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Vibrational Properties of Bioprotectant Mixtures of Trehalose and Glycerol

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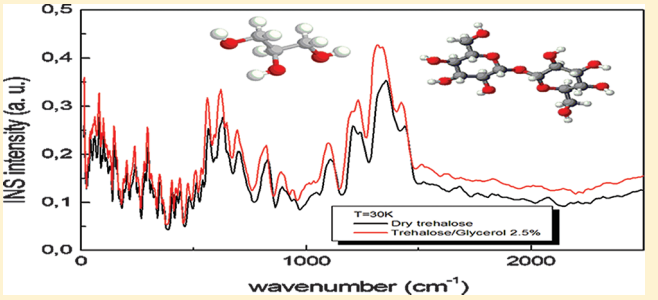
ABSTRACT: In this work vibrational spectra of mixtures of two glass-forming bioprotectant systems, i.e., trehalose and glycerol, are collected at very low temperature by using the indirect geometry time-of-flight (t.o.f.) TOSCA spectrometer at the ISIS Pulse Neutron Facility (Rutherford Appleton Laboratory, Oxford, U.K.). The main aim of this work is to investigate, through inelastic neutron scattering (INS), the vibrational behavior of trehalose and its mixtures with glycerol at different concentration values in order to characterize the changes induced by glycerol on the trehalose hydrogen bonded network. The obtained experimental findings, which are discussed and interpreted in the framework of previous INS, quasi elastic neutron scattering (QENS) and molecular simulation data obtained on trehalose/glycerol mixtures at different concentration and temperature values, will be linked to the different mixtures bioprotectant effectiveness.



I. INTRODUCTION

Understanding the molecular mechanisms responsible for biological processes is one of the goals of modern biophysics. In this frame, elucidating the strategies used by several organisms to survive under environmental stress conditions has both scientific and applicative interest.[1−3]

Among these survival stratagems, there are the capability to balance the osmotic pressure of hypersaline environments,[4] the thermotolerance of psychrophiles, thermophiles, and hyperthermophiles,[5−7] and the DNA repair mechanisms activated by radiation-resistant organisms.[8] Remarkable examples of extraordinarily surviving organisms are represented by polyextremophiles capable of effectively combining different responses to various environmental stresses, such as the archaea *Sulfolobus acidocaldarius* living at low pH and high temperature[9] and tardigrades surviving at very low and very high temperatures, very high pressures, and high radiation levels.[10,11]

The accumulation and interplay of compatible solutes such as trehalose and glycerol have been demonstrated to occur in desiccation and freezing conditions, so revealing a cryo- and lyoprotectant role of both carbohydrate and alcohol.[12−14] To cite an example, the natural synthesis of trehalose and glycerol has been found to be linked to the changes in membrane composition and to the prevention of the damage from dehydration in *Megaphorura arctica*.[12,13,15]

This work is aimed at obtaining information about the effect of glycerol on the hydrogen-bonded arrangement of the trehalose molecule at different glycerol content. These results provide precious information which can help the interpretation data of present and previous results obtained by using the OSIRIS and IRIS spectrometers at the ISIS facility (Rutherford Appleton Laboratory, Oxford, U.K.), which have shown the existence, at very low glycerol content, of a switching-off maximum for the fast and diffusive dynamics of trehalose/glycerol mixtures.[16,17] These findings showed that, in the investigated concentration range, at the concentration value of 2.5% by weight of glycerol in trehalose, a minimum exists in the mean square displacement, in the fragility parameter and in the translational line width.

On the other hand, Cicerone et al.[18,19] first had shown that the effect of addition of glycerol to trehalose is to reduce to the glass transition temperature of the disaccharide, by highlighting an anomalous behavior at the concentration value of 5% by weight of glycerol in trehalose in the mean square displacement.

