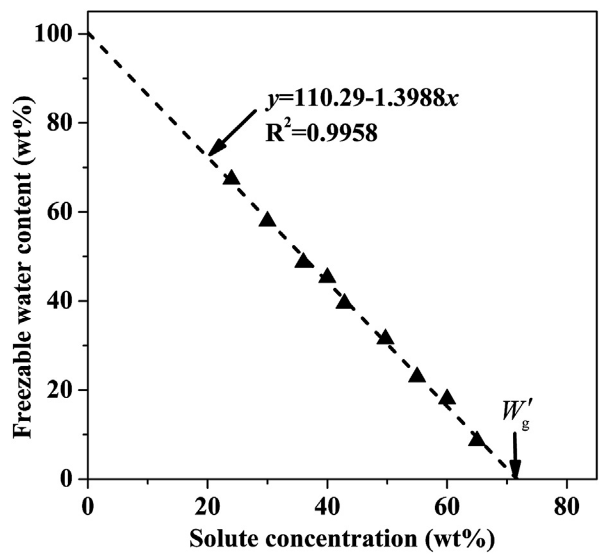

Figure 4. Plot of freezable water fraction versus solute concentration for HES/NaCl/water solutions at MR = 10. Dashed line presented the fitted regression line.

$$W_{un}/W_1 = a_1 + a_2 \cdot W_2/W_1 \tag{13}$$

The hydration ratio of each solute a_1 and a_2 could be obtained by a best linear fit of W_{un}/W_1 versus W_2/W_1. With regard to the fitting results, the hydration capabilities of different types of solute were discussed, it was found that a "universal" a_i for a given solute is possible which makes prediction of T'_g according to initial solution composition feasible. Finally, the prediction results of our proposed method were compared with those of Fonseca et al.[25]

Verification With Measured Data

It is well known that T'_g for solutions with fixed mass ratio of solutes at various concentrations are the same. For trehalose/NaCl/water solutions, the measured T'_g values are shown in Figure 2. T'_g for each mass ratio MR is an average of the values at different concentrations. It can be seen that T'_g increases monotonically with increasing MR. For MR = 2, 5, 10, 20, and ∞, W'_g obtained by "T'_g extrapolating" method are 63.69, 69.78, 72.63, 74.09, and 76.13 wt%, respectively, thus the corresponding $W_{un} = (100\text{wt\%} - W'_g)$ are 36.31, 30.22, 27.37, 25.91, and 23.87 wt%, respectively. According to Equation 13, plotting $W_{un}/W_{trehalose}$ versus 1/MR ($W_{NaCl}/W_{trehalose}$) will get hydration ratio parameter a_i for trehalose and NaCl, which are 0.3096 and 1.0849 g water/g solute, respectively, as shown in Figure 3.

For BSA/NaCl/water and HES/NaCl/water solutions, W'_g is determined by the "freezable water extrapolating" method, as shown in Figure 4. At MR = 4.88, 6.41, 9.58, 18.45, and 249.7, W'_g are 61.75, 66.87, 68.46, 70.24, and 71.99 wt%, respectively, for BSA/NaCl/water system; at R = 4, 5, 10, 20, and ∞, W'_g are 67.62, 69.65, 71.70, 72.73, and 74.64 wt%, respectively, for HES/NaCl/water system. Similarly, plotting W_{un}/W_{BSA} versus 1/MR will get parameter a_i for BSA and NaCl, and plotting W_{un}/W_{HES} versus 1/MR will get parameter a_i for HES and NaCl. The results are shown in Figure 5.

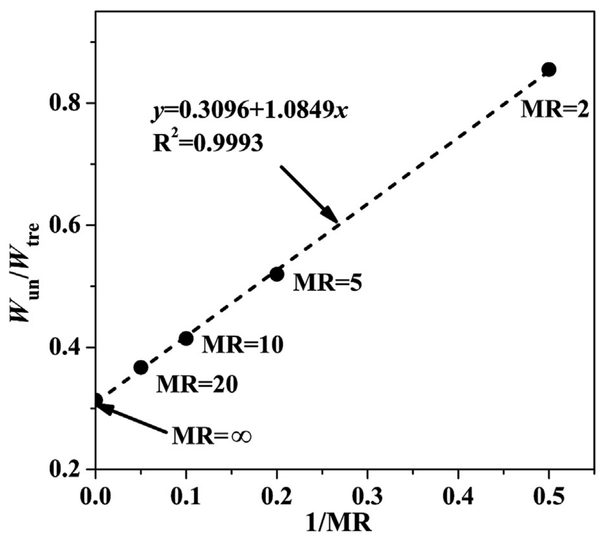

Figure 3. Mass ratio of unfrozen water fraction to trehalose $W_{un}/W_{trehalose}$ versus 1/MR ($W_{NaCl}/W_{trehalose}$) for trehalose/NaCl/water solutions. Dashed line presented the fitted regression line.

