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Current developments and future perspectives on biotechnology applications of natural deep eutectic systems

Ana Rita Jesus, Alexandre Paiva and Ana Rita C. Duarte

Abstract

Natural Deep Eutectic Systems (NADES) have emerged in the past years as alternative solvents to traditional organic solvents and ionic liquids. NADES are easy to produce, sustainable, biocompatible, eco-friendly, remarkable solubilizing agents, and highly task-specific. The outstanding properties of this new liquid media have attracted the attention of researchers in the last decade in many fields and biotechnology is probably one of the fields where NADES have gained more relevance. Nonetheless, the progress beyond the state of the art in this field is not yet fully explored. Most research papers regarding the use of NADES in biotechnology are related to their use as solubility enhancers for poorly soluble active ingredients, particularly for pharmaceutical applications. However, the applicability of NADES in applications such as cryopreservation, stabilization of proteins and DNA, as well as other biomedical applications, has only recently been explored and presents still a plethora of discoveries to be unravelled. The current developments in this scientific field and future perspectives will be discussed herein.

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Keywords

Natural deep eutectic systems, Biotechnology, Biomedical, Green chemistry.

Abbreviations

DES, Deep eutectic Systems; NADES, Natural Deep Eutectic Systems; CPA, Cryoprotectant agent; FO, Forward osmosis.

Introduction

During the last decade, deep eutectic systems (DES) have gained much attention from academia and industry as greener alternatives to toxic and volatile solvents, and as a result, the number of scientific papers has been growing significantly.

DES have been described as a mixture of two or more compounds that when mixed at a specific molar ratio suffer a high melting temperature depression. This decrease in the melting temperature of the mixture, compared to the individual components, is mainly attributed to hydrogen bond formation between the components [1]. When DES components are natural metabolites commonly found in animals and plants, such as sugars, polyols, and amino acids, they are the so-called natural deep eutectic systems (NADES) [2].

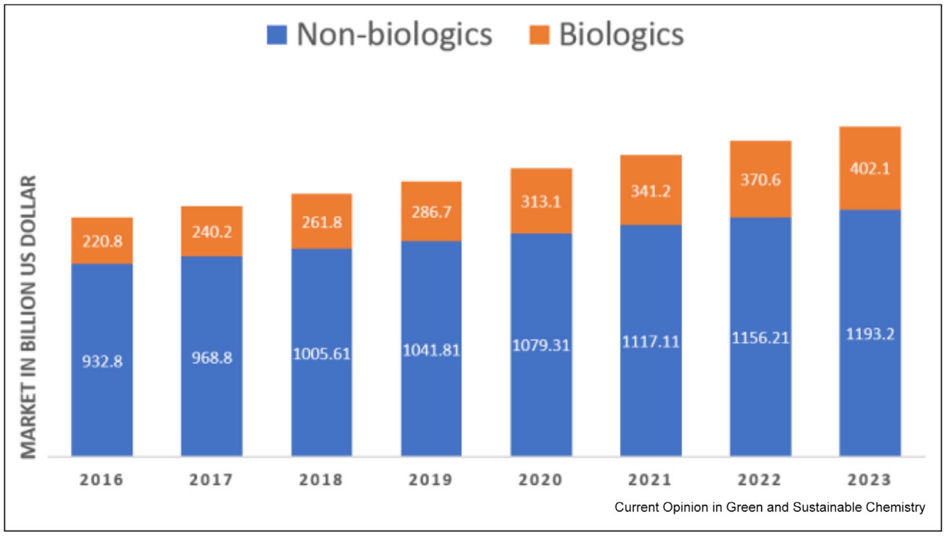
The simple and fast preparation of NADES, which does not require any synthesis and purification steps, is extremely attractive in many fields. The preparation of NADES can be performed by three main procedures: a) freeze-drying, b) vacuum evaporation, and c) heat and stirring [3]. They are truly versatile since a wide range of different compounds can be used for their preparation, leading to task-specific systems with optimized properties for a determined application.