In the present work trehalose/glycerol mixtures are investigated at the glycerol concentration values of 0.0, 2.5, and 25.0% in order to highlight the effects of glycerol on the intermolecular and intramolecular vibrational motions of trehalose and hence the changes induced by glycerol on the trehalose hydrogen bonded network.

II. EXPERIMENTAL SECTION

Vibrational spectra of trehalose and trehalose/glycerol mixtures at the glycerol concentration values of 0 (trehalose),

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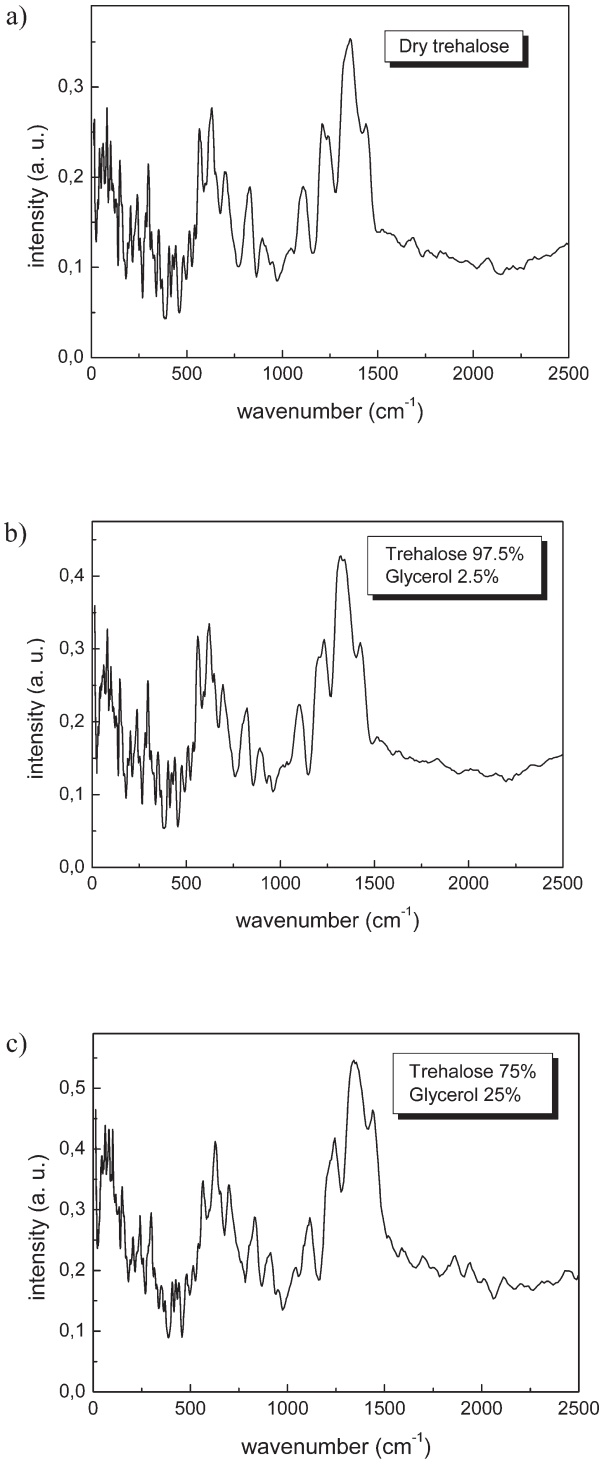

Figure 1. INS spectra of (a) trehalose, (b) trehalose/glycerol 2.5%, and (c) trehalose/glycerol 25%.