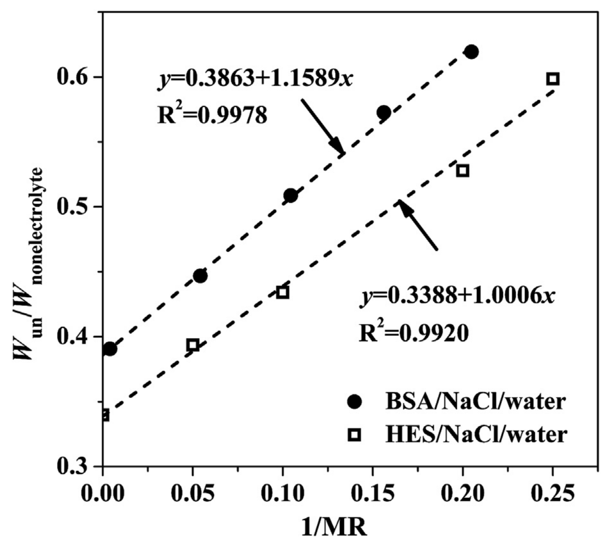

Figure 5. Mass ratio of unfrozen water fraction to nonelectrolyte solutes versus 1/MR. Dashed line presented the fitted regression line.

Table 1
W'_g or T'_g Values for Ternary Solutions Listed in Literature

Solutions	MR	T'_g (°C)	W_{un}/W_{BSA} (g/g)	W'_g (wt%)	W'_g Determination Method	Reference
Trehalose/$MgCl_2$/water	3.60			69.5	Calculating frozen water fraction from the latent heat released after storage at −26°C for 24 h, at solute concentration of 40 wt%	22
	7.19			73.0		
	17.98			74.4		
	∞			76.2		
BSA/NaCl/water	1		1.35	59.70	Calculating unfrozen water fraction by subtraction of ice and eutectic water content at solute concentration of 40 wt%	35
	1.5		1.13	59.59		
	4		0.77	61.88		
	9		0.63	63.96		
	∞		0.52	65.79		
HES (450/0.7-0.8)/NaCl/water	1 < R < 50			$\frac{(R+1)}{(R+1.3757)} \times 62.0309$	"Freezable water extrapolating" method. (The water and NaCl impurities in HES reagent was taken into account.)	21
Sucrose/NaCl/water	3.97	−67.2		72.22	"T'_g extrapolating" method. (Glass transition temperatures were fitted to C-K equation with $T_g = 65$°C, $k = 4.85$.)	6,24
	5	−63.7		73.70		
	7	−61.2		74.71		
	15	−54.7		77.16		
	∞	−48.2		79.39		
Glycerol/NaCl/water	1			65.3	"T'_g extrapolating" method.	21
	1.5			68.6		
	2.33			72.7		
	4			76.2		
	7.69			77.6		
	19			79.8		

W'_g or T'_g Values for Ternary Solutions Listed in Literature Data (Continued)

Solutions	MR[a]	T'_g(°C)	W'_g(wt%)	W'_g Determination Method	Reference
Sucrose/bovine plasma protein (BPP)/water	1.25	−51.48	79.18	"T'_g extrapolating" method. Glass transition curves were calculated by Equation 6 with T_g values fitted for sucrose, glucose, and BPP were 65°C, 31°C, and 65°C, and k values were 4.85, 4.52, and 5.5, respectively.	29
	2.50	−50.12	79.30		
	3.75	−48.42	79.71		
Glucose/BPP/water	1.25	−62.50	77.60		
	2.50	−61.06	78.48		
	3.75	−59.82	79.11		
Trehalose/surimi/water[b]	0.5	−48.5	68.16	"T'_g extrapolating" method. Glass transition curves were calculated by Equation 6 with T_g values fitted for trehalose, glucose, and surimi were 115°C, 31°C, and 167°C, and k values were 5.18, 4.52, and 4.6, respectively.	42
	1.17	−46.8	70.45		
	2.57	−45.0	72.57		
	4	−44.2	73.53		
	6.71	−42.5	74.68		
	∞	−41.1	76.28		
Glucose/surimi/water	1.08	−60	69.55		
	2.57	−59	73.76		
	6.71	−58	77.30		
	∞	−57	80.62		

[a] For ternary solutions composed of sugar and protein, MR value was defined as the mass ratio of sugar to protein.
[b] Surimi was rich in myofibrillar proteins.