Biotechnology is a scientific field of research that uses biological systems and living organisms to develop new products [4]. During the last decade, NADES have been introduced in the field, mainly due to their properties and their non-toxic nature. NADES are known to present very low toxicity, depending on their components, towards human and animal cells, and the cytotoxic profile of different systems has already been reported [5–8]. Overall, the use of natural metabolites, such as sugars and polyols, leads to less cytotoxic systems, but natural organic acids can induce certain associated toxicity to the NADES due to acidity [7].

Some examples of the use of NADES are the extraction of active compounds from medicinal plants, solubilization of pharmaceuticals, and formulation of pesticides [1]. Nevertheless, the interest in using NADES in other biotechnological applications is increasing every day. One of them is the development of biologic drugs or biologics field (e.g., stem cells and biomolecules) [9,10], which have a significant increase in their global market (Figure 1).

NADES can have a huge impact on this field overcoming the fact that these therapeutics are highly sensitive to

Figure 1



Global pharmaceutical market of chemical drugs and biologics from 2014 to 2023. (Adapted from https://www.statista.com/statistics/1085563/revenue-chemical-drugs-and-biologics-global-pharmaceuticals/(accessed Nov 11, 2021).

temperature, requiring a very well-established cold-chain system during shipping, storage, and handling to maintain their bioactivity [11,12].

NADES in cryopreservation of cells, tissues, and organs

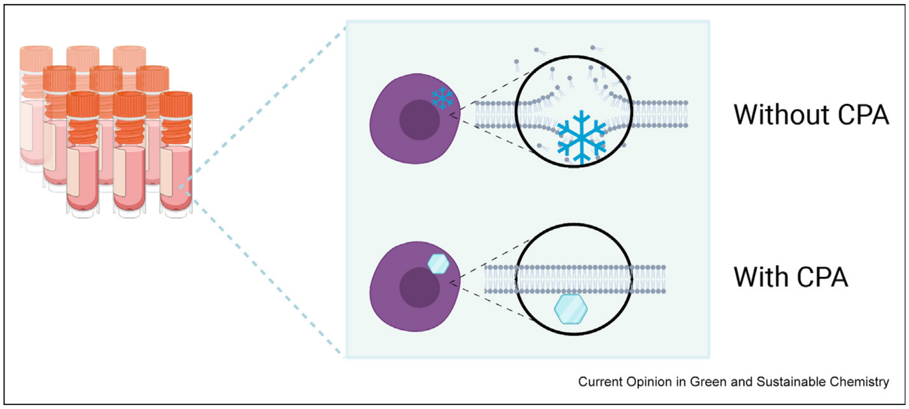
Cryopreservation is the most effective way to preserve cells, but upon freezing, deadly water crystals are formed inside the cell. These crystals once formed disrupt the cell membrane leading to cell death. The only way to avoid this is by using acryoprotectant agent CPA, i.e., a compound that has the ability to decrease the crystallization temperature of water, change the shape of ice crystals, or inhibit ice crystal formation (Figure 2) [13]. The most common CPA is dimethyl sulfoxide (DMSO); however, its toxicity at the most effective concentration (10%) is well documented and it is subject of intense discussion in literature [14,15]. For this reason, active research has been carried out in an attempt to design new CPA's at least as effective as DMSO.

Recently, it has been shown that many animals can survive extreme thermal amplitudes in their natural habitats. This is only possible due to the fact that these animals produce certain metabolites that can significantly decrease the crystallization temperature of water [16]. These metabolites are mostly sugars, organic acids, choline derivatives, and polyols, and have been studied individually as CPA. Most of them were reported to vitrify oocytes and sperm but still require the addition of small amounts (1.5%) of DMSO or glycerol [17–20] for optimal results. Major advances rely on the use of combinations of these molecules as is the case of NADES. Hence, NADES composed of these metabolites should be, perfect candidates to substitute the toxic DMSO for the cryopreservation of cells. Unfortunately, up to date, there are only a few reports exploring NADES as CPAs [8,21,22], which show promising results for the cryopreservation of different cell lines, namely, L929, HacaT, Jurkat, and T-Cells. NADES have been shown to be much less toxic than DMSO, even at higher concentrations (10–20%), and provide high post-thawing viabilities, high proliferation rates, and normal cell morphology [8,23]. Moreover, in some cases, NADES worked fairly better than DMSO, especially for keratynocytes and T-cells [10,24]. These results really open the opportunity window for NADES in the cryobiology field and may be a major breakthrough in stem cell therapies.

