Natural deep eutectic systems as alternative nontoxic cryoprotective agents

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Abstract

Natural deep eutectic systems (NADES) are mostly composed of natural primary metabolites such as sugars, sugar alcohols, organic acids, amino acids and amines. These simple molecules have been identified in animals living in environments with extreme temperature amplitudes, being responsible for their survival at negative temperatures during winter. Herein, we report for the first time the use NADES based on trehalose (Treh) and glycerol (Gly) in cryopreservation, as cryoprotective agents (CPA). The evaluation of the thermal behaviour of these eutectic systems, showed that NADES have a strong effect on the water crystallization/freezing and melting process, being able to reduce the number of ice crystals and hence ice crystal damage in cells, which is a crucial parameter for their survival, upon freezing. Using this NADES as CPA, it is possible to achieve similar or even better cellular performance when compared with the gold standard for cryopreservation dimethyl sulfoxide (DMSO). In this sense, this work relates the physical properties of the NADES with their biological performance in cryopreservation. Our comprehensive strategy results in the demonstration of NADES as a promising nontoxic green alternative to the conventional CPA's used in cryopreservation methods.

Keywords: Natural Deep Eutectic Solvents; Sugars; Cryoprotectants; Freeze-thawing

Introduction

In the last decade, deep eutectic solvents (DES) have emerged as a promising alternative to conventional solvents used in different areas. DES have been described as a result of intermolecular hydrogen bonds between two or more compounds, which at an adequate molar ratio lead to a strong depression in the melting point when compared with the ones of the individual components [15,37,48,50,60]. These eutectic systems fully comply with the green chemistry metrics. They present low toxicity, are often biodegradable and no waste is generated upon their production. Furthermore, in comparison with other designer solvents such as ionic liquids, DES are cheaper to produce, since the raw materials have a lower cost, their synthesis is quite simple and compounds with high purity and no by-products are obtained [13,32,46]. Nonetheless, some recent publications reported that DES appear to have some toxicity [32,34,40,46]. To overcome this drawback, the use of natural origin molecules to produce DES has been proposed and these are hence called natural deep eutectic systems (NADES) [33,34,40].

NADES are mostly composed by natural primary metabolites such as sugars, sugar alcohols, organic acids, amino acids, and amines and additionally often contain water in certain molar ratios [13,18,46,54]. Such as it happens in DES, the position and the number of the hydrogen bonds, the hydrogen bond donor (HBD) and the hydrogen bond acceptor (HBA), have a significant influence in the stability of NADES [9,16,17,22]. Some studies have demonstrated that the stability and properties such as viscosity, conductivity, toxicity and biocompatibility can be influenced by the addition of water [3,10,16,22,23,34,45]. The introduction of water as a tertiary component primarily leads to strong hydrogen bond interactions between water and the components of NADES. In addition, it decreases the

65 overall viscosity of the eutectic mixture and, consequently, enhances its process ability and
66 decreases the cytotoxic profile of NADES [18,40]. Recently, the presence of NADES in
67 animal and plants who survive in extreme conditions and temperature amplitudes has been
68 discussed in the literature [2,10,11,42,52,58]. These publications have demonstrated that the
69 presence of considerable amounts of simple molecules (i.e., sugars, polyols and amino acids)
70 in all these microbial, mammalian and plant cells may be crucial for their survival
71 [10,19,30,34,58]. NADES have been pointed out as essential for the dissolution of
72 intercellular solutes of intermediate polarity, in the storage of metabolic products, in the
73 germination, and in the resistance of various organisms to extreme temperature conditions
74 due to the vitrification of water inside the cells [40].
75 Our work, herein described, focuses on the possible application of NADES in
76 cryopreservation process as new cryoprotective agents (CPA).The cryopreservation process
77 can be defined as the use of extreme cold temperatures to maintain and preserve biologic
78 material, usually below -140 °C [4,30,41,55]. In this process, the formation/presence of ice
79 crystals, formed upon freezing, in the biological systems has several consequences for cell
80 survival, hampering their viability. Unprotected freezing is normally lethal to cells and thus
81 the use of a CPA plays an important role in the survival of cells, as it inhibits the
82 crystallization of water and, thereby, the formation of ice [27,35,38,49,55]. NADES may play
83 the same role as a CPA, i.e., they are able to vitrify water, reducing the crystallization
84 temperature of water and changing the crystallization phenomena [13,24,60]. For this reason,
85 NADES have been suggested as the liquid media, responsible for the survival of cells during
86 the winter, in animals living in extreme temperature amplitude environments
87 [8,11,12,30,52,58]. In a previous work, our group reviewed and listed the compounds
88 reported to be present in different organisms which survive to extreme temperature

amplitudes [30], and with such collected information, we herein tested various combinations of these compounds.

In this work, we studied an eutectic system based on trehalose:glycerol (Treh:Gly) (molar ratio 1:30) as an alternative CPA to DMSO (Fig.1), known as the gold standard. This system was selected since the pure individual components have already been reported as CPA in several publications [5,6,44,51,53,55,7,14,20,21,27,35,36,43]. However, up to now, to the best of our knowledge, the use of eutectic mixtures as CPA has not yet been reported. The use of eutectics as CPA may present enhanced properties and advantages caused by a synergistic effect between the components that form the eutectic system and can provide interesting features for new developments in this field.

Materials and Methods

Preparation of NADES

NADES were prepared using D-trehalose dihydrate (Treh, 99% purity, CAS 6138-23-4, Sigma Aldrich) and glycerol (Gly, 99.9% purity, CAS 56-81-5, Sigma Aldrich) at different molar ratios (see Table 1, Results). Briefly, the eutectic systems were prepared by gently mixing both components and heating the mixture at 70 °C, with a constant stirring, until a clear liquid was formed.

Polarized light microscopy (POM)

The optical characterization of the formulations of NADES was acquired at room temperature by POM, using an Olympus transmission microscope (Olympus, UK) coupled with a Leica digital camera DFC 280 (Leica, UK. The microscopic slides containing 1-2 droplets of NADES were observed on a microscope.

Preparation of NADES with different amounts of water

In order to study the influence of Treh:Gly (1:30) in the thermal properties of water, several different NADES and water mixtures were prepared. Individual samples of Treh:Gly (1:30) with 40, 50, 60, 70, 80 and 90% (wt %) of deionized water were prepared, and stored in a desiccator, until DSC analysis.

Differential scanning calorimetry analysis (DSC)

DSC experiments of Treh, Gly and Treh:Gly (1:30) were carried out in a DSC Q2000 from TA Instruments Inc. (Tzero DSC technology) operating in heat Flow T4P mode. The measurements were carried out under anhydrous high purity nitrogen at a flow rate of 50 mL min^{-1}. The calibration was carried out in the temperature range from -90 °C to 200 °C. When deionized water was studied, it was submitted to several cooling and heating runs between -90 and 120 °C, at a rate of 10 °C/min. All the samples were encapsulated in aluminium pans with a hermetic lid containing a pinhole, which allow the water evaporation, except in the case of deionized water. When mixtures of Treh:Gly(1:30) and water were analysed by DSC, several cooling runs between -90 and 40 °C were performed, also at a rate of 10 °C/min, but

in this case the hermetic pans did not have a pinhole, to avoid any water loss. In some samples, the scan rate was varied in order to see the influence in the resulting thermal behaviour of the water and NADES mixture. In those cases, the heating scan rate was 5 °C/min or 20 °C/min.

NMR studies

The NADES and raw materials were dissolved in dimethyl sulfoxide-d6 (DMSO-d6, 99.9 atom %, LOT. STBH4385, Sigma Aldrich). NMR experiments were recorded at a 400 MHz Bruker Advance II. ^{1}H chemical shifts are expressed in ppm with the residual HOD solvent signal as reference. *Mestrenova 9.0* software was used for spectral processing.

Viscosity measurements

The viscosity of the different systems was measured using a Kinexus Prot Rheometer (Kinuxus Prot, MaL 1097376, Malvern) fitted with parallel plate geometry with 20 mm of diameter (PU20 SR1740 SS) and 1 mm of gap. The effect of water content was studied using eutectic systems containing different percentages of water (10, 20, 50, 80, 90, 95 and 99%). In addition, the viscosity of the gold standard CPA (DMSO) was also assessed by using 10% of DMSO in FBS or water. The viscosity of 10% of NADES in FBS solution was also tested. All the measurements were performed under controlled stress conditions and at a constant shear rate of 10 s^{-1}. After equilibrating the sample temperature at 20 °C during 5 min, a temperature scan was performed from -20 °C to 40 °C. at 2 °C/min.

Cell culture

The cell culture studies were performed with L929 cell line (European collection of cell culture (ECCC), UK) which are mouse fibroblasts of connective tissues. L929 cells were grown in DMEM (Sigma, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochron AG, Germany) and 1% antibiotic – antimycotic (Gibco, USA). The cells were cultured in a humidified atmosphere of 37 °C, with 5% CO_2.

Indirect – contact of NADES with cell cultured monolayers

The cytotoxicity was initially studied by analysing the effect of NADES on the cell's metabolism, which is in accordance with ISO/EN 10993 guidelines. Briefly, the cells were seeded onto a 96-well plate ($1x10^4$ cells/well) and after 24 hours of attachment, the medium was removed and extracts with different percentage of NADES (1, 5, 10 and 20%) and DMSO (1, 5, 10 and 20%) were added. A latex rubber extract was used as positive control for cell death, while the culture medium was used as negative control representing the ideal situation for cell proliferation. The cell viability was determined after 24 hours of incubation with extracts by CellTiter 96® Aqueous One Solution Cell Proliferation Assay which is based on tetrazolium active component ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) – MTS). After 24 hours, the cell monolayers were washed with PBS and immersed in a mixture consisting of serum-free cell culture medium and MTS reagent in a 5:1 ratio and incubated for 3 hours. Afterwards, 100 µL of each well were transferred to a 96-well plate. The amount of formazan product was measured by absorbance at a wavelength of 490 nm using a microplate spectrophotometer

(Bio-TEK, USA). Cell viability was expressed as percentage of cells exposed to extracts versus control cells.

