Development of pseudo-formulations for therapeutic solutions to assess cryoprocessing parameters at industrial scale

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ABSTRACT
Monoclonal antibodies (MAb) are therapeutic proteins that are ideally stored and transported in frozen solutions to optimize production and extend the shelf life. However, this cycle of freezing, storing and thawing induces much stress on the proteins, which can compromise the physical stability and consequently their compliance and market specifications. Thus, the parameters inherent in freezing and thawing must be well defined in order to avoid degradation of the products during the cryopreservation process.
Normally, the parameters are studied under scale-down conditions, especially for optimization of MAb which are very expensive proteins, during early stage trials. The main goal of this work was to develop pseudo-formulations using relatively inexpensive proteins that can mimic the rheological and thermodynamic properties of the therapeutic molecules.
Towards this aim an exhaustive study was carried to model the viscosity of aqueous solutions containing model proteins and trehalose in different concentrations, in a temperature range of $-7{,}5$ to $25°C$. Mathematical models were applied to correlate viscosity as a function of temperature and concentration.
Several of the mathematical models were able to describe the viscosity of the selected proteins. The models anticipated that a solution with about $4\ g/dL$ of Ovalbumin and $10\ g/dL$ of trehalose, $pH = 4{,}6$ or a solution with approximately $12\ g/dL$ of BSA and $10\ g/dL$ of trehalose, $pH = 4{,}7$, should replicate the viscosity change with temperature of a MAb formulation, which was confirmed experimentally.
It is concluded that it is possible to mimic the viscosity of MAb solutions with the model proteins studied and a mathematical model is proposed to calculate the composition of BSA or Ovalbumin to match the viscosity curve of a given MAb formulation.

Keywords: *viscosity, proteins, monoclonal antibody, cryopreservation, temperature*

INTRODUCTION

Therapeutic Proteins

Proteins study is considered an important area of research in science and technology, being therapeutic proteins - such as monoclonal antibodies (MAb) - a prominent position in pharmaceutical and biotechnology industry with a rapid increase in market share (1), with high production costs.
Therefore, there is an increase in the mass production of this type of therapeutic proteins, associated to the need for optimization of processes, such as batch production (2) and improvement of storage capacity for relatively long periods of time. (1)(2)
Ideally, frozen solutions are used for storage the solutions (and also for transport). (1)(2)(3)(4)(5) Freezing favors stability of solutions, extending their service life (4); minimizing the risk of microbial contamination (1)(5) and reducing the risk of inactivation of the compounds. (5) Frozen transport is facilitated, when compared to liquid state (5), because agitation and consequent foaming are avoided. (1)(4)
On the other hand, storing large volumes of frozen protein solutions presents as a challenge (4) since this process can lead to chemical and physical changes that result in the denaturation such as: protein unfolding caused by low temperatures (6), formation of irreversible aggregates over time. (4)(7)
One of the methods used to overcome the aggregation and particle formation problem is the addition of surfactants or sugars - trehalose, for example, acts as a vitrifying agent, increasing the glass transition temperature (Tg) of the solution.(8) These additives stabilize proteins reducing aggregate formation; however, they can lead to an increase in the viscosity of the solution. Viscosity has a significant impact on most handling and production processes. For this reason, it is necessary that separation, transport or freeze processes are optimized taking into account the rheological properties of therapeutic protein solutions. However, the production cost of these proteins is very high, and it is therefore not convenient to carry out trials at the production scale. Typically two alternatives are followed; develop the processes using scale down models (5) or use model solutions containing inexpensive proteins with similar properties for pilot or production scale trials.

Viscosity

Viscosity is an internal resistance of the fluid to the flow. Molecularly, the viscosity is explained by the attraction and repulsion forces between the molecules of the fluid and is also correlated with the molecules size and shape, having Einstein relating the viscosity to the volume fraction of solute in solution. (9)

It is possible to affirm that the viscosity of a liquid decreases with increasing of temperature - the opposite is also true - because it is necessary to overcome intermolecular interactions. (10)

Mathematical Models

Over the years, several mathematical models have been developed to describe the viscosity of solutions as a function of temperature variation and solutes concentration.