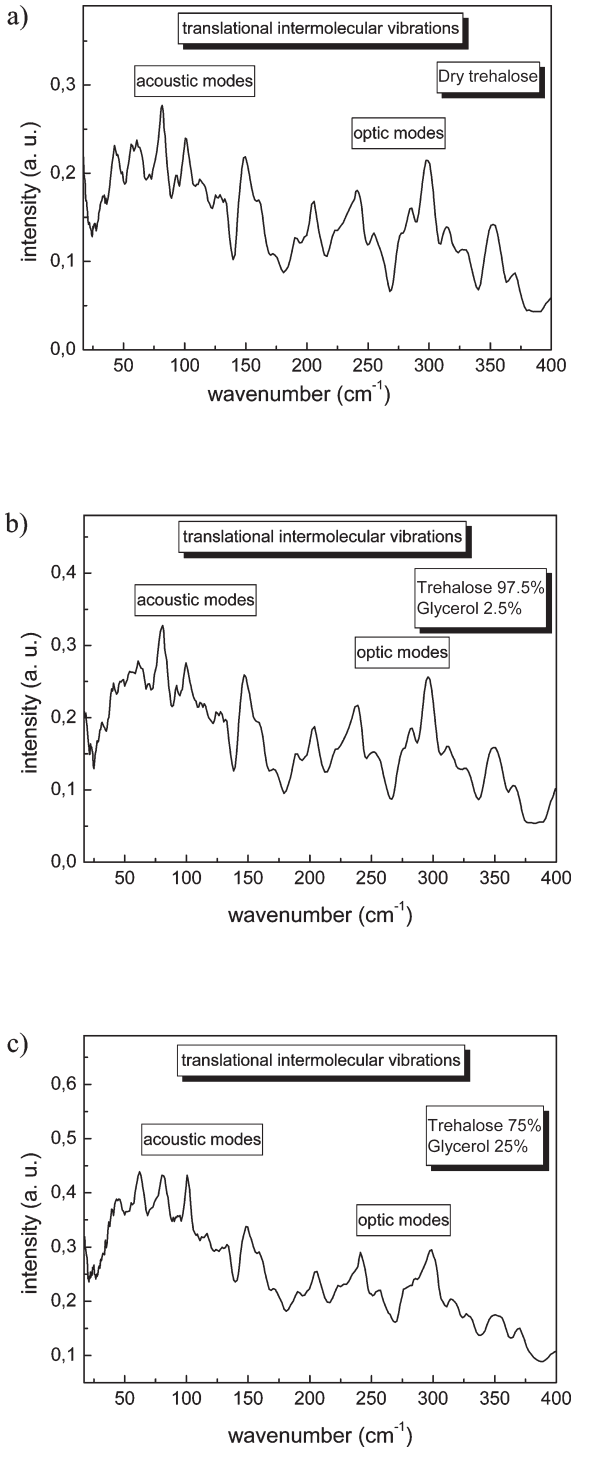

Figure 2. INS translational intermolecular vibrational modes of (a) trehalose, (b) trehalose/glycerol 2.5%, and (c) trehalose/glycerol 25%.

2.5 (trehalose/glycerol 2.5%), and 25.0% (trehalose/glycerol 25%) by weight have been collected at the temperature value of 30 K by the indirect geometry tof TOSCA spectrometer at the ISIS Facility (Rutherford Appleton Laboratory, Oxford, U.K.).[20−23] Ultra pure (99%) dihydrated trehalose and glycerol, purchased by Aldrich-Chemie, were used for sample preparation. The samples, contained in thin walled aluminum cells, were cooled to 30 K by a liquid helium cryostat and measurements were performed for 12 h for each sample.

TOSCA covers an energy range of 0÷4000 cm^{-1}, the vibrational bands being better defined in the 0÷2000 cm^{-1} with a resolution of $\Delta E/E \approx 1.5 \div 2\%$ and an uncertainty in the peak position shifts of ∼1 cm^{-1}. As described in ref 21, TOSCA has

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Table 1. Frequency Positions of the Acoustic and Optic Peaks for Trehalose and Its Mixtures with Glycerol

	dry trehalose	trehalose/glycerol 2.5%	trehalose/glycerol 25%
first acoustic peak position (cm^{-1})	76.78	74.64	76.48
second acoustic peak position (cm^{-1})	151.37	149.36	151.35
first optic peak position (cm^{-1})	238.21	236.01	238.30
second optic peak position (cm^{-1})	297.58	295.93	297.61

revealed itself to give detailed results combined to optical spectroscopic techniques such as Raman spectroscopy due to its design associating a single momentum transfer with each energy transfer. Furthermore TOSCA has been successfully used for investigating biological systems, such as globular proteins,[24−27] collagen and model polypeptides[28,29] and interfacial water.[30,31]