Freezing and thawing of cells with NADES

The freezing and thawing of cells was performed according to methods elsewhere reported [25,26,59]. Briefly, for the freezing process, the confluent cells were trypsinized and collected by centrifugation (300 rpm, 5 min). Afterwards, the cells ($1x10^6$ cells/mL) were re-suspended in different formulations, including: a mixture of FBS with DMSO (10 vv.%) and a mixture of FBS with Treh:Gly(1:30) (10 wt.%). Additionally, the role of the individual counterparts of NADES were also tested, using Treh and Gly either at 10 wt % or in the same concentration as in NADES form. The cryotubes (Nunc®, Sigma Aldrich) containing the different formulations were placed in a freezing container "Mr Frosty" (Nalgene®, Height 86 mm, diameter 117 mm, Sigma Aldrich) which was directly transferred to -80 °C for 24 hours. After this period, the cryotubes were transferred to liquid nitrogen (-196 ºC) for 1, 2 and 3 months.

For the thawing process, the cryotubes were warmed up in a 37 °C water bath for approximately 1 min, until the ice disappears. After re-suspension in D-MEM, the cell suspension was centrifuged (300 rpm, 5 min). Upon discarding the supernatant solution, the cells were re-suspended in fresh culture medium and seeded in a 96-well plate in order to evaluate the changes on cell's viability, proliferation and morphology. The cells were sub-cultured up to 3 passages. The cell behaviour upon exposure to different CPA was compared with the gold standard, which is a solution of 10% (v/v) DMSO in FBS and a storage temperature of -196 ºC.

Cell viability assay

The influence of the CPA on cell viability was determined by MTS assay after thawing and replanting cells. At each time point (i.e., 24 hours and 72 hours) the cells were washed with PBS and MTS assay was performed as aforementioned. In addition, the live/dead assay was also performed by using calcein AM/propidium iodide (PI) staining. Briefly the cell monolayers were incubated for 20 min with calcein AM (2 μg mL^{-1}, Molecular Probes, Invitrogen) and PI (3 μg mL^{-1}, Molecular Probes, Invitrogen). Afterwards, the cell monolayers were washed with PBS to remove residual fluorescent and visualized by inverted microscope (Axio Observer, ZEISS, Germany).

Cell morphology

At predetermined time points the cells were fixed with 10% (v/v) formalin (Thermo Fisher Scientific) and stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, Sigma Aldrich,) and phalloidin–tetramethylrhodamine B isothiocyanate (Phalloidin, Sigma-Aldrich) to visualize F-actin filaments and cell nuclei, respectively. At each time point DAPI (2 μg mL^{-1}) and phalloidin (5 μg mL^{-1}) staining was performed for 40 min at room temperature and protected from light. Upon PBS washing the cells were visualized in the dark by an inverted microscope (Axio Observer, ZEISS, Germany). The morphologic changes on cells was also observed by optic microscopy (AxiovertA1, Zeiss, Germany).

Cell proliferation

The Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen Life Technologies, Scotland) was used for DNA quantification according to the instructions from the manufacturer. Prior to the quantification, the cells were lysed by osmotic and thermal shock and the supernatant was collected for the assay. Triplicates were performed for each sample or standard assay. The absorbance was read in a microplate reader (Bio-TEK, USA), using 485 and 528 nm as excitation and emission wavelengths, respectively, according to the spectroscopic properties of the dye. The DNA amounts were calculated from a standard curve.

Statistical analysis

The experiments were carried out in triplicate unless otherwise stated. The results were presented as the mean ± standard deviation (SD). Statistical analysis was performed by Shapiro Wilk normality test using Graph Pad Prism 7 for Windows. After this analysis, non-parametric (Kruskal Wallis test) or parametric tests (one-way Anova followed by Tukey test) were used depending on if the samples were from normally distributed populations or not, respectively.

Results

Preparation of NADES

In present work, glycerol (Gly) and trehalose (Treh) were mixed in different molar ratios, in order to obtain an eutectic mixture. The formation of the liquid mixture was defined from

direct observation of the different molar ratios (Table 1). Using a molar ratio of 1:30, a transparent viscous liquid was obtained at room temperature (RT). The other molar ratios lead to a white solid mixture and/or liquid mixture with some crystals and were thus discarded. The successful production of an eutectic mixture (Treh:Gly (1:30)) was confirmed by polarized optical microscopy (POM) (Fig. 2). The full back colour of the POM image shows that no crystals were presented in the solution. Additionally, the DSC thermogram of NADES (Fig. 2) also demonstrate a depression of the melting point of the individual counterparts.

Nuclear magnetic resonance (NMR) spectroscopy was also used to prove the existence of hydrogen bonds in NADES [51–54]. In this study, 1H NMR was applied to Gly (Fig. 3A). , Treh (Fig. 3B) and Treh:Gly (1:30) (Fig. 4). On other side, 2D NMR (HMBC) was only applied to Treh:Gly (1:30) (Fig. 5).

In the ^{1}H NMR spectrum, the main difference is a shift on the chemical signals of the hydroxyl groups (-OH) of Treh. In the Treh spectrum (Fig. 3B) the signal of OH groups is larger presenting one shift between 3.71 ppm and 4.30 ppm, whereas in the Treh:Gly (1:30) spectrum (Fig. 4) the signals of OH groups are well defined in different shifts (4.31-4.32 ppm, 4.54-4.58 ppm, 4.71-4.75 ppm and 4.85-4.88 ppm). This chemical displacement demonstrates that OH groups are affected by hydrogen bonds that were created between Gly and Treh. Overall, the results indicate (Fig. 4 and Fig. 5) the existence of hydrogen bonding between Treh and Gly in the Treh:Gly (1:30) demonstrating the successful formation of an eutectic mixture.

Assessment of the viscosity of DMSO and Treh:Gly(1:30) with different percentages of water and fetal bovine serum (FBS)

We studied the viscosity of the Treh:Gly (1:30) system with different percentages of water (10, 20, 50, 80, 90, 95 and 99%) in a temperature range from -20 ^{o}C to 40 ^{o}C, being -20 ^{o}C the lower limit of the rheometer used. The viscosity profile of Treh:Gly (1:30) presented a distinct behaviour at negative temperatures, which included the presence of oscillations and an unstable behaviour during the temperature ramp specially for higher percentages of water (i.e., 95% and 99%). In the positive range of temperatures (Fig. 6 A), the profile is more stable and no evident variations of viscosity along the temperature axis can be seen. In addition, the results also indicate a decrease on the viscosity of Treh:Gly (1:30) system upon increasing the water content. Such results can indicate the establishment of hydrogen bonds between water and the NADES.

To mimic the conditions used during cryopreservation procedures, the behaviour of a mixture of 10% Treh:Gly (1:30) in 90% water was compared with the one of the gold standard CPA-dimethyl sulfoxide (DMSO) (Fig. 6 B). A comparison between the performance of the systems in FBS and water was also performed (Fig. 6 B). The results indicate a similar trend on viscosities between Treh:Gly (1:30) and DMSO using water as solution. When FBS is used as solution the trend is different; at negative temperatures the Treh:Gly (1:30) system presents values of viscosity higher than DMSO. Only at positive temperatures (Fig. 6 B1) the values of viscosity between these two systems present some similarity.

286 **Assessment of the thermal behaviour of CPA**

287 In order to evaluate the influence of the NADES Treh:Gly (1:30) mixed with water, several
288 formulations were prepared, with different percentages of water and NADES (see
289 experimental section). In order to evaluate the potential of NADES to prevent or alter the
290 water thermal behaviour, and its possible CPA role, the thermal behaviour of each of these
291 mixtures was analysed by DSC.

292 The thermogram of the NADES Treh:Gly (1:30) (Fig. 7A) shows that with the exception of
293 glass transition, no other thermal events occur in the range of temperatures under study.
294 When higher amounts of Treh:Gly (1:30) are present, no thermal events are registered, but
295 when water and Treh:Gly (1:30) are mixed at 40:60 wt % or above, both melting events and
296 glass transition are observed in the DSC experiments (Fig. 7 B and C). Table 2 presents the
297 values of the correspondent glass transition temperatures (T_g) and melting temperatures (T_m)
298 as well as for the thermodynamic properties heat capacity (ΔC_p) and melting enthalpy (ΔH_m).
299 For samples with more than 60 wt % of Treh:Gly (1:30), the only thermal event registered is
300 the glass transition, meaning that the mixtures behave as amorphous material with no
301 evidence of water crystals presence. Using higher amounts of water (from 50 to 90 wt %) an
302 exothermic peak associated with melting is always observed. From this peak a melting
303 temperature-Tm- can be determined, and the melting enthalpy variation (ΔH_m) can be
304 determined from the area under the peak.

305 From these results, it is clear that the NADES has an influence on the thermophysical
306 properties of water, and that the thermal behaviour is dependent on the amount of NADES
307 present. Thereby, higher wt % of NADES decreases the melting temperature of the mixture,
308 and consequently increases the melting enthalpy. Even in the case of 10% of NADES in 90%

309 of water (wt %), the melting temperature is decreased ca. 6 °C, when compared to deionized
310 water alone. The T_g and ΔC_p of the mixtures of water and Treh:Gly (1:30) follows the same
311 trend, meaning that the NADES can have a plasticizing effect to some extension. Below 30
312 wt% of water no thermal events are detected, and up until 40% of water the glass transition
313 is inexistent or below the temperature detection limit of the DSC. A different behaviour is
314 observed when 40 wt% of water is present. In this case, no glass transition is observed and
315 only a cold crystallization peak is detected. These results show that this NADES Treh:Gly
316 (1:30) can act as a CPA, since it can promote water vitrification, increasing the temperature
317 range where water is not crystallized, even at sub-zero temperatures.

318

319 **Contact of NADES and DMSO with cell culture during 24h**.

320 The cytotoxic profile of different percentages of Treh:Gly (1:30) and DMSO (1, 5, 10 and
321 20%) was evaluated *in vitro* against L929, which is a reference cell line commonly used in
322 cytocompatibility tests (Fig. 8). The interactions of NADES and DMSO with L929 were
323 evaluated through the previously described indirect – contact of NADES with cell cultured
324 monolayers, i.e, a D-MEM cell culture media extract test, which is designed to measure the
325 rate of cytotoxicity of the systems. Cells cultured for 24 hours in the presence of extracts
326 containing different NADES concentrations have a higher viability. That is to say, under
327 these conditions the results indicate that the eutectic system Treh:Gly (1:30) presents lower
328 cytotoxicity at all the tested percentages when compared with DMSO. In addition, it is also
329 important to point out that cell viability decreased as the percentage of NADES in extracts
330 increased, being the lowest value of cell viability obtained for a percentage of 20% Treh:Gly

331 (1:30). On the other hand, the cells in contact with lower concentrations of DMSO present
332 values of viability of ca. 2%.