It was Einstein who first presented a theoretical approximation (11)(12) relating the viscosity (η) with the diffusion coefficient (D) and with temperature (T), in the Stokes-Einstein relation - **Eq.1**

$$\boldsymbol{\eta = \frac{k_B\, T}{A\, D\, r_h}} \qquad \textbf{Eq.1}$$

Were k_B is the Boltzmann constant, r_h is the hydrodynamic radius of the molecule and A assumes a value which depends on the type of friction on the solute surface; 4π for a slip boundary condition or 6π for a stick boundary condition. (13)

This equation was based on an approximation to a rigid and relatively large sphere - sugar molecule - to diffuse into a continuous fluid of small molecules of solvent, without specific interactions. (14) However, according to some authors (11), this equation is only applicable to diluted solutions, and there is also some who defend that even in this situation. (13) Later that, his equation was extended to take into account the molecular interactions that appear when the solute concentration exceeds the infinite dilution limit.

<u>Viscosity Concentration Dependence</u>

Vand (15) proposed a suspension model, derived from hydrodynamic considerations, in which ν is the solute concentration in $\mathrm{g.cm^3}$, η_w is the viscosity of water, q_0 is related to the shape of the molecules and the hydration at infinite dilution, and q_1 and q_2 are related to the hydrodynamic and intermolecular interactions - **Eq. 2**. (11)

$$\boldsymbol{ln\, \frac{\eta}{\eta_w} = \frac{\nu}{q_0 + q_1\nu + q_2\nu^2}} \qquad \textbf{Eq.2}$$

Génotelle used an equation proposed by *Kaganov* - **Eq. 3** - to describe the viscosity of aqueous solutions of sucrose with concentrations up to 85% by mass, in a temperature range between 20 and 80°C. (11)

$$\boldsymbol{ln\, \frac{\eta}{\eta_w} = A + Bc} \qquad \textbf{Eq.3}$$

In this equation, c is the molar concentration and A and B are constants.

<u>Viscosity Temperature Dependence</u>

It is important to highlight the purpose of Tg. This temperature corresponds to a reversible transition, in glassy (non-crystalline) materials, from a state where they are rigid to a very high viscosity state and is used in some equations.

In these cases, where it is possible to find Tg, this temperature should be taken into account in the viscosity models as a "limitation" (in a positive sense). This is because as the temperature approaches the Tg, the viscosity tends to infinity. For this reason, the viscosity should not only depend on T but on a conjugation of T with Tg, so that the model can be limited to the physical phenomenon.

One of the proposed equations that fit well for a wide range of viscosities of liquids is the *Vogel-Fulcher-Tammann* equation (VFT) - **Eq. 4** – where η_0, T_0 and D are constants. (16) The D Parameter is related to brittleness and decreases with increasing curvature of the Arrhenius plot. (17)

$$\boldsymbol{\eta = \eta_0 exp\left(\frac{DT_0}{T - T_0}\right)} \qquad \textbf{Eq.4}$$

Another model, which was developed to describe the viscosity of polymers on a scale between Tg and Tg + 100 K is a *Williams-Landel-Ferry* (WLF) equation - **Eq. 5**. (18)

$$\boldsymbol{log_{10}\left(\frac{\eta}{\eta_g}\right) = -\frac{C_1(T - T_g)}{C_2 + (T - T_g)}} \qquad \textbf{Eq.5}$$

In this equation, C_1 and C_2 are constants and η_g corresponds to the viscosity at Tg, which can assumes the value of $10^{14}\, Pa.s$ (19) or $10^{12}\, Pa.s$. (13)

WLF and VFT equations are convertible into each other (20) and mathematically equivalent, however T_0 and Tg don't have the same physical meaning. (11)

Another author (21) observed that for low viscosity values, the behavior of organic liquids is fairly well described by an expression that translates into a power equation - **Eq. 6**.

$$\boldsymbol{\eta = \eta_0 (T - T_c)^{\gamma}} \qquad \textbf{Eq.6}$$

η_0 is the pre-exponential factor, T_c is the critical temperature and γ is the critical exponent. As an approximation, the critical temperature assumes the value $T_c = 1{,}18\ T_g$. (22)

The following model was proposed for viscosity in liquids and appeared in a random way - **Eq. 7**. (11)

$$\boldsymbol{\eta = \eta_0 exp\ \left(\frac{T_0}{T}\right)^2} \qquad \textbf{Eq.7}$$

Being η_0 and T_0 constant values and $T_g < T < T_c$ (T_c represented in **Eq. 6**).