III. RESULTS AND DISCUSSION

In Figure 1 the total INS spectra in the 0÷2500 cm^{-1} region of trehalose and its mixtures with glycerol at T = 30 K for different concentration values are shown. Taking into account the different spectral regions, the following features can be observed.

i. 0÷400 cm^{-1} Region. This spectral region, shown in Figure 2, concerns the low energy collective modes. More specifically, in the INS spectrum of dry trehalose below 150 cm^{-1} the bands are linked to the intermolecular acoustic modes, whereas above 150 cm^{-1} the features of the intermolecular optic modes are identified.[32−34] In each region two peaks are present: the acoustic peaks at ∼76 cm^{-1}, which can be attributed to the first Van Hove singularity in the dynamics of acoustic phonons, and ∼151 cm^{-1} and the optic peaks at ∼238 and ∼297 cm^{-1}.[32−34]

In the presence of glycerol, in respect to pure trehalose these peaks are shifted to lower frequency values for the trehalose/glycerol mixture with a glycerol content of 2.5%, whereas they are unchanged for the trehalose/glycerol mixture with a glycerol content of 25%. In Table 1 the frequency positions of the acoustic and optic peaks for all the investigated systems are shown.

Such findings are in line with previous results,[32,33] where it is observed that at the glycerol content of 2.5% the hydrogen bonded network strength of trehalose is mostly affected by the presence of glycerol, while a higher amount of glycerol does not have remarkable effects. In particular, the registered frequency shift of these modes to lower values, at low glycerol content, points to a mass effect due to the hydrogen-bonded interaction between the disaccharide and the alcohol and, in turn, to a plunge of the "fragility" of the glass forming system trehalose.

ii. 400÷1060 cm^{-1} Region. Figure 3 shows the INS spectral region between 400 and 1060 cm^{-1} of trehalose/glycerol mixtures. By taking into account the vibrational spectrum of trehalose obtained by simulation,[34] the relevant bands present in this region, which is still linked to intermolecular modes, can be assigned. By a comparison with simulation findings,[34] we are able to assign the peaks observed at ∼423 and ∼569 cm^{-1} to the oscillations of the OH groups around the minimum of the