333

334 **Cell recovery after cryopreservation with freezing medium containing different CPA.**

335 Once the cytotoxic profile of NADES was established, the potential of NADES as CPA was
336 evaluated using L929 as a model cell line. Cells were cryopreserved in different freezing
337 media, containing a mixture of 90% (vv.) of FBS with 10% (vv.) of DMSO or 10% (wt.)
338 Treh:Gly (1:30).

339 The individual components (Treh and Gly) were also used as control to get insights on the
340 effect of each one as CPA. To evaluate such effect the individual components were used in
341 the same amount that is presented in 10% (w/v) of Treh:Gly (1:30). The vials from each
342 formulation were thawed at different periods of time 1, 2 and 3 months. The cell behaviour
343 was evaluated in terms of viability, morphology and proliferation after 24 or 72 hours of
344 culture. The results indicate a similar cellular response when DMSO or Treh:Gly (1:30) are
345 used as CPA (Fig. 9). In both treatment groups, the cells are viable, presented a stretched
346 morphology and no clusters can be seen after 24 or 72 hours of the thawing process. However,
347 when pure Treh or Gly were used a different cell behaviour was observed. Using Treh the
348 morphology, viability and proliferation are strongly compromised, whereas the same
349 behaviour does not occur for Gly. Combining both components, the cells were highly
350 attached to the surfaces, viable, and no disruption on cell membrane occurred. Moreover, the
351 cell proliferation rate was higher using Treh:Gly (1:30) which is an indicative of the
352 synergistic effect between these compounds when in NADES form. The impact of the CPA

on cells was also quantitatively evaluated by MTS assay and by DNA quantification after 24 and 72 hours of the thawing. As such, it is possible to verify that a statistical enhancement on cell viability was observed after 72 hours of thawing using Treh:Gly (1:30) as CPA (Fig. 9D and E). Regarding the DNA content no changes were found between the cells cryopreserved with NADES and with DMSO. A similar behaviour was also observed, when thawing the vials of the different formulations for periods up to 3 months (Fig. 9). In which concerns the cell viability, the cells cryopreserved with NADES remain viable with values similar to the ones obtained with DMSO, in all the evaluated storage time. These results were corroborated by Live/Dead assay (Fig. 8 A). Regarding cell morphology, no alterations were observed using Treh:Gly (1:30) (Fig. 8 B and C) and the cells remain able to proliferate along with time.

The cell behaviour was also evaluated using different passages (P) after the thawing of the cells. The results obtained in the NADES case indicate a similar trend between the passages, and a better cell performance on P1 than P0, while between P1 and P2 no differences were observed. This trend also occurs on the control (DMSO) (Fig. 10, Fig. S1 and Fig. S2). This is in agreement with the conventional observations post-thawing studies. After thawing cells are recovering and their cellular activity has not yet reached a maximum level. Hence despite their good viability it is only after the first passage that cells present higher values of viability and a boost in proliferation is observed.

Discussion

The main aim of this study was the development of new and "greener" CPA, for cryopreservation processing. A new CPA should overcome toxicity problems of the conventional ones and should not compromise, at the same time, the viability of cryopreserved cells or organisms when drastic negative temperatures are applied. To achieve such goal, an eutectic system based on Treh and Gly was devised. These components have been previously reported to be present in some animals that survive to drastic temperature amplitudes throughout the year. For the preparation of this eutectic system, different molar ratios of Treh and Gly have been tested until a clear transparent liquid was obtained and no crystals were observed by POM- see Fig 2 (i.e., molar ratio 1:30, respectively).The evaluation of the thermal behaviour, confirmed the formation of an eutectic mixture (Fig. 2), since the thermogram of Treh:Gly (1:30) presents a different behaviour from the pure starting components (it exhibits no characteristic peaks), whereas for the other molar ratios tested this behaviour is not observed. The DSC results obtained for Treh:Gly (1:30) show a strong depression on the melting temperature of the individual components due to the establishment of hydrogen bonds between both counterparts. DSC also shows that for systems with 80% water and 20% Treh:Gly (1:30) (the cytotoxicity limit of this NADES), the thermal events are very distinct from the ones of deionized water alone (Fig 7.). The melting temperature is significantly decreased and the thermal behaviour is clearly altered. This suggests that the NADES is able to prevent crystallization at lower temperatures, and that it can have a role in the vitrification process of water.

^{1}H NMR spectra of the NADES (Fig. 4) shows sharp and well defined chemical shift signals of hydroxyl protons from Treh, comparing with respective ^{1}H NMR spectrums of raw

materials (Fig. 3) which arise as large signals. The HMBC (Fig. 5) spectrum revealed the existence of interactions between the protons of hydroxyl groups from Treh and the hydroxyl groups from Gly. These protons also show to have interactions with CH_2 from Gly. These observations suggest that hydrogen bonds were formed between hydroxyl groups from Treh and Gly. These screening tests allow the preparation of a system where it will be possible to take advantage of the synergistic effect between Gly and Treh. Gly and also the ability to limit the formation of ice and, thereby, preventing salts from reaching harmful concentrations during cooling step [15,56]. Treh presents an ability to protect denaturation of protein in nature, which has been explained by the mechanism of ultra-high trehalose content and glass-forming liquid states [57]. Moreover, in many yeasts, Treh might play a role in protecting the cells during freezing and against heat stress [35].

The characterization of the physical properties of NADES, such as viscosity, plays an important role in the cryopreservation of biological systems. High values of viscosity are reported to have a negative effect to the cell viability [28,31,40,43]. Very viscous systems could affect the highly organized molecular machinery supporting the cell, whereas, systems with lower viscosity present an higher stabilization capacity of the cells integrity than more viscous systems [18,22,27,40]. The viscosity of a water and FBS solution, with 10 (wt.) % Treh:Gly (1:30) system is higher than with 10 (wt.)% DMSO (Fig. 6). Nevertheless, the results of the cell viability for 10 (wt.) % of Treh:Gly (1:30) were higher than with 10 (wt.)% of DMSO, which means that the viscosity of the Treh:Gly(1:30) appears to be tolerated by the cells.

The standard cryopreservation protocol often requires a rapid thawing accomplished by placing the sample in a 37 °C stirred water bath until most of the ice melts [4]. The use of DMSO as CPA, above certain concentrations proved to be toxic and to affect the

differentiation of some types of cells [1,47]. Thus, DMSO needs to be rapidly eliminated after thawing, and its replacement still remains a challenge [4,6,29,39]. In this sense, the use of Treh:Gly (1:30) as CPA appears as a promising alternative, that can diminish the toxic effects of the CPA on cell viability.

During the cryopreservation protocols, it is well-known that cells begin with hypothermic exposure which persists during the period of active extracellular ice growth until the equilibrium is reached in the glassy-state (vitrified) [4]. Herein, the cryopreservation process was studied with 5 (wt.) % and 10 (wt.) % of Treh:Gly (1:30) (Fig. 1). However, it was observed that the cryoprotectant properties were not the most favourable in the lowest concentration. This phenomenon is attributed to the lower amounts of CPA which lead to a decrease in the cryoprotectant properties [35,39,49]. Therefore, this study focused on 10 (wt.)% of CPA and 90 (wt.)% of FBS as it presents a higher cell viability (Fig. 8), being the amounts commonly used in standard protocols [25,26,59].

The experiments illustrated in Fig. 9 show that freezing medium containing 10 (wt.) % Treh:Gly (1:30) present a higher cryoprotectant ability. In this sense, it is observed that the cell viability and cell morphology were not affected. In addition, the results demonstrate a similar trend when DMSO is used. It should also be pointed out that in all the formulations (i.e., NADES and controls) the post-thaw viabilities were obtained after centrifugation and plating, which may lead to cell loss.

Using Treh:Gly (1:30) as CPA, the damage induced by cooling process is reduced, which protects cells from apoptosis. On the other hand, when cells are frozen with individual components (Treh and Gly), the cell behaviour is changed as it is shown in Fig. 10, Fig. S1 and Fig. S2, especially when Treh is used. This is evidenced by a progressive decrease on cell viability, cell proliferation and cell morphology, which can be caused by the formation

of ice crystals during freezing. When these components are individually used in the cryopreservation process, the properties as CPA are compromised, demonstrating the benefit of the use of NADES as a superior CPA.

Conclusions

In conclusion, in this work we propose a new family of CPAs which presents advantages over the traditional ones, allowing the use of natural and biocompatible molecules. The advantage of NADES acting as CPA, is mainly related with the thermal stability in the working temperature of the cryopreservation process. Despite the evaluation of the efficiency of this CPA has been only performed using L929 as a model cell line, it is assumed that Treh:Gly (1:30) will be efficient in the cryopreservation of other kinds of cells and even in other range of temperatures. Thus, we believe that the use of NADES are an interesting alternative in in the cryopreservation of human cells and tissues.

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References

[1] S. Adler, C. Pellizzer, M. Paparella, T. Hartung, S. Bremer, The effects of solvents on embryonic stem cell differentiation, Toxicol. Vitr. 20 (2006) 265–271.

[2] M.C.F. Do Amaral, R.E. Lee, J.P. Costanzo, Enzymatic regulation of glycogenolysis in a subarctic population of the wood frog: Implications for extreme freeze tolerance, PLoS One. 8 (2013).

[3] I.M. Aroso, A. Paiva, R.L. Reis, A.R.C. Duarte, A. Rita, C. Duarte, Natural deep eutectic solvents from choline chloride and betaine – Physicochemical properties, J. Mol. Liq. 241 (2017) 654–661.

[4] J.G. Baust, D. Gao, J.M. Baust, Cryopreservation: An emerging paradigm change., Organogenesis. 5 (2009) 90–6.

[5] G.M. Beattie, J.H. Crowe, A.D. Lopez, V. Cirulli, C. Ricordi, A. Hayek, A. Hayek, TVehalose: A Cryoprotectant That Enhances Recovery and Preserves Function of Human Pancreatic Islets After Long-Term Storage, Diabetes. 46 (1997) 519–523.

[6] B.P. Best, Cryoprotectant Toxicity: Facts, Issues, and Questions, Rejuvenation Res. 18 (2015) 422–436.

[7] S. Bhattacharya, B.G. Prajapati, A. Professor, A Review on Cryoprotectant and its Modern Implication in Cryonics, Asian J. Pharm. 10 (2016) 154–159.