Stem cells are considered the future of medicine as they may cure many diseases such as cancer, Parkinson's, Alzheimer's, and other degenerative diseases [25]. However, after their retrieval from the donor, stem cells have a very low lifespan, unless they are frozen. In the current procedure, DMSO is used as the CPA per excellence, however, 1 in 70 people suffer side effects from DMSO toxicity after being exposed to this compound following cell transplant [26].

In a study reported by Jesus *et al*, [8] it has been showed that after thawing cells, using NADES as CPA, there was no need to remove the CPA prior the cell culture as these systems do not hinder the cell viability. These results may suggest that it would be possible to inject

Figure 2



Role of the cryoprotectant agent during the freezing process. (Created with BioRender.com).

them along with stem cells directly into the patient, mainly due to the fact that NADES are composed of metabolites recognized by human cells.

Besides the use of a CPA, the cryopreservation of cells also requires ultra-low temperatures, generally - 196 °C, by the use of liquid nitrogen, which can be a problem, during transportation and storage of cells in remote areas, especially n developing countries.

Studies found in literature [8,22] suggest that it may be possible to use NADES as CPA to freeze immortalized cells at −20 °C, but further investigation is required to truly know the impact of NADES in the recovery of stem cells, particularly in which concerns the question whether they retain their stemness after thawing.

Literature [8,21–24] reports highlight the relevance of NADES in cryobiology and NADES can change the future of cryopreservation of cells. But, what about tissues and organs? Can NADES be used for their cryopreservation? In literature, there are still no studies regarding the vitrification of tissues and/or organs with NADES, but we consider that this could be an interesting perspective in the field. In this sense, future research will foresee more complex strategies to prevent ice formation and eliminate the toxicity factor from the CPA. Furthermore, it will be necessary to fully understand the interaction between NADES and the cell membrane so that it is possible to design an optimal and eventually universal CPA.

NADES as stabilizing agents of therapeutic biomolecules

Biomolecules are any molecules produced by living organisms, which are classified according to their size in small molecules, such as primary and secondary metabolites and natural products or large molecules, namely proteins, polysaccharides, lipids, and nucleic acids [27]. Biomolecules belong to the growing group of active compounds which has been studied for therapeutic purposes. Despite their unquestionable therapeutic action they possess inherent challenges to their delivery and administration.

Proteins

Therapeutic proteins are one of the most important biomolecules used in biotechnology. Common therapeutic proteins include vaccines, enzymes, hormones, and growth factors [28].

Although most proteins are water-soluble, the presence of water can have a detrimental impact on their integrity, causing chemical degradation (deamidation, oxidation, hydrolysis) [29] but also physical degradation (thermal degradation, aggregation) [30,31]. Lyophilization is the most common method to maintain protein stability, but the process itself may cause significant denaturation [32]. The properties of NADES have attracted the attention of researchers for their potential applications in this field [9,33]. For example, the stability of lysozyme in neat and aqueous solutions of choline chloride-based eutectic systems was evaluated and the secondary structure of proteins is minimally affected when the solvent varies from phosphate buffer to pure NADES [34–38]. Moreover, the protein folding is similar when the protein is in buffered conditions or in NADES/water mixtures [39]. In a study carried out by Hossain *et al*, it has been suggested that NADES are able to replace water molecules around the amino acids in the active site of cytochrome C, promoting a partial folding conformation and reducing the protein activity through the combination of specific binding and reduction of solvophobic effects. When water is present, water molecules will surround the active sites allowing the protein to fold in the same manner as when the protein is in the buffer. However, the diffusion of the components is hindered by the presence of NADES and the activity of cytochrome C is decreased [40]. This is the reason why the use of NADES or NADES/water mixtures for the stabilization of proteins remains a challenge.