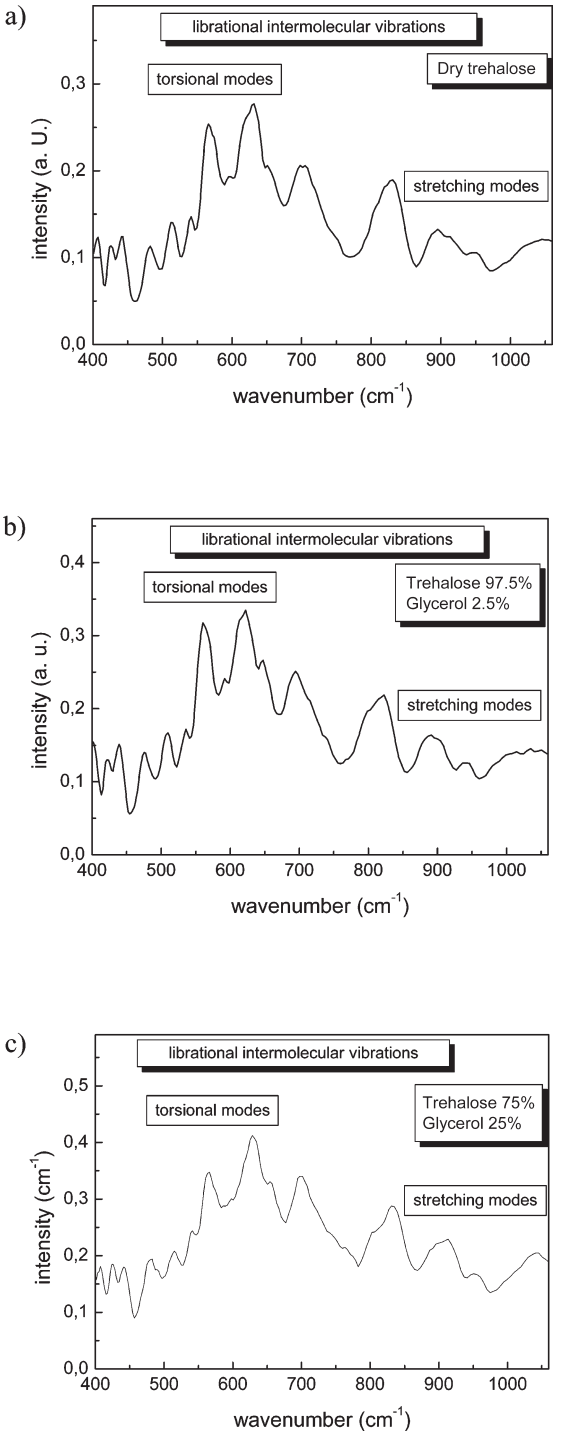

Figure 3. INS librational intermolecular vibrational modes of (a) trehalose, (b) trehalose/glycerol 2.5%, and (c) trehalose/glycerol 25%.

C−C−O−H torsional potential, with the exception of the OH group giving rise to the intramolecular hydrogen bond, whose torsional mode has an energy of about 700 cm^{-1}. Other peaks related to librational modes are found at ∼627, ∼824, and ∼903 cm^{-1}. The stretching of the C−O bonds occurs in a range between 970 and 1100 cm^{-1} where a large band centered at ∼1042 cm^{-1} is present.[34]

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Table 2. Frequency Positions of the Librational Peaks for Trehalose and Its Mixtures with Glycerol

	dry trehalose	trehalose/glycerol 2.5%	trehalose/glycerol 25%
OH-oscillation first peak position (cm^{-1})	422.94	415.75	423.87
OH-oscillation second peak position (cm^{-1})	569.04	563.87	569.13
first librational peak (cm^{-1})	627.52	621.33	629.30
OH-torsional peak position (cm^{-1})	702.45	696.80	702.32
second librational peak (cm^{-1})	824.60	813.05	828.69
third librational peak (cm^{-1})	903.74	891.85	903.56
C−O stretching peak position (cm^{-1})	1042.70	1023.80	1042.80

In such a vibrational spectral region, the effect of glycerol is again to systematically shift the peaks to lower frequency values for the trehalose/glycerol mixture with a glycerol content of 2.5%; furthermore, this shift is more and more important by increasing frequency. On the other hand, no shifts or very slight shifts occur to higher frequency values for the trehalose/glycerol mixture with a glycerol content of 25%. The frequency positions of the librational peaks for all the investigated systems are reported in Table 2.

What clearly emerges is that, similarly to what happens in the previous intermolecular vibrational region, the addition of an amount corresponding to 2.5% of glycerol in trehalose has the effect of creating a privileged interaction between trehalose and glycerol.

iii. 1060÷2000 cm^{-1} Region. The spectral intensity profiles of trehalose and of its mixtures with glycerol in this spectral region, corresponding to the range of the bending intramolecular vibrational modes, are reported in Figure 4.