Concentration and Temperature Dependences

The next hypothesis presented was proposed for the particular case of disaccharides solutions - **Eq.8** - and in this x is the molar fraction of sugar, t is the temperature in $°C$ and η^* represents a standard viscosity which makes the logarithm dimensionless. (11)

$$\boldsymbol{log_{10}\left(\frac{\eta}{\eta^*}\right) = x\left(\frac{a}{t+b} - c\right) - d} \qquad \textbf{Eq.8}$$

There is an empirical model represented in **Eq.9**, in which Φ the reduced temperature (Eq.10) is, x is the molar fraction of sugar e η^* is a standard viscosity. (11)

$$\boldsymbol{log_{10}\left(\frac{\eta}{\eta^*}\right) = a_1 + a_2 x + \Phi(b_1 + b_2 x^n)} \qquad \textbf{Eq.9}$$

$$\boldsymbol{\Phi = \frac{30-t}{91+t}} \qquad \textbf{Eq.10}$$

Finally, the viscosity logarithm was adjusted as a $\frac{T_g}{T}$ polynomial and it was found that a cubic equation is adequate to represent the viscosity of the solutions with four constants (a, b, c and d) - **Eq.11**. The main objective is to assume that for each solute, the effect of its concentration on viscosity is implicitly taken into account by the $\frac{T_g}{T}$ quotient, which determines the distance of the system to Tg. The approach is very reasonable for sucrose and for trehalose, especially in the supercooled regime. (11)

$$\boldsymbol{log_{10}\eta = a\left(\frac{T_g}{T}\right)^3 + b\left(\frac{T_g}{T}\right)^2 + c\left(\frac{T_g}{T}\right) + d} \qquad \textbf{Eq.11}$$

MATERIALS

Reagents

In the present work, the following reagents were used: hydrochloric acid (*Sigma-Aldrich*), distilled water Milli-Q (*Merck Millipore*), bovine serum albumin (BSA) (*Sigma-Aldrich*), a monoclonal antibody (MAb), L-Arginine (*Sigma-Aldrich*), L-Histidine Base (*AppliChem*), Ovalbumin (*Sigma-Aldrich*), Polysorbate 20 (*Fluka*), D-(+)-Trehalose Dihydrate (*TCI*) and Tris(hydroxymethyl)aminomethane (*Sigma-Aldrich*).

Equipment

The experimental work was only possible using the following materials: analytical balance (*Mettler Toledo*), ultra sound bath (*Sonorex*), vacuum pump (*Vacuubrand*), differential scanning calorimeter (DSC) (*F3 Maia 200, Netzsch*), cryostat (*Julabo*), freeze-drier (*Christ*), pH meter (*Nahita*), distillation system (*Milli-Q Integral 3, Merck Millipore*), Viscometer (DV-*II+ Pro, Brookfield*), stirring and heating plates (*Ovan*) and current laboratory material.

METHODS

Solutions

The solutions used for the study of viscosity were prepared and the solutes concentrations are given below, in mass/volume fraction.

There were prepared aqueous solutions with 10, 20 and 30 g/dL of trehalose; solutions in a histidine and arginine buffer (pH~4.7), 10 g/dL of trehalose and different concentrations of BSA: 15, 17.5, 19, 20, 22.5, 25, 27.5 and 30 g/dL; solutions in a Tris-HCl buffer (pH~7), 10 g/dL of trehalose and different concentrations of BSA: 10, 20, 25 and 30 g/dL; solutions in a buffer of histidine and arginine (pH~4.7), 20 g/dL BSA and different concentrations of Trehalose: 5, 10, 15, 20, 21, 22, 25, and 30 g/dL; solutions in a histidine and arginine buffer (pH~4.6), 10 g/dL of trehalose and different concentrations of Ovalbumin: 5, 10, 15, 30 and 40 g/dL; solutions in a histidine and arginine buffer (pH~4.6), 10 g/dL of Ovalbumin and different concentrations of trehalose: 5, 10, 20 and 30 g/dL.

Differential scanning calorimetry

The DSC technique was used for experimental determination of Tg.

The sample to be analyzed was prepared by weighing a mass of 2 to 10 mg, which was introduced into a Netzsch aluminum crucible. This was placed on the equipment as well as an empty and closed one used as a reference. The apparatus contains a built-in cooling system and the entire analysis is performed under a nitrogen atmosphere continuously purged to a maximum pressure of 0.6 bar.