As can be seen from the R^2 values in Figure 3 and Figure 5, very good linear relationship between W_{un} and mass contents of solutes exists.

Verification With Literature Data

To further testify the proposed relationship (Eq. 1) between unfrozen water content and solute contents in multicomponent solutions and to obtain parameters a_i for each solute, more articles studying ternary solutions were also analyzed: for some solutions, W'_g values were determined by the latent heat released by unfrozen water and displayed directly, whereas for others, because only T'_g values and glass transition curves were given, W'_g values were calculated by "T'_g extrapolating" methods. All the data were listed in Table 1.

The W'_g values obtained from literature data were transformed to unfrozen water content and displayed in Figures 6 and 7, the relationships between $W_{un}/W_{nonelectrolyte}$ and 1/MR or W_{un}/W_{sugar} and $W_{protein}/W_{sugar}$, could be fitted to linear lines perfectly. The fitted results were summarized in Table 2 (our experimental results are also included), it was noted that all R^2 values were greater than 0.99, which demonstrated that Equation 1 could be applied to various systems.

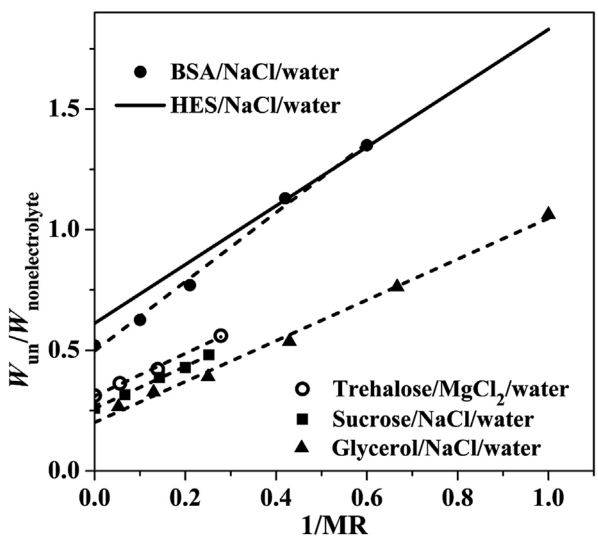

Figure 6. Mass ratio of unfrozen water fraction to nonelectrolyte solutes versus 1/MR for ternary solutions listed in references (Table 1). Dashed line presented the fitted regression line.

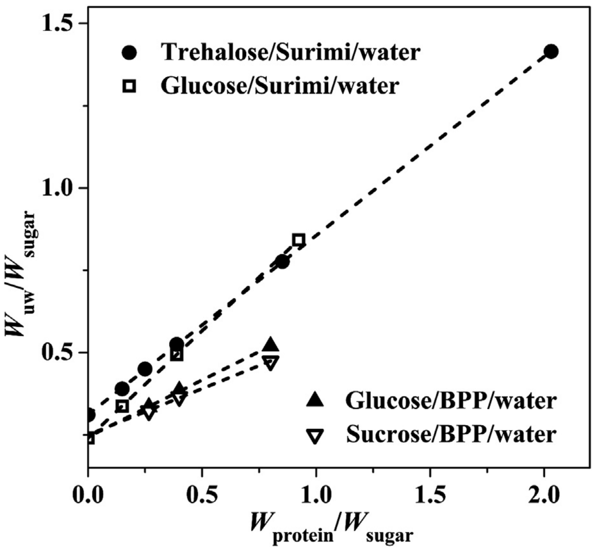

Figure 7. Mass ratio of unfrozen water to sugars versus mass ratio of proteins to sugars for ternary solutions listed in references (Table 1). Dashed line presented the fitted regression line. BPP, bovine plasma protein.

Discussion of a_i

From Table 2, variation in the fitted a_i for the same solute in different systems can be noticed. The most obvious is for NaCl, its a_i varies in the range of 0.8461-1.4344 g water/g NaCl or 2.7470-4.6570 mol water/mol NaCl. The difference may be due to different ways used to determine W'_g values, for example, the largest a_i value was obtained from Inoue,[28] where fast cooling and heating rates (50 K/min and 30K/min) were used in DSC measurements, which may lead to more unfrozen water than maximally freeze-concentrated solution. The average a_i is 1.0880 ± 0.2059 g water/g or 3.5323 ± 0.6684 mol water/mol, which is less than the hydration value of about 6 mol water/mol determined by experiments or molecular dynamics simulation for dilute solution at room temperature.[28,35] This shows that hydration may be seriously influenced by concentration and temperature.