[8] D.S. Bolintineanu, Y.N. Kaznessis, Computational studies of protegrin antimicrobial peptides: A review, Peptides. 32 (2011) 188–201.

[9] F. Chemat, H.J. You, K. Muthukumar, T. Murugesan, Effect of l-arginine on the

physical properties of choline chloride and glycerol based deep eutectic solvents, J. Mol. Liq. 212 (2015) 605–611.

[10] Y.H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I.W.C.E. Arends, G.-J. Witkamp, R. Verpoorte, Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology?, Plant Physiol. 156 (2011) 1701–5.

[11] T.A. Churchill, K.B. Storey, Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance, Am. J. Physiol. Integr. Comp. Physiol. 265 (1993) R1324–R1332.

[12] J.P. Costanzo, A.M. Reynolds, M. Clara, F. Do Amaral, A.J. Rosendale, R.E. Lee, Cryoprotectants and extreme freeze tolerance in a subarctic population of the wood frog, PLoS One. 10 (2015) 1–23.

[13] R. Craveiro, I. Aroso, V. Flammia, T. Carvalho, M.T. Viciosa, M. Dionísio, S. Barreiros, R.L. Reis, A.R.C. Duarte, A. Paiva, Properties and thermal behavior of natural deep eutectic solvents, J. Mol. Liq. 215 (2016) 534–540.

[14] J.H. Crowe, F. Tablin, W.F. Wolkers, K. Gousset, N.M. Tsvetkova, J. Ricker, Stabilization of membranes in human platelets freeze-dried with trehalose, in: Chem. Phys. Lipids, 2003: pp. 41–52.

[15] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, Green solvents for green technologies, J. Chem. Technol. Biotechnol. 90 (2015) 1631–1639.

[16] Y. Dai, J. van Spronsen, G.-J. Witkamp, R. Verpoorte, Y.H. Choi, Natural deep eutectic solvents as new potential media for green technology., Anal. Chim. Acta.

766 (2013) 61–68.

[17] Y. Dai, J. van Spronsen, G.-J. Witkamp, R. Verpoorte, Y.H. Choi, Ionic liquids and deep eutectic solvents in natural products research: mixtures of solids as extraction solvents., J. Nat. Prod. 76 (2013) 2162–73.

[18] Y. Dai, G.J. Witkamp, R. Verpoorte, Y.H. Choi, Tailoring properties of natural deep eutectic solvents with water to facilitate their applications, Food Chem. 187 (2015) 14–19.

[19] R.C. Deller, M. Vatish, D.A. Mitchell, M.I. Gibson, Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing., Nat. Commun. 5 (2014) 3244.

[20] S.-P. Ding, J. Fan, J.L. Green, Q. Lu, E. Sanchez, C.A. Angell, Vitrification of Trehalose by water loss from its crystaline dihydrate, J. Therm. Anal. 47 (1996) 1391–1405.

[21] a Eroglu, M.J. Russo, R. Bieganski, a Fowler, S. Cheley, H. Bayley, M. Toner, Intracellular trehalose improves the survival of cryopreserved mammalian cells., Nat. Biotechnol. 18 (2000) 163–167.

[22] M. Espino, M. de los Ángeles Fernández, F.J. V Gomez, M.F. Silva, Natural designer solvents for greening analytical chemistry, Trends Anal. Chem. 76 (2016) 126–136.

[23] R. Esquembre, J.M. Sanz, J.G. Wall, F. del Monte, C.R. Mateo, M.L. Ferrer, Thermal unfolding and refolding of lysozyme in deep eutectic solvents and their aqueous dilutions, Phys. Chem. Chem. Phys. 15 (2013) 11248–11256.

[24] M. Francisco, A. Van Den Bruinhorst, M.C. Kroon, Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents, Angew. Chemie - Int. Ed. 52 (2013) 3074–3085.

[25] R.I. Freshney, Culture of Specific Cell Types, Cult. Anim. Cells. (2005) 375–420.

[26] R.I. Freshney, A. Capes-Davis, C. Gregory, S. Przyborski, Culture of animal cells : a manual of basic technique and specialized applications, n.d.

[27] B. Fuller, S. Paynter, Fundamentals of cryobiology in reproductive medicine, Reprod. Biomed. 9 (2004) 680–691.

[28] B.J. Fuller, N. Lane, E.E. Benson, Life in the frozen state, CRC Press, 2004.

[29] Georges da Violante, Naima Zerrouk, Isabelle Richard,Gérard Provot, J.C.C. and P. Arnaud, Evaluation of the Cytotoxicity Effect of Dimethyl Sulfoxide (DMSO) on Caco2/TC7 Colon Tumor Cell Cultures, Biol. Pharm. Bull. 25 (2001) 1600–1603.

[30] A.C. Gertudes, R. Craveiro, Z. Eltayari, R.L. Reis, A. Paiva, A.R.C. Duarte, How do animals survive extreme temperature amplitudes ? The role of Natural Deep Eutectic Solvents, ACS Sustain. Chem. Eng. (2017) 1–32.

[31] A. Hayyan, F.S. Mjalli, I.M. AlNashef, Y.M. Al-Wahaibi, T. Al-Wahaibi, M.A. Hashim, Glucose-based deep eutectic solvents: Physical properties, J. Mol. Liq. 178 (2013) 137–141.

[32] M. Hayyan, M.A. Hashim, A. Hayyan, M. a Al-Saadi, I.M. AlNashef, M.E.S. Mirghani, O.K. Saheed, Are deep eutectic solvents benign or toxic?, Chemosphere. 90 (2013) 2193–5.

554 [33] M. Hayyan, C.Y. Looi, A. Hayyan, W.F. Wong, M.A. Hashim, In Vitro and In Vivo
555 toxicity profiling of ammonium-based deep eutectic solvents., PLoS One. 10 (2015)
556 1–18.

557 [34] M. Hayyan, Y.P. Mbous, C.Y. Looi, W.F. Wong, A. Hayyan, Z. Salleh, O. Mohd-
558 Ali, Natural deep eutectic solvents: cytotoxic profile, Springerplus. 5 (2016) 913.

559 [35] Z. Hubálek, Protectants used in the cryopreservation of microorganisms,
560 Cryobiology. 46 (2003) 205–229.

561 [36] A. Hubel, T.B. Darr, A. Chang, J. Dantzig, Cell partitioning during the directional
562 solidification of trehalose solutions, Cryobiology. 55 (2007) 182–188.

563 [37] G. Imperato, S. Hoger, D. Lenoir, B. Konig, Low melting sugar-urea-salt mixtures as
564 solvents for organic reactions-estimation of polarity and use in catalysis, Green
565 Chem. 8 (2006) 1051.

566 [38] H. Kim, J. Lee, Y. Hur, C. Lee, S.-H. Park, B.-W. Koo, Marine Antifreeze Proteins:
567 Structure, Function, and Application to Cryopreservation as a Potential
568 Cryoprotectant, Mar. Drugs. 15 (2017) 1–27.

569 [39] K. Matsumura, J.Y. Bae, S.H. Hyon, Polyampholytes as cryoprotective agents for
570 mammalian cell cryopreservation, Cell Transplant. 19 (2010) 691–699.

571 [40] Y.P. Mbous, M. Hayyan, W.F. Wong, C.Y. Looi, M.A. Hashim, Unraveling the
572 cytotoxicity and metabolic pathways of binary natural deep eutectic solvent systems,
573 Sci. Rep. 7 (2017) 41257.

574 [41] H.T. Meryman, Cryopreservation of living cells : principles and practice, Tranfusion.

47 (2007) 935–945.

[42] H. Monhemi, M.R. Housaindokht, A.A. Moosavi-Movahedi, M.R. Bozorgmehr, How a protein can remain stable in a solvent with high content of urea: insights from molecular dynamics simulation of Candida antarctica lipase B in urea : choline chloride deep eutectic solvent., Phys. Chem. Chem. Phys. 16 (2014) 14882–93.

[43] G.J. Morris, M. Goodrich, E. Acton, F. Fonseca, The high viscosity encountered during freezing in glycerol solutions: Effects on cryopreservation, Cryobiology. 52 (2006) 323–334.

[44] F. Mosca, M. Madeddu, A.A. Sayed, L. Zaniboni, N. Iaffaldano, S. Cerolini, Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm, Cryobiology. 73 (2016) 343–347.

[45] S. Nardecchia, M.C. Gutierrez, M.L. Ferrer, M. Alonso, I.M. Lopez, J.C. Rodriguez-Cabello, F. del Monte, Phase Behavior of Elastin-Like Synthetic Recombinamers in Deep Eutectic Solvents, Biomacromolecules. 13 (2012) 2029–2036.

[46] A. Paiva, R. Craveiro, I. Aroso, M. Martins, R.L. Reis, A.R.C. Duarte, Natural deep eutectic solvents - Solvents for the 21st century, ACS Sustain. Chem. Eng. 2 (2014) 1063–1071.

[47] R. Pal, M.K. Mamidi, A.K. Das, R. Bhonde, Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells, Arch. Toxicol. 86 (2012) 651–661.

[48] K. Radošević, N. Ćurko, V. Gaurina Srček, M. Cvjetko Bubalo, M. Tomašević, K. Kovačević Ganić, I. Radojčić Redovniković, Natural deep eutectic solvents as

beneficial extractants for enhancement of plant extracts bioactivity, LWT - Food Sci. Technol. 73 (2016) 45–51.

[49] P.Q. Ri-Cheng Chian, Fertility Cryopreservation, 2010.

[50] E.L. Smith, A.P. Abbott, K.S. Ryder, Deep eutectic solvents (DESs) and their applications., Chem. Rev. 114 (2014) 11060–82.

[51] B.T. Storey, E.E. Noiles, K.A. Thompson, Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation., Cryobiology. 37 (1998) 46–58.

[52] K.B. Storey, J.M. Storey, Physiology B mental Physiology Biochemical adaption for freezing tolerance in the wood frog, Rana sylvatica, J Comp Physiol B. 155 (1984) 29–36.

[53] T. Takahashi, J.B. Bross, R.E. Shaber, R.J. Williams, Effect of cryoprotectants on the viability and function of unfrozen human polymorphonuclear cells, Cryobiology. 22 (1985) 336–350.

[54] B. Tang, K.H. Row, Recent developments in deep eutectic solvents in chemical sciences, Monatshefte Für Chemie - Chem. Mon. 144 (2013) 1427–1454.

[55] W.F. Wolkers, Cryopreservation and Freeze-Drying Protocols, 2015.