DNA

Besides proteins, there is also a growing interest in new alternative solvents for the stabilization and preservation of DNA [36]. In general, DNA requires an anhydrous environment, hence, water-free NADES have been used as alternative media. It has been confirmed the stability of all the secondary structures of DNA (duplex, triplex, and G-quadruplex) in neat choline-based NADES [41,42].

Eutectic systems have also been explored as a draw agent for the enrichment of low-abundance DNA and proteins using forward osmosis (FO) [43]. This green technique is commonly used to concentrate biomolecules, such as proteins and DNA, after their extraction. Because it does not require pressure, thermal, or any other external force, it is highly attractive if we consider the increasing demand for pure proteins and DNA in biotechnology, molecular biology, food sciences, and clinical research. Neat NADES possess distinct colligative properties, such as high osmotic pressure ($\pi > 300$ atm) and low water activity ($a_w \leq 0.1$) which are quite interesting properties as draw agents in the FO process. Even at 80% dilution, where the viscosity no longer poses a problem, there is a significant retention of the osmotic pressure ($\pi > 60$ atm), highlighting the potential of eutectic systems for maintaining the high osmotic flux [43].

Vaccines

Vaccination is by far the most efficient approach to prevent widespread disease with massive global impact on the reduction of suffering and death. However, the lack of storage conditions for vaccines in developing countries hinders the delivery of immunization, causing millions of deaths every year [44].

The appearance of SARS-CoV-2 virus in early 2020 and the development of new vaccines raised some questions regarding the cold-chain vaccines transportation [45]. In late 2020, the first vaccine, from Pfizer-BioNTech was finally approved and the vaccination programme started almost immediately. To fast-forward the vaccination, countries had to create conditions to store millions of doses of vaccines, but most vaccines against SARS-CoV-2 require very low temperatures (−80 °C or below), meaning that conditions should be created to ensure that no electric failures occur during the vaccination programme. More critical is the situation in developing countries, in which the success of the vaccination programme is not only constrained by challenges in the health systems, including the lack of trained medical personnel but also inadequate vaccine storage facilities [46]. This situation showed that the design of new and improved formulations for long-term vaccine stabilization is, unequivocally, the frontline of vaccine research and development.

Side by side with vaccine development, different strategies are being pursued. This is the case of virus-like particles (VLPs), which are structures composed of one or more proteins able to mimic native viruses, though lacking genetic information of their infectious counterparts, which makes them safe and effective vaccine candidates. For example, influenza VLPs, accommodating multiple hemagglutinin (HA) subtypes, can stimulate a stronger immunologic response than subunit or recombinant proteins immunogens [47], but also offer a broadened recognition and cross-protection against circulating viruses, thus extending vaccine life-span [48]. However, the efficiency of a vaccine depends not only on the ability of the antigen to elicit immunologic memory but also, and as important, its stability.

NADES have been proven to be stabilizing agents for proteins and enzymes [49]. Therefore, it is hypothesized that NADES can also effectively protect VLPs during storage; hence, potential candidates for new formulations of vaccines. In fact, it has been shown that NADES can be considered as extractants and excipients able to stabilize the final formulation of biopharmaceutics, avoiding the additional step of VLPs extraction [50]. Still, can we use NADES and VLPs to create a new vaccine system able to be stored at room temperature, so that issues with the cold-chain during transportation or long-term storage are no longer present? Recently a study using NADES composed of trehalose and glycerol (TGly) which was able to preserve the activity and the physical integrity of influenza hemagglutinin (HA)-displaying virus-like particles (VLPs) has been published. It has been suggested that it is possible to store VLPs for longer periods and at higher temperatures than the commonly used buffered formulations [51]. Although it is only one study, the results are very promising and highlight the possible use of NADES as new storage media for VLP-based vaccines, using non-refrigerated conditions and short-term thermal stress. This may have a notable impact on vaccines' cold chain and consequently a huge economic and environmental impact. If successful with other vaccine modalities (e.g., DNA, r-subunit or r-vector) it would be a game changer for the pharmaceutical companies.