The average value of fitted unfrozen water hydrated by trehalose was 0.3104 ± 0.0012 g water/g, with corresponding W'_g for trehalose/water binary solution 76.31 wt%. Similarly, average a_i value for sucrose and glucose were 0.2545 ± 0.0062 g water/g and 0.2483 ± 0.0081 g water/g, respectively. Corresponding W'_g values for sucrose/water and glucose/water binary solutions were 79.71 and 80.11 wt%, which were consistent with the reference data.[3,31] Average hydrated water for trehalose, sucrose, and glucose were 5.9033, 4.8402, and 2.4852 mol water/mol. Comparing with the hydration number calculated by thermodynamic computation, (5.80, 3.13, and 1.93 mol water/mol[36]), the hydration numbers of sucrose and glucose determined in this article were slightly larger.

Fitted a_i for BSA was 0.3863 g water/g, close to the value of 0.35 g water/g obtained by Ling et al.,[37] whereas smaller than the value of 0.4976 g water/g obtained by Inoue,[28] which was due to the large cooling and heating rates used in DSC measurement. The corresponding W'_g value for BSA/water solutions was 72.13 wt%, which was comparatively larger than the value of 70 wt% obtained by Hottot et al.[38] as the intersection of ice melting and glass transition curve.

The fitted unfrozen water hydrated by HES (200/0.5) in this article was 0.3388 g water/g, converting to W'_g value of HES/water solutions was 74.69 wt%, which was close to the value of 75.3 wt% obtained by Sun et al.[39] for HES (262.6/0.46)/phosphate-buffered saline/water solutions, where the mass ratio of HES to NaCl was 63.21, whereas the large distinction from fitted a_i value for HES (450/0.7-0.8) may be attributed to large differences in molecular weight.

For sugar/protein/water ternary solutions, differences between the fitted a_i for the same protein (bovine plasma protein or surimi) should be noticed. Singh and Roos[31,32,40] studied ternary solutions of egg albumin (molecular weight: 44.3 kDa) and disaccharide at mass ratio of 1:1, W'_g was determined by the "T'_g extrapolating" method, and corresponding a_i for albumin were 0.3446, 0.2191, and 0.2342 g water/g when the disaccharide was lactose, sucrose, or trehalose, respectively. The large variation of a_i may be due to the interaction forces between protein and disaccharide molecules, which affect the hydration degree of protein molecules. More experiment data involving W'_g for sugar/protein/water solutions are needed to further analyze the effect of molecular interactions to the hydrated water a_i by each solute.

Prediction of T'_g *for Multicomponent Solutions*

Although a_i shows variance for the same solute in different systems, these variances are not so obvious for most solutes we investigated as mentioned previously. Because more interests focus on prediction of T'_g for practical consideration, we will make such a try by using "universal" a_i, which is determined arbitrarily here through averaging a_i values for the same solute in different systems. Table 3 lists "universal" a_i and other values: T_{gi} and k_i that will be used in the prediction of T'_g for multicomponent solutions. Table 4 gives the calculated T'_g by Equation 10 for trehalose/BSA/NaCl/water and trehalose/HES/NaCl/water quaternary solutions and compared with the experimental values measured by ourselves.

Table 2
Fitted a_i for Various Ternary Solutions

Solutions	Salts g/g	Sugars or Glycerol g/g	Proteins or HES g/g	R^2	Reference
Trehalose/NaCl/water	1.0849	0.3096		0.9993	This article
BSA/NaCl/water	1.1589		0.3863	0.9978	
HES (200/0.5)/NaCl/water	1.0006		0.3388	0.9920	
Trehalose/$MgCl_2$/water	0.8848	0.3099		0.9921	22
BSA/NaCl/water	1.4345		0.4976	0.9931	28
HES (450/0.7-0.8)/NaCl/water	1.2178		0.6121	1	21
Sucrose/NaCl/water	0.8730	0.2589		0.9979	24
Glycerol/NaCl/water	0.8461	0.2010		0.9942	21
Sucrose/BPP/water[a]		0.2501	0.2804	0.9972	29
Glucose/BPP/water[a]		0.2439	0.3453	0.9993	
Trehalose/surimi/water		0.3118	0.5441	0.9999	42
Glucose/surimi/water		0.2404	0.6518	1	

[a] The average molecular weight of bovine plasma protein was 47.5 kDa.

