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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 of 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.

1. 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 overall viscosity of the eutectic mixture and, consequently, enhances its process ability and decreases the cytotoxic profile of NADES [18,40]. Recently, the presence of NADES in animal and plants who survive in extreme conditions and temperature amplitudes has been discussed in the literature [2,10,11,42,52,58]. These publications have demonstrated that the presence of considerable amounts of simple molecules (i.e., sugars,

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polyols and amino acids) in all these microbial, mammalian and plant cells may be crucial for their survival [10,19,30,34,58]. NADES have been pointed out as essential for the dissolution of intercellular solutes of intermediate polarity, in the storage of metabolic products, in the germination, and in the resistance of various organisms to extreme temperature conditions due to the vitrification of water inside the cells [40].

Our work, herein described, focuses on the possible application of NADES in cryopreservation process as new cryoprotective agents (CPA). The cryopreservation process can be defined as the use of extreme cold temperatures to maintain and preserve biologic material, usually below −140 °C [4,30,41,55]. In this process, the formation/presence of ice crystals, formed upon freezing, in the biological systems has several consequences for cell survival, hampering their viability. Unprotected freezing is normally lethal to cells and thus the use of a CPA plays an important role in the survival of cells, as it inhibits the crystallization of water and, thereby, the formation of ice [27,35,38,49,55]. NADES may play the same role as a CPA, i.e., they are able to vitrify water, reducing the crystallization temperature of water and changing the crystallization phenomena [13,24,60]. For this reason, NADES have been suggested as the liquid media, responsible for the survival of cells during the winter, in animals living in extreme temperature amplitude environments [8,11,12,30,52,58]. In a previous work, our group reviewed and listed the compounds 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 a 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 [5–7,14,20,21,27,35,36,43,44,51,53,55]. 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.

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

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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.

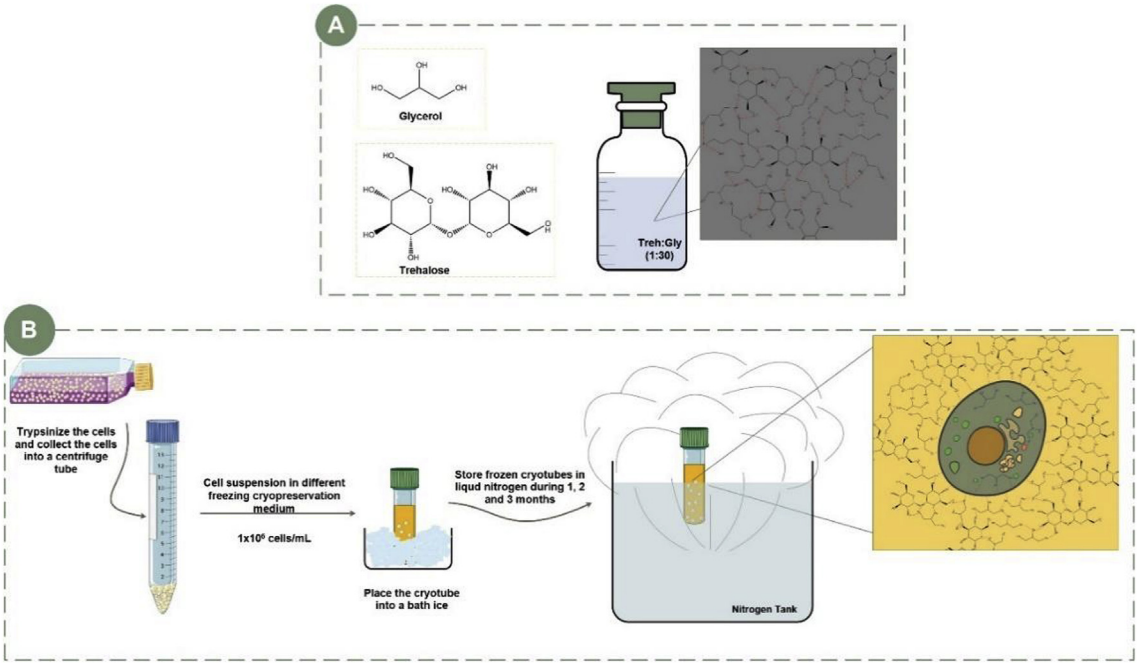

Fig. 1. Schematic representation of the different steps of the work developed; Chemical structure of the components used to prepare the natural deep eutectic solvent (A), cryopreservation process (B).