For this determination, a temperature program starting at 20℃ was used, followed by cooling to

$-50°C$ at a constant rate of $15\ K/min$ at which temperature was held for $10\ min$. Finally, the sample was heated up to $25°C$ at $5\ K/min$.

Determination of viscosity

The viscosity was measured using a Brookfield cone and plate viscometer. This equipment has a cell in which a spindle cone is placed with an appropriate diameter to the viscosity of the sample to be analyzed. In this case, it was used the spindle S40 which, once in operation, must be adjusted its rotation speed in such a way that the effort rate displayed by the equipment remains within the appropriate values. A sample of solution $(500\ \mu L)$ was introduced into the cell.

As the objective is the analysis of viscosity variation with temperature, the viscometer was coupled to a Julabo cryostat, which allowed the cell and, consequently, the sample temperature change. Thus, for the reading of the viscosity value, only the stabilization of the temperature value ranging from -7.5 to $25°C$ was required.

Numerical Methods

The analysis on the models and their adjustments was performed with the *Solver* tool of *Microsoft Office Excel* that is based on Newton's method.

RESULTS

Effect of protein concentration

The viscosity measurements show that for BSA the variation of the protein concentration, with constant trehalose has a strong impact on viscosity - **Fig. 1**. The same is observed for Ovalbumin, however, this isn't as pronounced as in the previous case - **Fig. 2**.

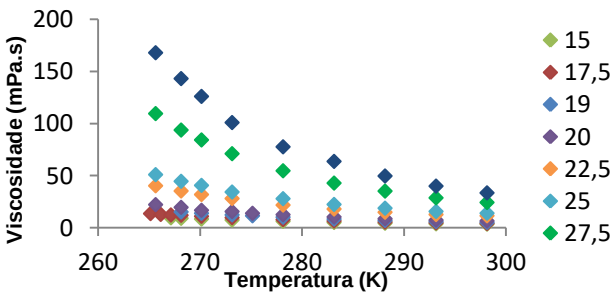

Fig. 1. Graphical representation of viscosity as a function of temperature for solutions with 10 g/dL trehalose and different concentrations of BSA (g/dL)

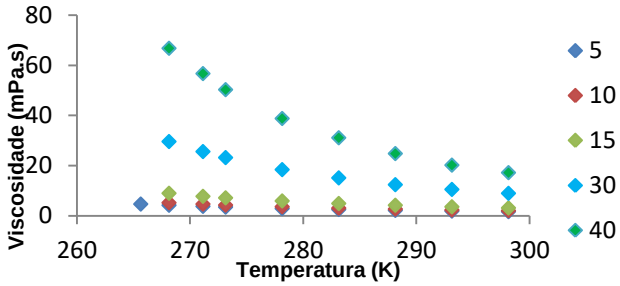

Fig. 2. Graphical representation of viscosity as a function of temperature for solutions with 10 g/dL trehalose and different concentrations of Ovalbumin (g/dL)

Effect of trehalose concentration

Figures 3 and 4 show the influence of trehalose variation on the solutions with BSA and Ovalbumin respectively.

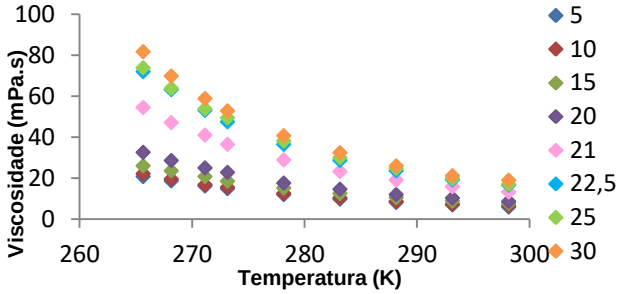

Fig. 3. Graphical representation of viscosity as a function of temperature for solutions with 20 g/dL of BSA and different concentrations of trehalose (g/dL)

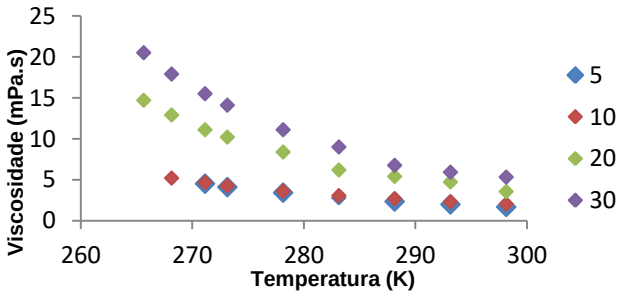

Fig. 4. Graphical representation of viscosity as a function of temperature for solutions with 10 g/dL of Ovalbumin and different concentrations of trehalose (g/dL)