Future perspectives

We have presented herein several examples that demonstrate the huge potential of NADES in the biotechnology field. Although the use of NADES as cryoprotectant agents or biomolecules stabilizing agents is little explored, the results obtained so far are quite promising. Work reported in the literature has described the use of NADES as CPA's for different cell lines; however, major breakthroughs would be expected if not only cells could be cryopreserved with this media but also tissues and organs. Advances in the management of chronic wounds and burns would be foreseen if keratinocyte cell membranes would be an off-the-shelf product that could be implanted directly in the patient. Furthermore, if whole organs could be preserved by the use of NADES the storage time could be increased, which would lead to an increase in the time span of the organs available for transplantation. In which concerns proteins, DNA, and/or vaccines the work reported in the literature demonstrated the great potential of this liquid system, which can act as storage and protectant media, preventing unfolding, degradation, or agglomeration. With the boost of new therapeutics based on biopharmaceutics, the progress beyond the state of the art also relies on new technologies for the stabilization of the bioactives. There is, however, still a long path ahead before NADES can start to be used in larger scale and in further biotechnological applications. Answering the questions raised in this manuscript would allow scientists predict and prepare the most suitable NADES for a specific application. The major goal of the manuscript is to trigger the curiosity of the scientific community and boost the research in this field leading to new developments and pushing forward alternative and more effective methodologies for cryopreservation of cells and for the development of vaccines which can be stored at room temperature, avoiding the need for a cold chain. Hopefully, the green features of NADES, together with their exceptional properties will contribute to an increase in their use, especially at the industrial level, avoiding toxic and dangerous organic solvents.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ana Rita C. Duarte reports financial support was provided by European Research Council. Ana Rita Jesus reports financial support was provided by Foundation for Science and Technology. Alexandre Paiva reports financial support was provided by Foundation for Science and Technology.

Data availability

Data will be made available on request.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Vanda H, Verpoorte R, Klinkhamer PGL, Choi YH: **Natural deep eutectic solvents: from their discovery to their applications**. *Deep Eutectic Solvents Synth. Prop. Appl.* 2019:61–81. https://doi.org/10.1002/9783527818488.ch4.

2. ** Choi YH, van Spronsen J, Dai Y, Verberne M, Hollmann F, Arends IWCE, Witkamp G-J, Verpoorte R: **Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology?** *Plant Physiol* 2011, **156**: 1701–1705.
This manuscript was the first showing the relatinship between the metabolites found in cells and natural deep eutectic systems. After this manusctipt was published many others were published regarding the use of natural deep eutectic systems for many biological applications. 10.1104/pp.111.178426.

3. Meneses L, Santos F, Gameiro AR, Paiva A, Duarte ARC: **Preparation of binary and ternary deep eutectic systems**. *JoVE* 2019, **152**, e60326. https://doi.org/10.3791/60326.

4. Hopkins MM, Kraft A, Martin PA, Nightingale P, Mahdi S. In *1.17 - is the biotechnology revolution a myth?*. Edited by Taylor JB, Triggle DJBT-CMCII, Eds, Oxford: Elsevier; 2007:591–613. https://doi.org/10.1016/B0-08-045044-X/00004-3.

5. Mbous YP, Hayyan M, Wong WF, Looi CY, Hashim MA: **Unraveling the cytotoxicity and metabolic pathways of binary natural deep eutectic solvent systems**. *Sci Rep* 2017, **7**, 41257. https://doi.org/10.1038/srep41257.