[56] B. Wowk, Thermodynamic aspects of vitrification, Cryobiology. 60 (2010) 11–22.

[57] R. Xin, S. Qi, C. Zeng, F.I. Khan, B. Yang, Y. Wang, A functional natural deep eutectic solvent based on trehalose: Structural and physicochemical properties, Food Chem. 217 (2017) 560–567.

618 [58] P.H. Yancey, M.B. Burg, Counteracting effects of urea and betaine in mammalian
619 cells in culture, Am. J. Physiol. 258 (1990) R198-204.

620 [59] Y. Yuan, Y. Yang, Y. Tian, J. Park, A. Dai, R.M. Roberts, Y. Liu, X. Han, Efficient
621 long-term cryopreservation of pluripotent stem cells at −80 °C, Sci. Rep. 6 (2016)
622 34476.

623 [60] Q. Zhang, K. De Oliveira Vigier, S.S. Royer, F. Jérôme, K. De, O. Vigier, S.S.
624 Royer, F. Jeoe, Deep eutectic solvents: syntheses, properties and applications.,
625 Chem. Soc. Rev. 41 (2012) 7108–7146.

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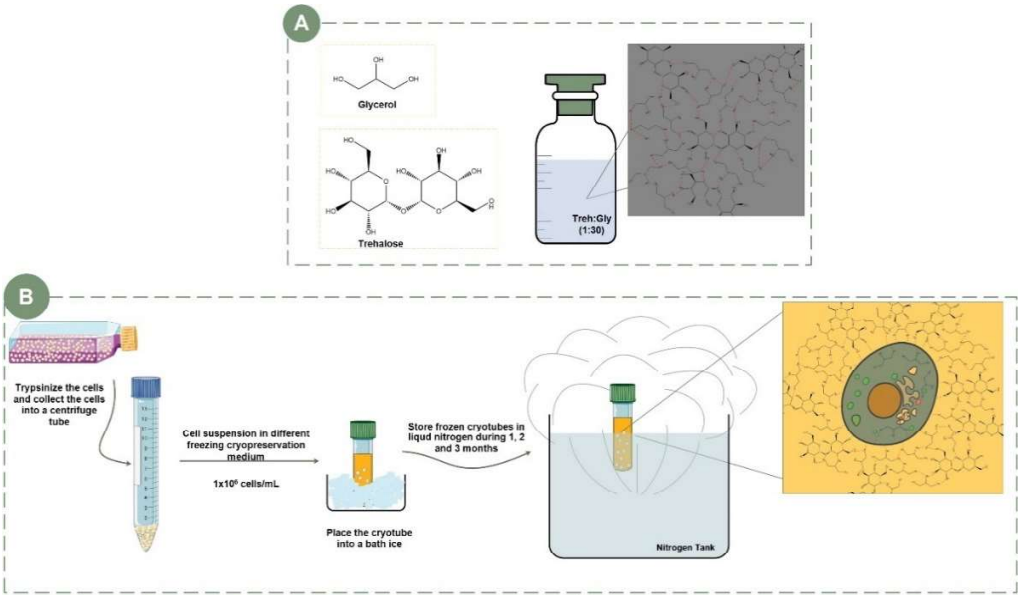


Fig. 1. Schematic representation of the different steps of the work developed; Chemical structure of the components used to prepare the natural deep eutectic solvent (A), cryopreservation process (B).

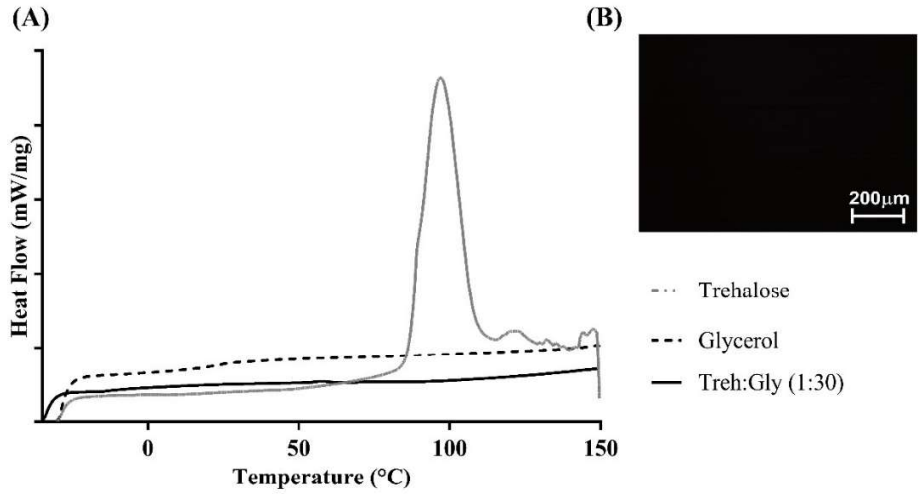


Fig. 2. DSC thermograms of Treh, Gly and Treh:Gly(1:30) for -30 °C to 150 °C (A), POM images at 22 °C of Treh:Gly(1:30) (B).

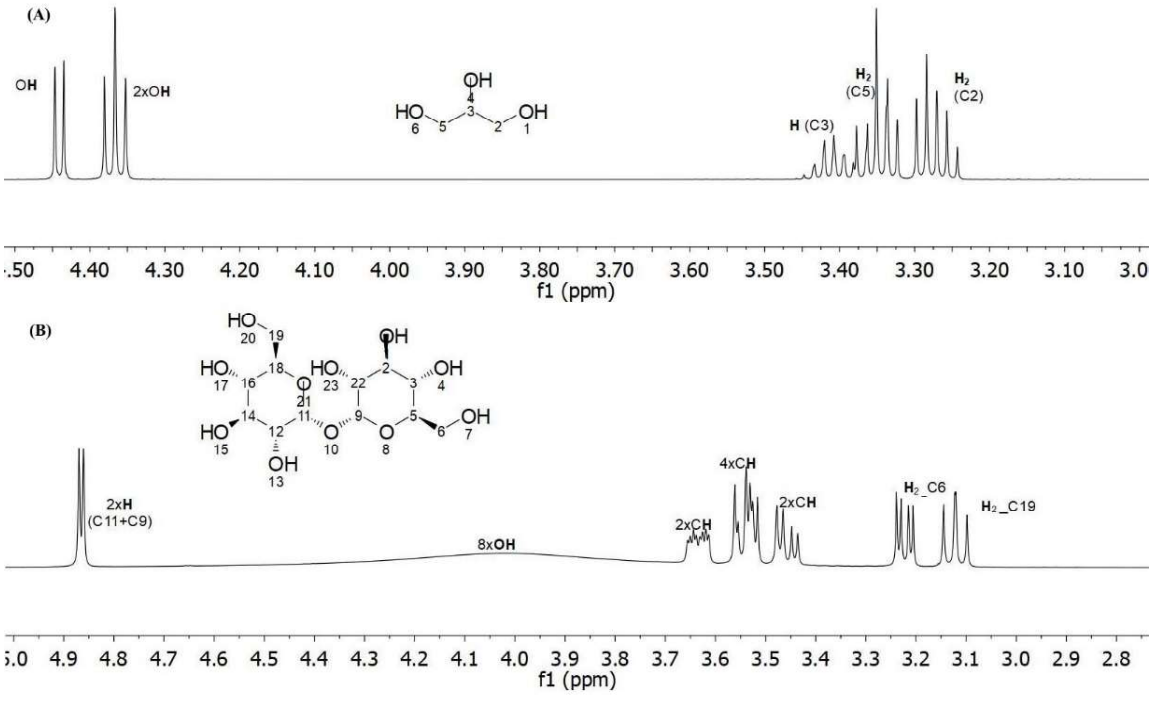


Fig. 3. ^{1}H NMR spectra of Gly (A) and Treh (B) system, all the resonances are attributed.

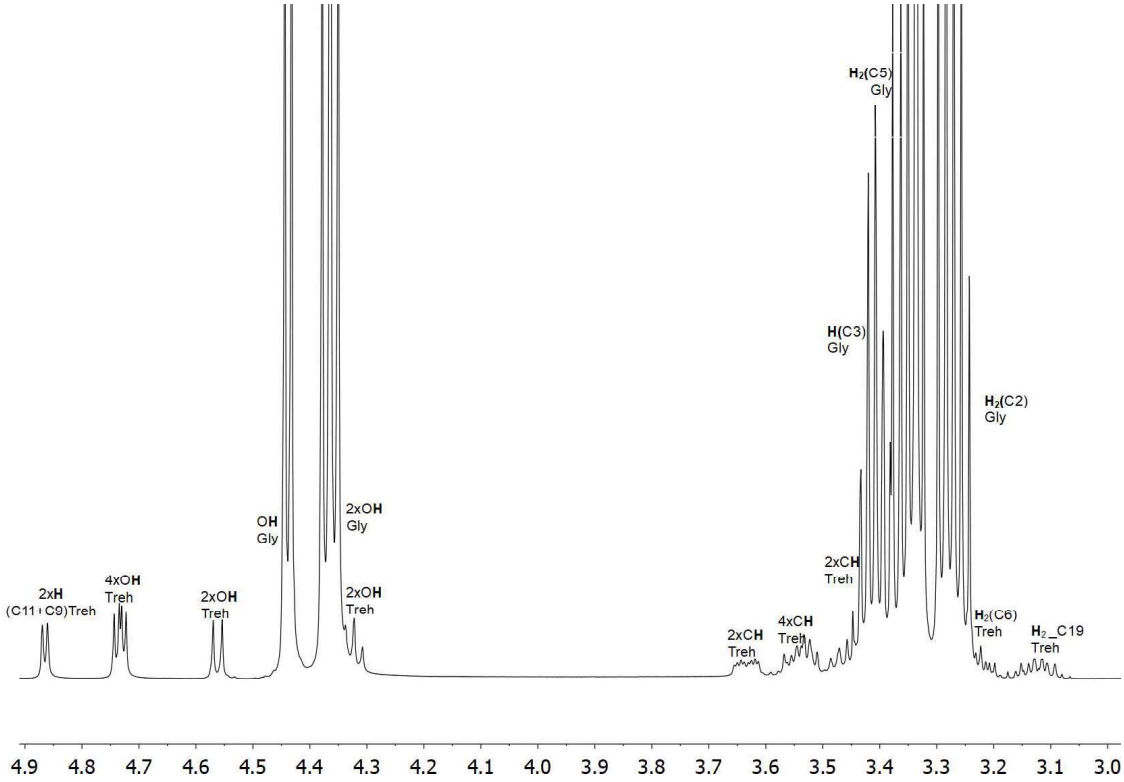


Fig. 4. ^{1}H NMR spectrum of Treh:Gly (1:30) system, all the resonances are attributed.