Table 3
Parameter Values for Solute/Water Binary Solutions

	T_{gi} (°C)	k_i[a]	T'_{gi} (°C)	W'_g (wt%)	a_i (g/g)[b]	a_i (g/g)[c]	β_i	Reference
Water	−138							17
Trehalose	115	5.18	−41.6	76.13	0.3135	0.3104 ± 0.0012	0.5035	17
Sucrose	65	4.85	−47.2	79.39	0.2591	0.2545 ± 0.0062	0.4607	31
Glucose	31	4.52	−57	80.0	0.2500	0.2483 ± 0.0081	0.4712	3
Lactose	101	6.56	−41	81.30	0.2300		0.3824	
Maltose	87	6.15	−42	81.6	0.2255		0.3881	
Galactose	30	4.49	−56	80.5	0.2422		0.4649	
Fructose	5	3.76	−57	82.5	0.2121		0.4781	
Raffinose	70	5.66	−36	84.1	0.1891		0.3658	43
BSA	150	3.76		72.13	0.3863			38
HES	133	6.5		74.69	0.3388			40
NaCl						1.0880 ± 0.2059		This article

[a] k_i was determined by fitting "modified" C-K equation to experimental glass transition temperatures.
[b] a_i was calculated from the concentration of maximally freeze-concentrated solution, $a_i = (100\% - W'_{gi})/W'_{gi}$.
[c] a_i was calculated as the average value (Table 2).

The differences between calculated and experimental T'_g were less than 2°C for trehalose/HES/NaCl/water solutions. It should be noticed that the C-K coefficient k of HES was determined by fitting Equation 7 to glass transition temperatures of trehalose/HES/NaCl/water quaternary solutions measured in this article, the value of 6.5 was much larger than the fitted G-T plasticization value of 4.75 by Sun et al.[39]; while the T'_g value for HES/water binary solutions calculated by extrapolating W'_g to C-K glass transition curve was −53.37°C, which was much smaller than the value of −16°C determined by Kresin et al.[13] There were 2 possible explanations: first, the measured T'_g was not the true glass transition temperature but the ante-melting temperature; second, the application of "modified" C-K (Eq. 6) using C-K coefficients determined from binary solutions, to calculate glass transition temperatures for multicomponent solutions was questionable, and more experimental data were needed to testify Equation 6.

Hottot et al. fitted glass transition temperatures of BSA/water binary solutions with G-T equation, G-T plasticization value for BSA was 3.44, assuming the "modified" C-K equal to G-T plasticization value, T'_g calculated by Equation 11 for trehalose/BSA/NaCl/water at different mass ratios were much larger than the measured ones, especially when mass ratio of BSA to trehalose equals to 0.55 and 1.10, the differences were larger than 15°C. If the k_i value for BSA was set to be 6.75, the calculated differences were less than 2.2°C. Again, the k_i values for nonelectrolytes in calculating glass transition curves of multicomponent solutions by Equation 6 were crucial to the determination of T'_g.

Equation 11 could also be applied to real complex biological materials. For example, Guizani et al.[41] measured the supplemented phase diagram for a sugar-rich fruit of data palm, of which 100 g fruit flesh consisted of 19.12 g sucrose, 18.35 g glucose, 17.50 g fructose, 19.14 g other carbohydrates, and 2.47 g protein. Assuming that the thermodynamic parameters of other carbohydrates and protein resembles sucrose and BSA, the values of W'_g, T'_g calculated by Equation 2 and Equation 11 were 80.16 wt%, −47.9°C, which were close to the measured values: 78 wt%, −48°C.

Fonseca et al.[25] also proposed an empirical equation to predict T'_g value for real complex biological medium, which was computed as the mass weighted mean value of T'_{gi} for each solute:

$$T'_g = \sum (W_i \cdot T'_{gi}) \Big/ \sum W_i \tag{14}$$

T'_g calculated by Equation 14 for bacterial fermented medium composed of lactose, galactose, lactic acid, and glucose, was about 5°C higher than measured value. Comparing Equation 14 with Equation 11, it was obvious that when $\beta_i (=1/k_i + a_i)$ value for each nonelectrolyte was the same, these 2 equations were equivalent.