Effect of pH variation

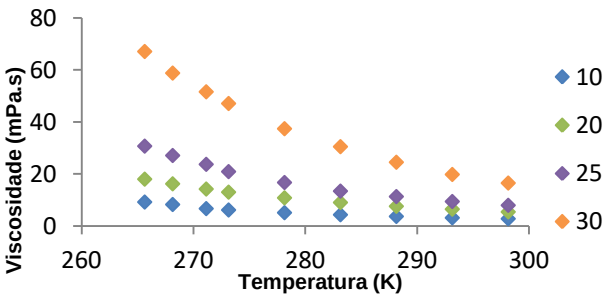

Fig. 5. Graphical representation of viscosity as a function of temperature for solutions with 10 g/dL of trehalose and different concentrations of BSA (g/dL). pH=7

DISCUSSION

Impact of pH and concentration on viscosity

By comparison of the graphics of **Fig. 6** and **Fig. 7** it is observed that, for the same concentrations and temperature, the viscosity value is significantly lower far from proteins isoelectric point. This is more pronounced for lower temperatures.

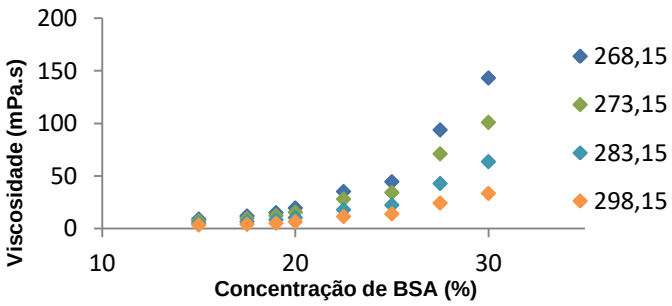

Fig. 6. Graphical representation of viscosity as a function of temperature for solutions with 10 g/dL of trehalose and different concentrations of BSA (g/dL) at different temperatures (K) at the isoelectric point

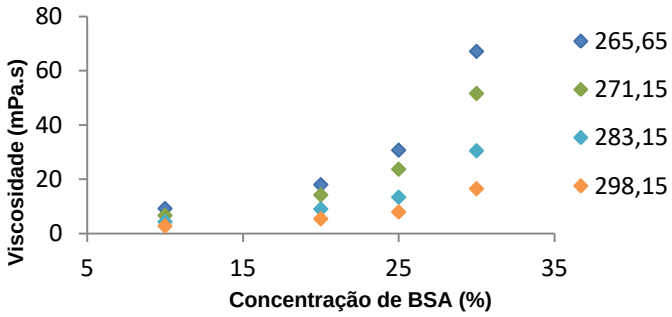

Fig. 7. Graphical representation of viscosity as a function of temperature for solutions with 10g/dL of trehalose and different concentrations of BSA (g/dL) at different temperatures (K) at pH=7

Another interesting result was the study of solutions with 20 g/dL of BSA and different concentrations of trehalose, in the protein isoelectric point - **Fig. 8**.

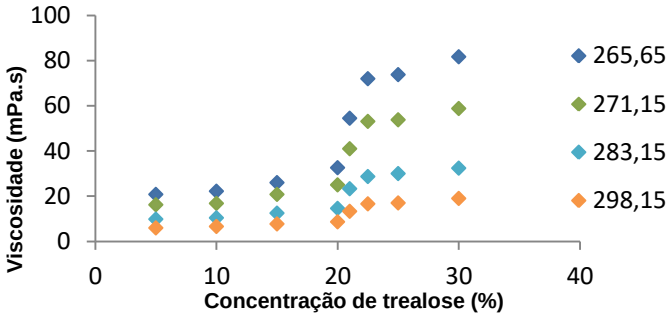

Fig. 8. Graphical representation of viscosity as a function of temperature for solutions with 20 g/dL of BSA and different concentrations of trehalose (g/dL) at different temperatures (K), in the protein isoelectric point

There is an abrupt increase in viscosity values when the trehalose concentration reaches 20g/dL. This rapid viscosity growth may be related to intermolecular interactions becoming stronger beyond this concentration.