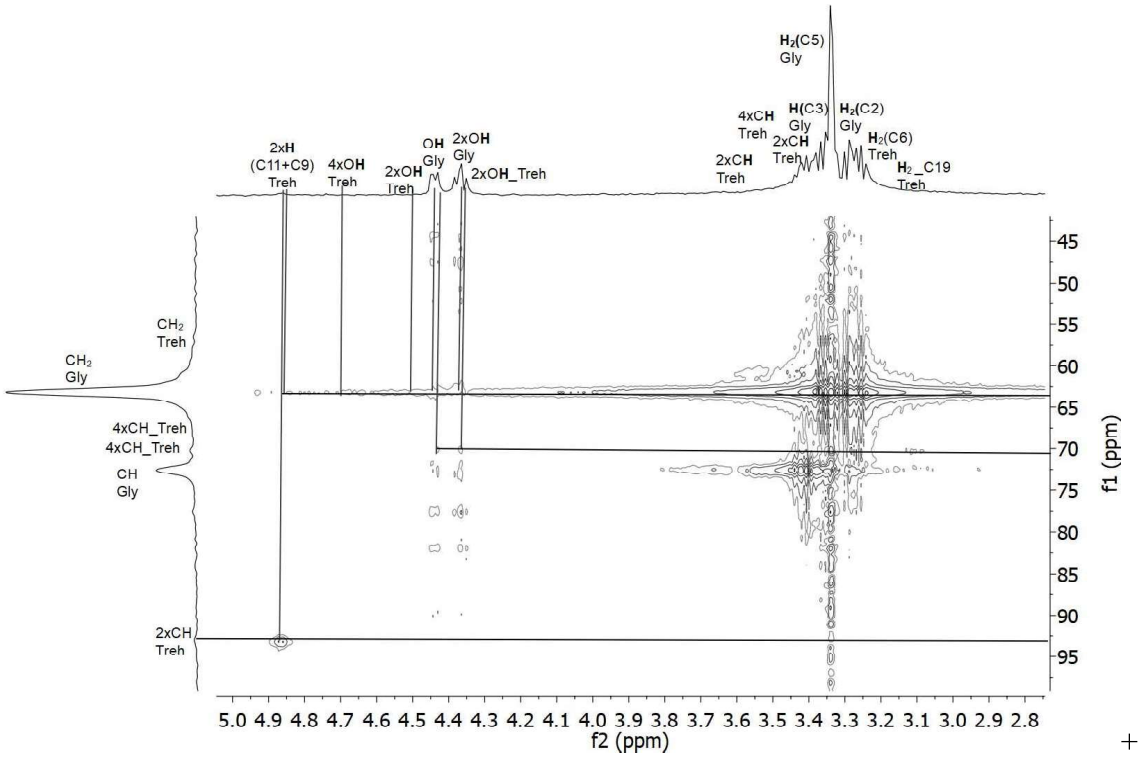


Fig. 5. 2D NMR (HMBC) spectrum of Treh:Gly (1:30) system, all the resonances are attributed.

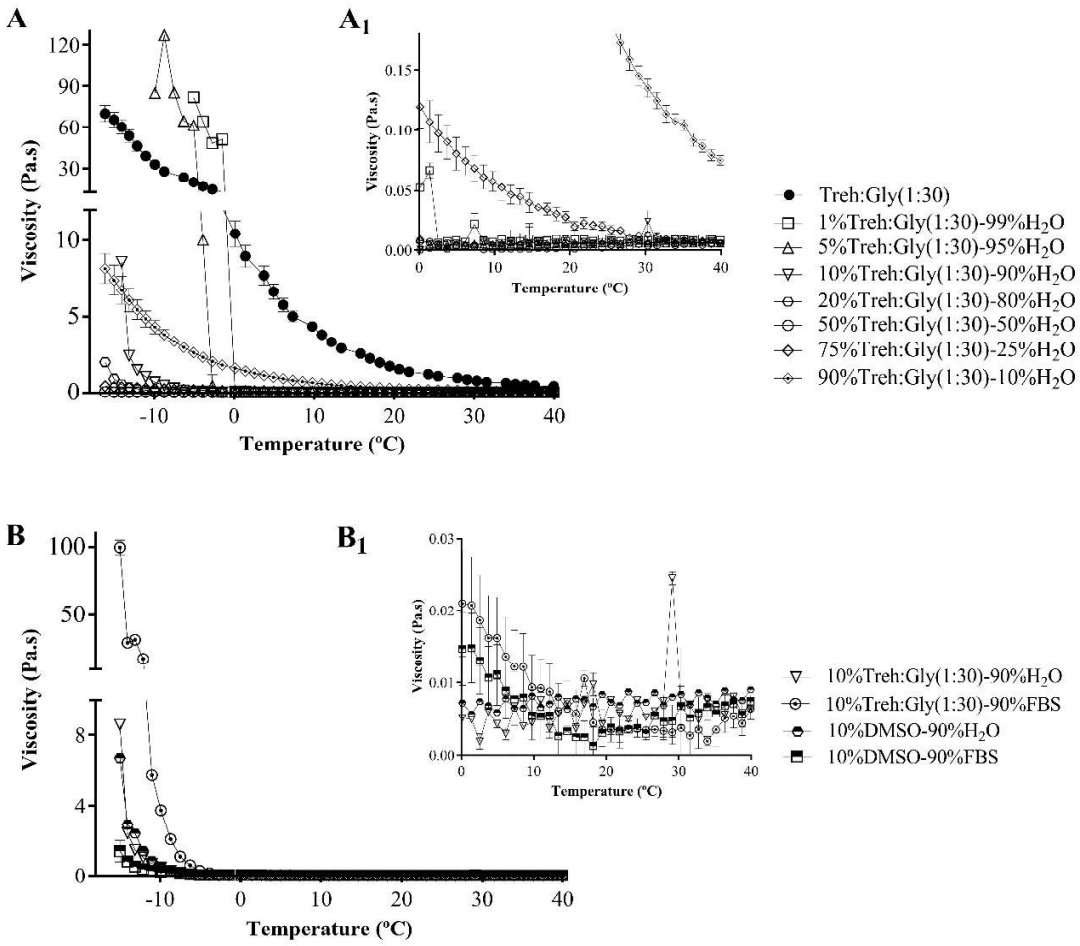


Fig. 6. Variation of viscosities as a function of temperature. Treh:Gly (1:30) with different percentages of water (A); extension of the positive temperatures ramp (A1); 10% of Treh:Gly (1:30) and DMSO with 90% water or FBS (B), extension of the positive temperatures ramp (B1).

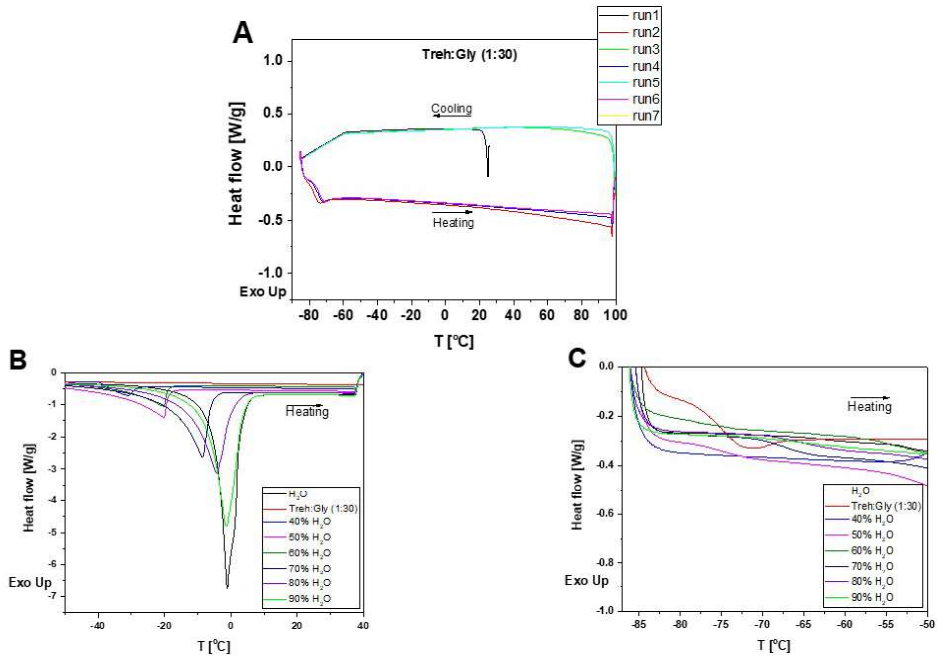


Fig. 7. Differential scanning calorimetric thermogram of the prepared NADES Treh:Gly (1:30) (A), and thermograms for deionized water and for the different mixtures of water and NADES, highlighting the melting region(B) and the glass transition region (C).

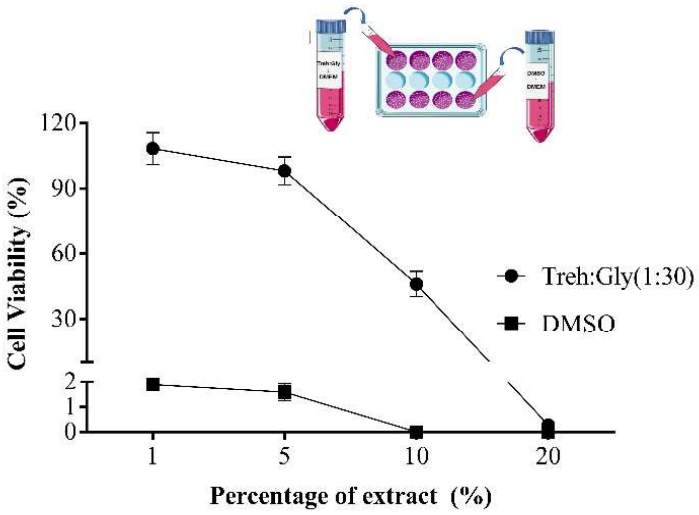


Fig. 8. Assessment of cell viability after 24 hours in contact with different percentages of NADES Treh:Gly (1:30) (●) and DMSO (■).