By the simulation results,[34] the hybridized H−C−H, C−C−H, and C−O−H bending modes are known to be responsible for the peaks at ∼1118, ∼1240, and ∼1365 cm^{-1}. By evaluating the conformational freedom in the trehalose molecule by simulation, it is observed that a relatively low energy is required for the torsions around the oxygen linking the two rings to occur, whereas the bending of the same angle is much less likely, being energetically unfavored.[34]

By an inspection of Figure 4, it appears that the bending modes are significantly influenced by the presence of 2.5% glycerol, with a shift to lower frequency values which increases with frequency, whereas the shift is to higher frequency for the other investigated mixture. This result suggests that the origin of the shift is to be attributed to a mass effect, namely to an added mass effect, which, in turn, is reflected in a higher rigidity for such a glycerol concentration value. Table 3 reports the frequency positions of the bending peaks.

In previous works,[32,33] the more "crystalline" spectral features of pure trehalose in comparison with those of its homologous pure disaccharides, maltose and sucrose, were linked to its more ordered structure on a nanoscopic scale, i.e., to its superior "cryptocrystalline" character; in particular these features were put in connection with its higher cryptobiotic effect, i.e., its capability to create a more rigid

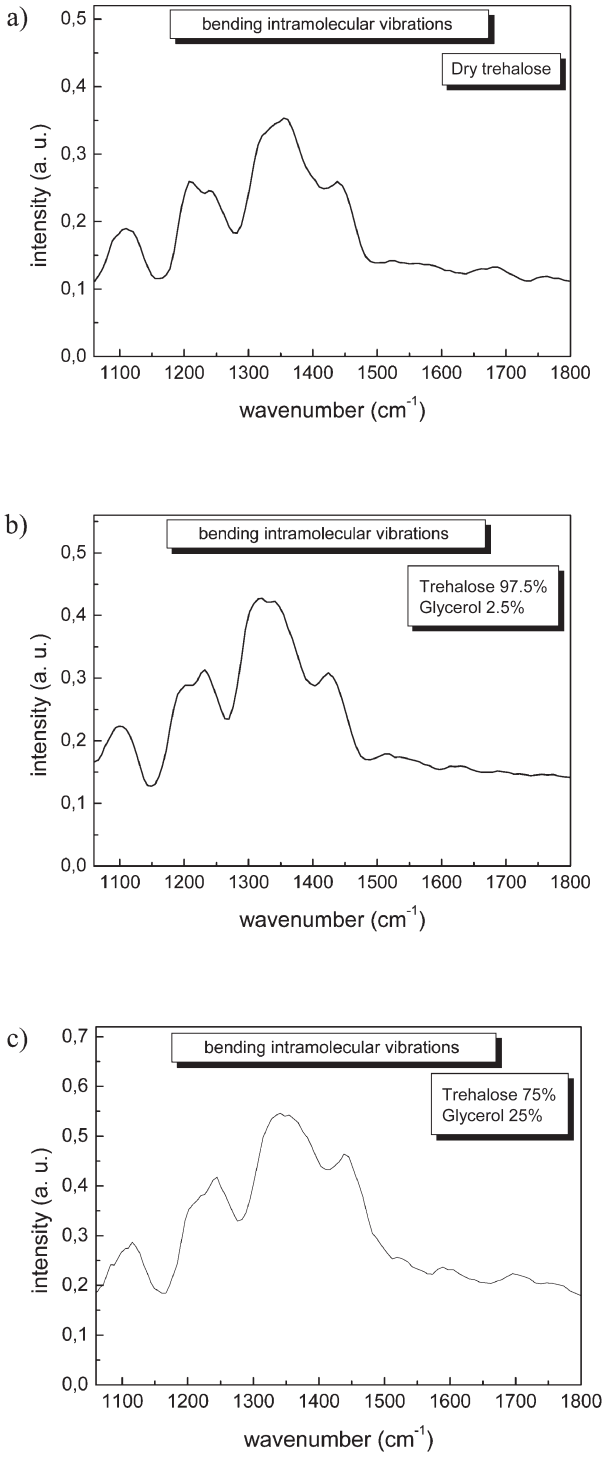

Figure 4. INS bending intramolecular vibrational modes of (a) trehalose, (b) trehalose/glycerol 2.5%, and (c) trehalose/glycerol 25%.

environment where biomolecules are better protected under stress conditions.[32,33,35]

By the present findings, it is possible to confirm previous results[16] indicating that the trehalose/glycerol mixtures at a concentration value of 2.5% show, as pointed out by frequency shifts which have been attributed to mass effects, a higher rigidity in respect to pure trehalose and to the mixtures containing a higher percentage of glycerol.