However, the models must only take into account the protein concentration as the trehalose concentration in solution has much lesser impact on viscosity. Moreover, biotherapeutic solutions usually contain less than 10 g/dL of saccharides. For this reason, it's not necessary that the models contemplate the most critical zone presented in **Fig. 8** nor have the results been explored in this sense.

Models

For the different modified and adjusted models, one was selected based on the calculation of the absolute errors between the experimental values and the points determined by the models. So, after modification and adjustment, **Eq.12** and **Eq.13** correspond to the model applied to Ovalbumin and BSA, respectively. In equations c is the molar concentration and η_w is the water viscosity.

$$\eta = e^{[\,0.26 + (-46.52 \times T + 21906)\,c\,]} \times \eta_w \qquad \textbf{Eq.12}$$

$$\eta = e^{[\,(0.01 \times T + -5.3) + (-268.3 \times T + 1.24 \times 10^5)\,c\,]} \times \eta_w \qquad \textbf{Eq.13}$$

The applicability of the model to a specific MAb was tested as a case study in order to determine the protein concentration required to replicate the solution with the MAb under study and the result is shown in the **Table 1**.

Table 1. Values of protein concentration (g/dL) determined by the model and the associated maximum absolute error

Protein	Concentration (g/dL)	Maximum absolute error (%)
Ovalbumin	3,8	3,9
BSA	12,0	8,6

For BSA, the maximum absolute error obtained has the value 8.6%, which, although not very high, is higher than the value obtained in the calculations with Ovalbumin. Nonetheless, both proteins fulfill its purpose of mimic the viscosity of therapeutic protein solutions.

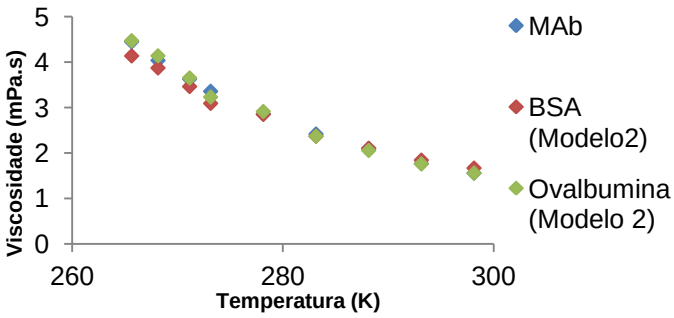

Fig. 9. Graphical representation of viscosity as a function of temperature for MAb solution and the predicted viscosities of the applied model

CONCLUSIONS

At the end of the developed work the following aspects are concluded:

- The increase protein concentration in solution has a greater impact on viscosity than the increase in trehalose concentration;
- It is confirmed that the pH variation of the solution and consequent separation from the protein isoelectric point influences the protein. A variation in pH of the BSA solution leads to significant variations in viscosity;
- It is possible to replicate the viscosity of MAb solutions with less expensive model protein solutions, namely Ovalbumin and BSA;
- It is possible to adjust mathematical models that relate viscosity variation to temperature and protein concentration;
- A solution with approximately 4 g/dL Ovalbumin and 10 g/dL trehalose at pH = 4.6 or a solution with about 12 g/dL BSA and 10 g/dL trehalose at pH = 4.7 is able to replicate the viscosity change with the temperature of the studied MAb, in a range between 25 and -7.5 °C

REFERENCES

(1) Miller, M.A., et al 2013
(2) Rodrigues, M. A., et al 2013
(3) Rodrigues, M. A., et al 2010
(4) Kolhe, P., et al., 2009
(5) Roessl, U., et al, 2013
(6) Rosa, M., et al 2013
(7) Cao, E. et al., 2003
(8) Corti, H., et al, 2008
(9) Einstein A., 1906
(10) Atkins, P., et al, Physical Chemistry
(11) Longinotti, M. P., et al, 2008
(12) Paznica, M., et al, 2012
(13) Corti, H. R., et al, 2008
(14) Inoue, K., et al, 2005
(15) Vand, V., 1948
(16) Angell, C.A., 2002
(17) Angell, C.A., 1988
(18) Williams, M. L. et al 1995
(19) Corti, H., et al, Diffusion of solutes in supercooled sugar aqueous solutions
(20) Angell, C.A., et al, 1994
(21) Rossler, E., 1990
(22) Richert, R., et al, 1990
(23) Peng, ZG, 2005
(24) Beeley, JA, et al., 1972