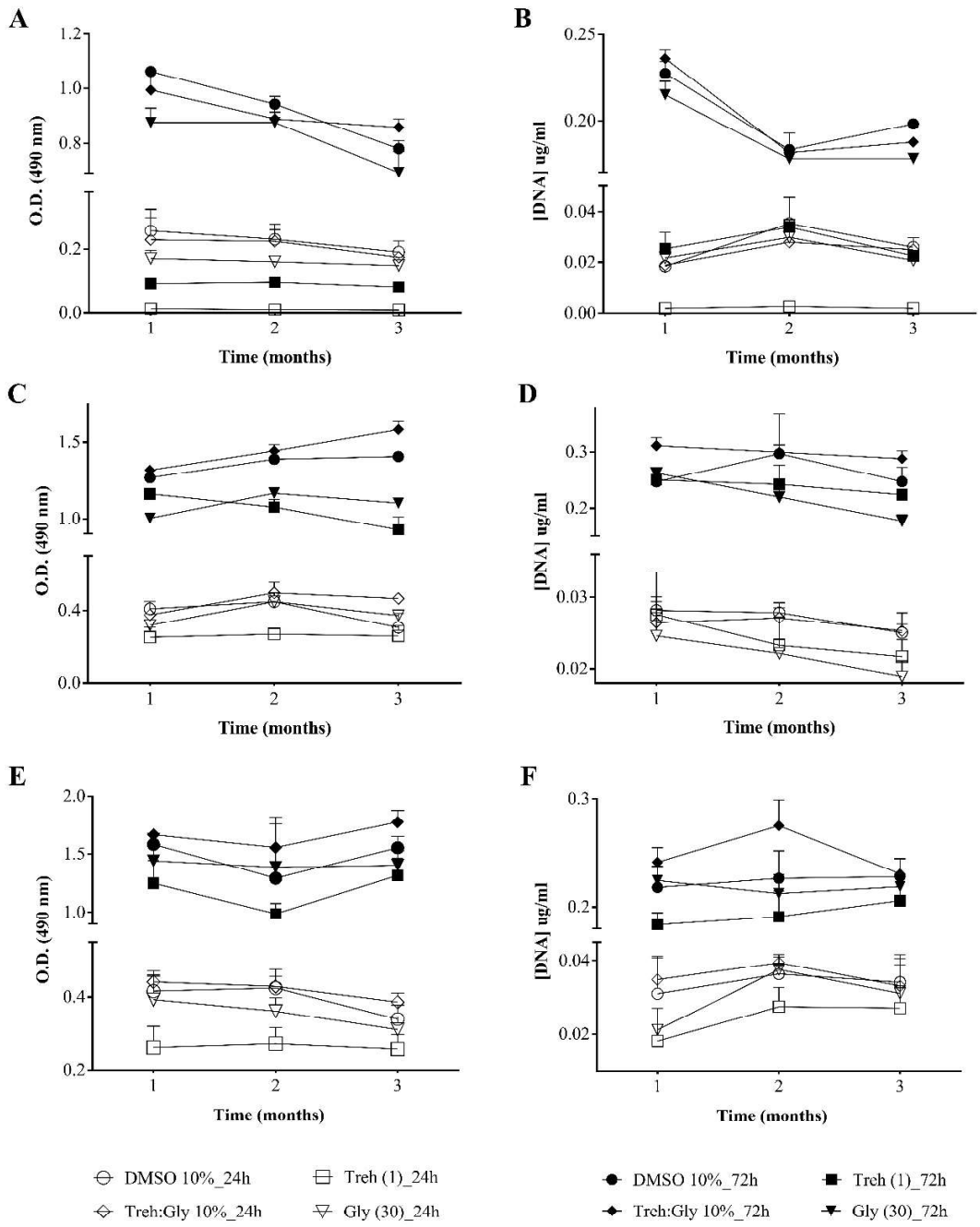
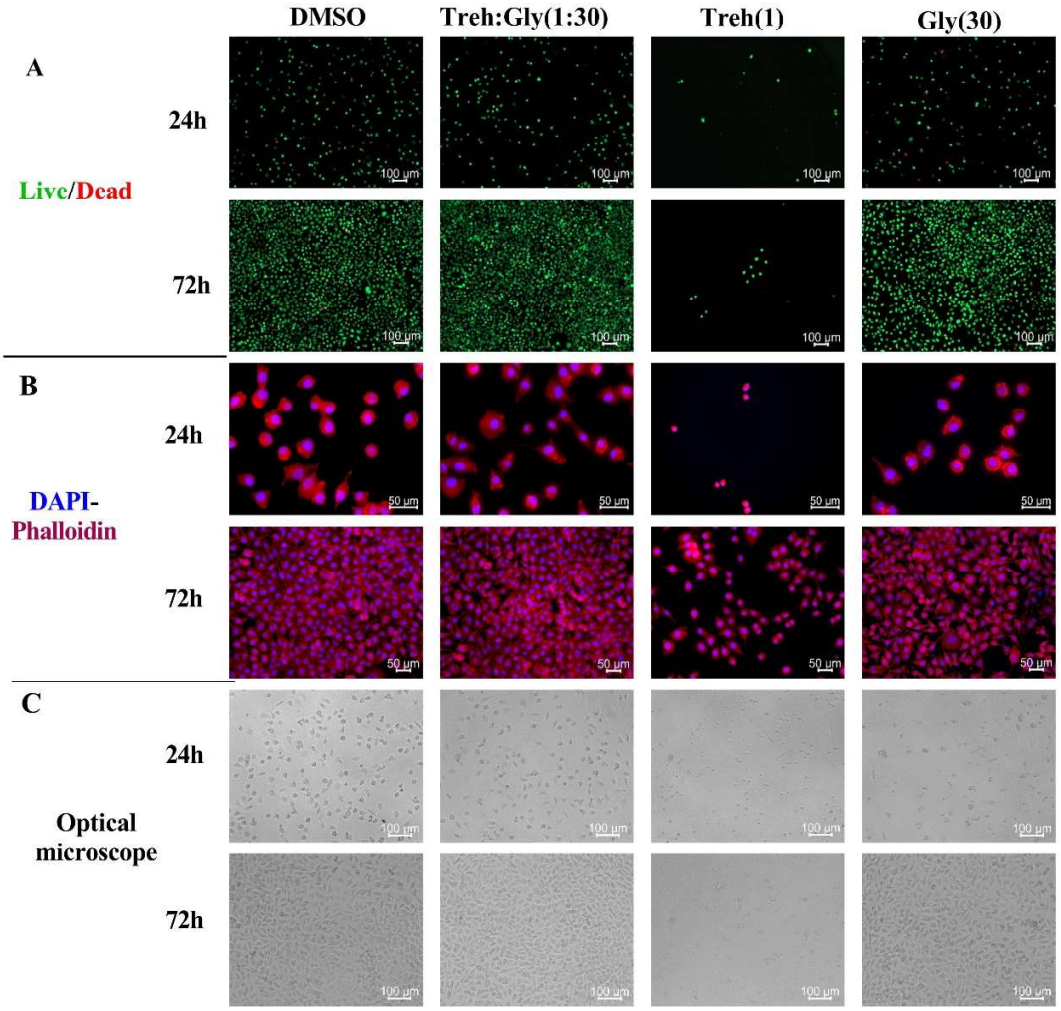
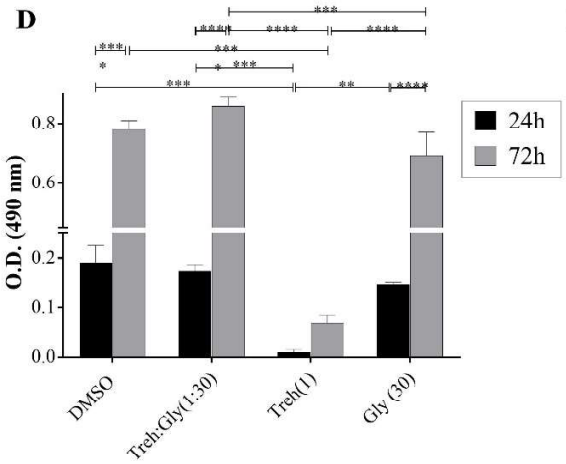


Fig. 9. Assessment of the post-thawing recovery of cells after different time cryopreservation (1, 2 and 3 months of storage) with freezing medium containing different CPAs (DMSO, Treh;Gly(1:30), Treh(1) and Gly(30)). MTS assay (A, C and E) and DNA assay (B, D and F) at 24 hours and 72 hours of culture for L929 cells in passage 0 (A and B), passage 1 (C and D) and passage 2 (E and F) after thawing


DMSO
Treh:Gly(1:30)
Treh(1)
Gly(30)
A
24h
Live/Dead
72h
B
24h
DAPI-
Phalloidin
72h
C
24h
Optical
microscope
72h
100 μm
50 μm


D
O.D. (490 nm)
0.0
0.1
0.2
0.6
0.8
24h
72h
DMSO
Treh:Gly(1:30)
Treh(1)
Gly (30)

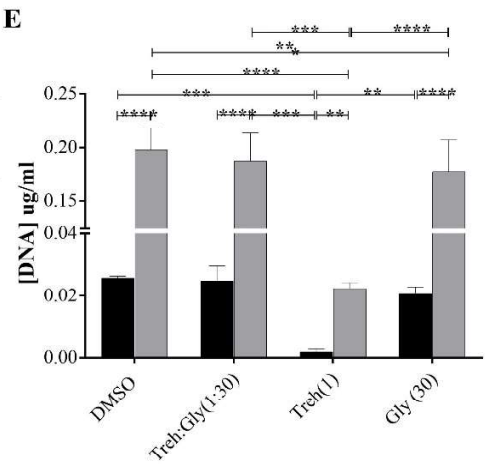

E
[DNA] ug/ml
0.00
0.02
0.04
0.15
0.20
0.25
DMSO
Treh:Gly(1:30)
Treh(1)
Gly (30)

Fig. 10. Assessment of the post-thawing recovery of cells after long term cryopreservation (3 months of storage) with freezing medium containing different CPA (DMSO, Treh:Gly (1:30), Treh (1) and Gly (30)) L929 cells were used in passage 0 (P0) after thawing. (A) Live/Dead fluorescence assay (living cells were stained green by calcein AM and dead cells red by PI) (B) DAPI/Phalloidin assay (cells nuclei were stained blue by DAPI and F-actin filaments in red by phalloidin), (C) Optic microscopy, (D) MTS assay and (E) DNA assay at 24 hours and 72 hours of culture. Data represent means ± SD (n=3), with different *, indicating statistical differences, values within same CPA at different time points and values within different CPA at same time points were found for (**) $p < 0.005$, (***) $p < 0.001$ and (****) $p < 0.0001$, one-way ANOVA.