It was noticed that Equation 14 could not be applied to multicomponent solutions composed of electrolytes, as the T'_{gi} values for most electrolytes were not known. In Equation 10, when the mass ratio of electrolytes to nonelectrolytes was small, such as $\sum W_e / \sum W_i$ <0.1, which is the case for most lyophilization solutions, Equation 10 could be reduced to:

Table 4
The Measured and Calculated T'_g (°C) for Quaternary Solutions

Trehalose/HES/NaCl/Water

Mass Ratio	T'_g by Experiment	T'_g by Equation 10	Mass Ratio	T'_g by Experiment	T'_g by Equation 10
2.26/2.50/1	−65.9 ± 0.2	−66.5	8.23/0.91/1	−54.6 ± 0.7	−54.9
3.02/1.67/1	−64.6 ± 0.9	−65.1	9.05/10.00/1	−52.1 ± 0.8	−53.9
3.77/0.83/1	−63.4 ± 0.7	−63.6	12.07/6.67/1	−51.4 ± 0.7	−52.0
4.52/5.00/1	−57.3 ± 0.4	−58.9	15.08/3.33/1	−50.3 ± 0.7	−50.2
6.03/3.33/1	−56.3 ± 0.5	−57.3	16.45/1.82/1	−49.6 ± 0.6	−49.3
7.54/1.67/1	−55.4 ± 0.7	−55.6			

Trehalose/BSA/NaCl/Water

Mass Ratio	T'_g by Experiment	T'_g by $k_i = 3.44$	T'_g by $k_i = 6.75$	Mass Ratio	T'_g by Experiment	T'_g by $k_i = 3.44$	T'_g by $k_i = 6.75$
2.24/2.47/1	−69.8 ± 0.9	−45.3	−67.7	8.20/0.90/1	−55.1 ± 0.7	−50.4	−55.0
3.00/1.65/1	−67.7 ± 0.8	−50.3	−66.0	8.70/9.58/1	−54.1 ± 1.1	−31.91	−56.3
3.76/0.83/1	−64.3 ± 0.7	−55.9	−64.1	11.75/6.47/1	−52.9 ± 0.6	−36.66	−53.7
4.44/4.88/1	−59.9 ± 0.7	−37.2	−60.9	14.88/3.28/1	−51.8 ± 0.5	−42.05	−51.1
5.95/3.28/1	−59.0 ± 0.7	−42.1	−58.6	16.33/1.80/1	−50.4 ± 1.0	−44.76	−49.8
7.49/1.65/1	−57.8 ± 0.4	−47.6	−56.3				

Table 5
The Measured and Calculated T'_g (°C) for Ternary or Quaternary Solutions

Solutes	Mass Ratio	T'_g by Experiments	T'_g by Equation 15 [a,b]
Trehalose/NaCl	20/1	−48.0 ± 0.6	−46.19
(this article)	10/1	−52.3 ± 0.8	−50.36
Sucrose/NaCl[24]	15/1	−54.7	−52.88
	7/1	−61.2	−58.55

[a] The T'_g of NaCl used in Equation 14 was set to be T_{gw}.
[b] The T'_g of BSA was set to be −14°C.[3]

$$T'_g \simeq \frac{\sum(\beta_i \cdot W_i \cdot T'_{gi}) + \sum(T_{gw} \cdot a_e \cdot W_e)}{\sum(\beta_i \cdot W_i) + \sum(a_e \cdot W_e)} \tag{15}$$

Equation 15 resembles Equation 14 except that weight coefficients are added and the T'_g of electrolyte is set to T_{gw}. Table 5 lists the calculated T'_g values applying Equation 15 for ternary and quaternary solutions containing NaCl. The differences between calculated and measured values were small especially when mass ratio of amorphous solute to NaCl was close to 1.

It should be mentioned that all the results concluded previously are only suitable for “miscible” multicomponent solutions, not for the “phase separated” solutions.[39] For the latter, multiple freeze-concentrated domains will form, leading to multiple T'_g.

Conclusion

The glass transition temperature for maximally freeze-concentrated solution T'_g is an important property of a lyoprotectant formula. Its prediction can make the selection of lyoprotectant and development of freeze drying protocol easier. We made such an attempt here by calculating W'_g first with the assumption that each mole of solute hydrated a certain amount of water that cannot be frozen, which seems to be true for solutes we investigated, trehalose, NaCl, BSA, HES (200/0.5), and other solutes from literature, such as $MgCl_2$, sucrose, glycerol, glucose, bovine plasma protein, and surimi. The predicted values of T'_g for ternary or quaternary solutions were fairly closer to experimental values from literature. For systems containing solutes that do not appear in this article, the present finding still awaits verification.

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