Table 3. Frequency Positions of the Bending Peaks for Trehalose and Its Mixtures with Glycerol

	dry trehalose	trehalose/glycerol 2.5%	trehalose/glycerol 25%
first bending peak position (cm^{-1})	1109.32	1098.70	1116.51
second bending peak position (cm^{-1})	1231.74	1220.81	1242.00
third bending peak position (cm^{-1})	1355.61	1333.94	1361.65

IV. CONCLUSIONS

The present INS findings point out the effects of glycerol on the trehalose vibrational properties and allow us to get precious information about the changes induced by the alcohol on the disaccharide hydrogen-bonded network.

By the analysis of both the intramolecular and intermolecular motions of pure trehalose and of trehalose/glycerol mixtures at two glycerol concentration values, it emerges that the glycerol content of 2.5%, due to the trehalose/glycerol interaction, makes less weak the trehalose hydrogen-bonded structure by means of stronger attractive forces between glycerol and trehalose molecules.

The detailed assignment of the translational and librational intermolecular and bending intramolecular vibrational bands provides an explanation to the previous investigations performed on trehalose/glycerol mixtures.[16−19,36−38] By these studies and in particular by the performed simulations,[18,19,36−38] the hydrogen bonding behavior of the trehalose/glycerol system was determined using the geometric criterion providing information about the distance between the donor and acceptor oxygens and the angle formed by the donor oxygen, the acceptor hydrogen and the acceptor oxygen and evaluating the occurrence of the hydrogen bonding in trehalose molecules in presence of glycerol.[16−19,36−38] The origin of the trehalose fast dynamics suppression by glycerol was hypothesized to be found in the change of the hydrogen bonded network of the system as it appears in the average lifetime and the occupancy of the hydrogen bonds.[16−19,36−38] More specifically, the average number of intermolecular hydrogen bonding formed between trehalose molecules and either other trehalose or glycerol molecules and the average number of intermolecular hydrogen bonding formed between glycerol molecules and either other glycerol or trehalose molecules highlighted a strengthening of the hydrogen bonded network of trehalose/glycerol mixtures at very low glycerol content compared to pure trehalose. At higher glycerol concentrations, glycerol is no longer capable of creating stronger hydrogen-bonding with trehalose than those existing among trehalose molecules, and then the trehalose dynamics is not strongly affected.[16−19,36−38]

The present findings confirm and justify the previous evidence, highlighting the effect of the 2.5% glycerol amount on the specific vibrational modes of trehalose. The shifts to lower energies shown by the analysis of the different INS spectral regions of the trehalose/glycerol 2.5% mixture increase by increasing frequency, so revealing that the effect of glycerol is more marked on the intramolecular modes than on the intermolecular modes.

Furthermore it is to be observed that these INS data are collected at very low temperature; therefore, they can have interesting implications about the described combined role of the trehalose/glycerol system as a cryo- and lyoprotectant system. The occurrence of a large amount of trehalose and small amount of glycerol in several organisms capable of activating a cryoprotective dehydration process can find a physical elucidation by the present findings. In fact, it is known that the dynamics slowing down due to a strong hydrogen bonded interaction can be linked to the decreased fragility and then to the increased bioprotective effectiveness of the system.[16−19,35−38]

This experimental evidence can have a considerable applicative interest, since today the use of bioprotectant systems is more and more addressed to the long-term stabilization of biological systems by cryopreservation and drying processes. The application fields include for example cell preservation,[39] transplantation organ storage and delivery,[40] and blood conservation.[41]

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