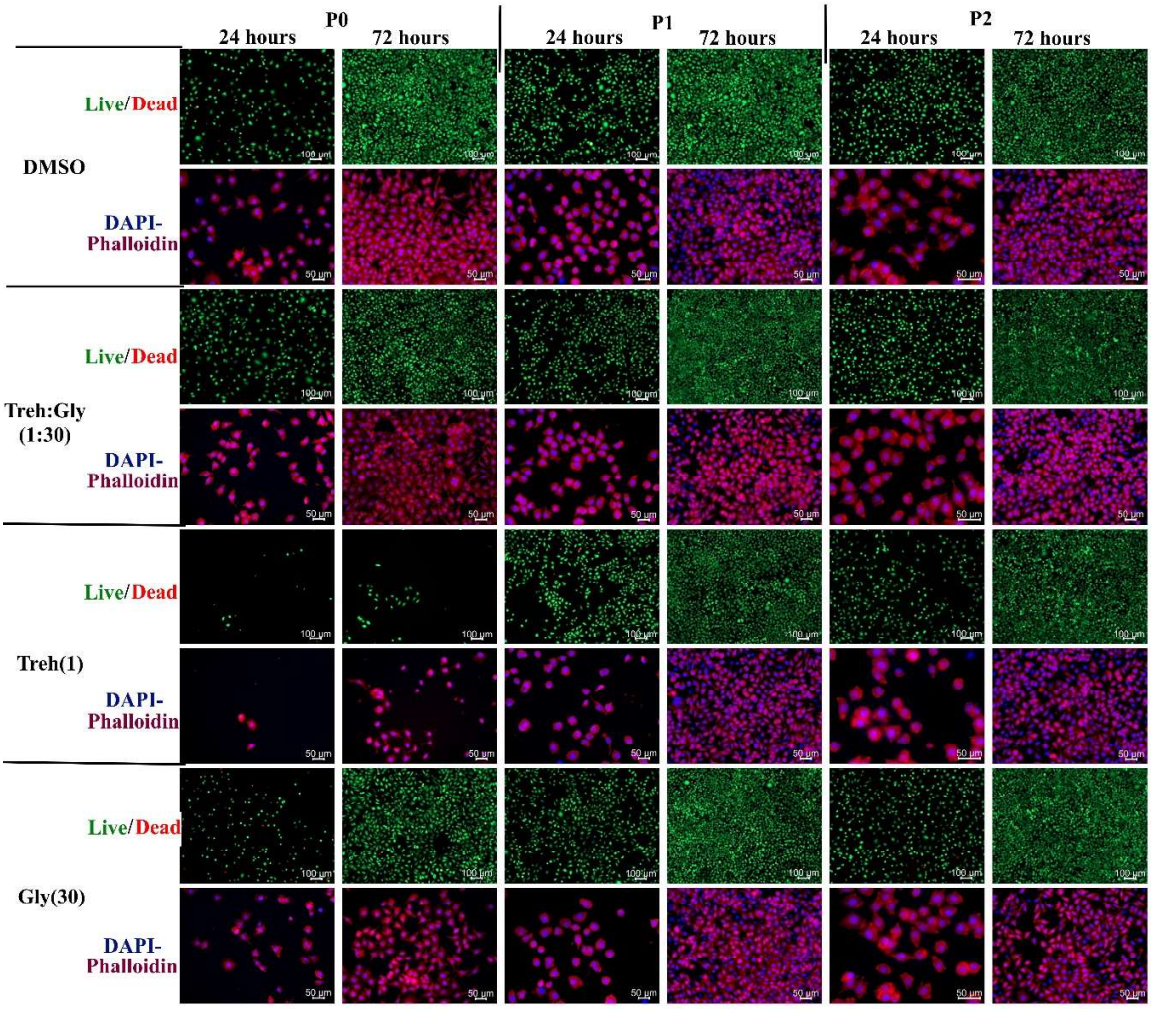


Fig. S1. Assessment of the post-thawing recovery of cells after long term cryopreservation (1 month of storage) with freezing medium containing different CPAs (DMSO, Treh:Gly(1:30), Treh(1) and Gly(30)) L929 cells were used in passage 0 (P0), passage 1 (P1) and passage 2 (P2) after thawing. Live/Dead fluorescence assay (living cells were stained green by calcien AM and dead cells red by PI); DAPI/Phalloidin assay (cells nuclei were stained blue by DAPI and F-actin filaments in red by phalloidin).

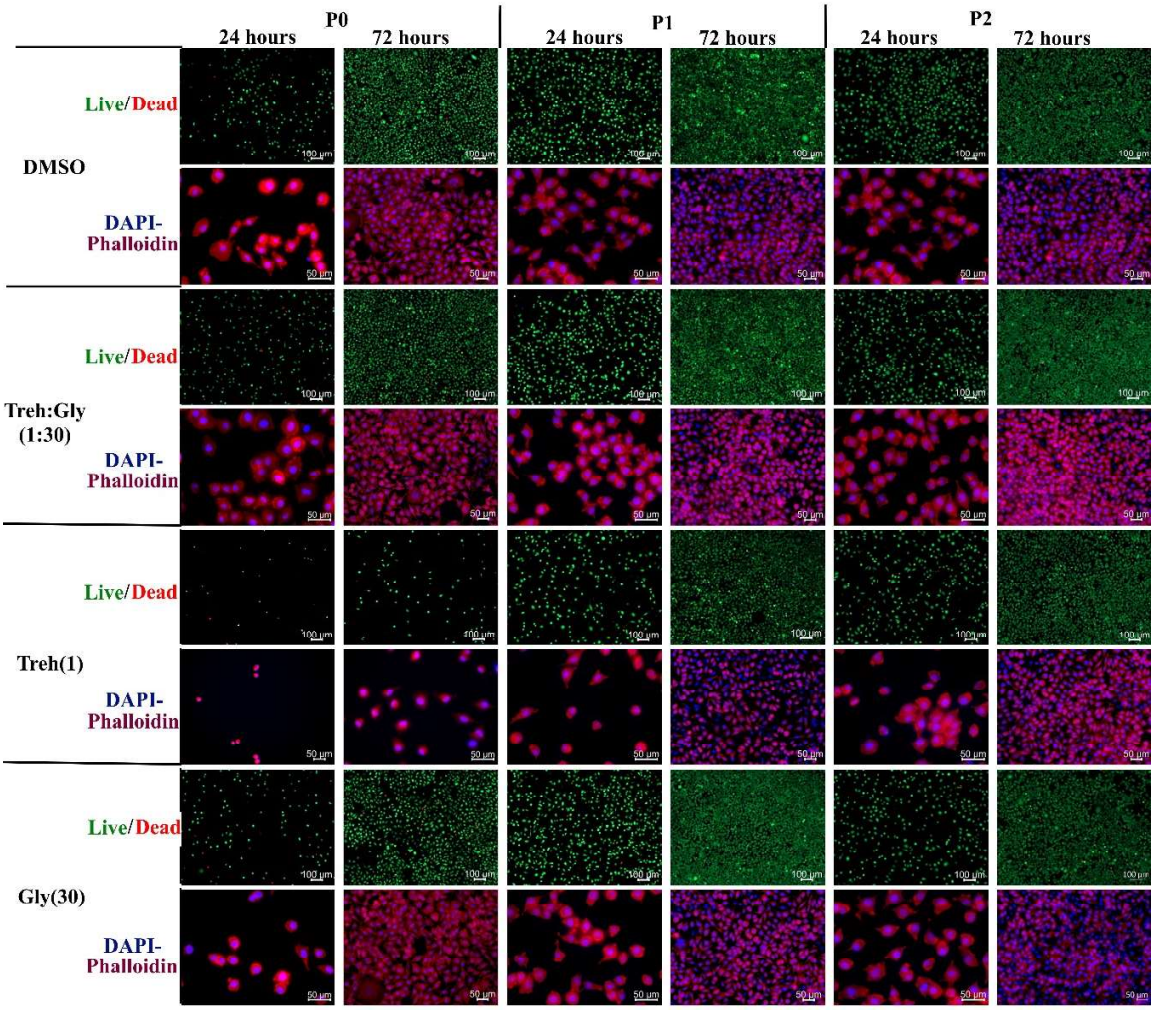


Fig. 1. Assessment of the post-thawing recovery of cells after long term cryopreservation (2 months of storage) with freezing medium containing different CPAs (DMSO, Treh:Gly(1:30), Treh(1) and Gly(30)) L929 cells were used in passage 0 (P0), passage 1 (P1) and passage 2 (P2) after thawing. Live/Dead fluorescence assay (living cells were stained green by calcein AM and dead cells red by PI); DAPI/Phalloidin assay (cells nuclei were stained blue by DAPI and F-actin filaments in red by phalloidin).

Table 1. Summary of the different molar ratio prepared for Treh:Gly system and its visual appearance at RT.

Molar ratio	Appearance
1:1	White solid
1:2	White liquid
1:10	Transparent liquid with some crystals
1:20	Transparent liquid with some crystals at RT
1:25	Transparent liquid with some crystals at RT
1:30	Transparent viscous liquid at RT

Table 2. Melting temperatures and glass transition temperatures for deionized water, NADES Treh:Gly (1:30) and for mixtures of water with different wt% of NADES (taken at mid-point). Melting enthalpy and heat capacity values are also presented. All these values refer to the ones obtained in the second heating scan of each DSC experiment.

Sample	T_m (°C)	ΔH_m (J.g^{-1})	T_g (°C)	ΔC_p (J.g^{-1}.°C^{-1})
Treh:Gly (1:30)-NADES	-	-	-75.14	0.846
60% NADES + 40% H_2O	-36.97*	8.22*	-	-
50% NADES + 50% H_2O	-33.08	59.27	-76.94	0.3014
40% NADES + 60% H_2O	-33.21	48.95		
30% NADES + 70% H_2O	-17.4	118.8	-70.15	0.3893
20% NADES + 80% H_2O	-12.33	143.8	-68.74	0.3283
10% NADES + 90% H_2O	-7.06	223.4	-69.04	0.1867
H_2O	-1.08	210.0	-	-

* these values refer to a melting peak preceded by a peak referent to cold crystallization.