6. Macário IPE, Oliveira H, Menezes AC, Ventura SPM, Pereira JL, Gonçalves AMM, Coutinho JAP, Gonçalves FJM: **Cytotoxicity profiling of deep eutectic solvents to human skin cells**. *Sci Rep* 2019, **9**:3932. https://doi.org/10.1038/s41598-019-39910-y.

7. Hayyan M, Mbous YP, Looi CY, Wong WF, Hayyan A, Salleh Z, Mohd-Ali O: **Natural deep eutectic solvents: cytotoxic profile**. *SpringerPlus* 2016, **5**:913. https://doi.org/10.1186/s40064-016-2575-9.

8. * Jesus AR, Meneses L, Duarte ARC, Paiva A: **Natural deep eutectic systems, an emerging class of cryoprotectant agents**. *Cryobiology* 2021, **101**:95–104. https://doi.org/10.1016/j.cryobiol.2021.05.002.
The authors have shown that different NADES can be used to cryopreserve different cell lines

9. Lee MS, Lee K, Nam MW, Jeong KM, Lee JE, Kim NW, Yin Y, Lim SY, Yoo DE, Lee J, Jeong JH: **Natural deep eutectic solvents as a storage medium for human interferon-A2: a green and improved strategy for room-temperature biologics**. *J Ind Eng Chem* 2018, **65**:343–348. https://doi.org/10.1016/j.jiec.2018.05.005.

10. Hornberger K, Li R, Hubel A: **Natural deep eutectic systems for improved cryopreservation of cells**. *Cryobiology* 2020, **97**: 256–257. https://doi.org/10.1016/j.cryobiol.2020.10.033.

11. Tan LJ, Barnett MA, Eisenberg A, Fox Fields H, Hackell JM, Léger MM, Meadows B, Moore KL, Rehm SJ, Rothholz MC, Smith C, Talkington K, Wexler DL: **From refrigerator to arm: issues in vaccination delivery**. *Vaccine* 2014, **32**:2389–2393. https://doi.org/10.1016/j.vaccine.2014.02.045.

12. Ammann C: **Stability studies needed to define the handling and transport conditions of sensitive pharmaceutical or biotechnological products**. *AAPS PharmSciTech* 2011, **12**: 1264–1275. https://doi.org/10.1208/s12249-011-9684-0.

13. Bozkurt SBE-Y: *Cryoprotectants and their usage in cryopreservation process*. Rijeka: IntechOpen; 2018. https://doi.org/10.5772/intechopen.80477. Ch. 2.

14. Galvao J, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF: **Unexpected low-dose toxicity of the universal**

solvent DMSO. *Faseb J* 2014, **28**:1317–1330. https://doi.org/10.1096/fj.13-235440.

15. Hanslick JL, Lau K, Noguchi KK, Olney JW, Zorumski CF, Mennerick S, Farber NB: **Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system**. *Neurobiol Dis* 2009, **34**:1–10. https://doi.org/10.1016/j.nbd.2008.11.006.

16. ** Gertrudes A, Craveiro R, Eltayari Z, Reis RL, Paiva A, Duarte ARC: **How do animals survive extreme temperature amplitudes? The role of natural deep eutectic solvents**. *ACS Sustainable Chem Eng* 2017, **5**:9542–9553.

This paper is the first reference on the role of Natural Deep Eutectic systems by animals to survive extreme enviroment conditions. https://doi.org/10.1021/acssuschemeng.7b01707.

17. Jesus AR, Meneses L, Duarte ARC, Paiva A: **Chapter fifteen - natural deep eutectic systems—a new era of cryopreservation**. In Verpoorte R, Witkamp G-J, Choi YHBT-A, Eds., *Eutectic solvents and stress in plants*, **97**. Academic Press; 2021: 385–409. https://doi.org/10.1016/bs.abr.2020.09.015.

18. Zhang L, Xue X, Yan J, Yan L-Y, Jin X-H, Zhu X-H, He Z-Z, Liu J, Li R, Qiao J: **Cryobiological characteristics of L-proline in mammalian oocyte cryopreservation**. *Chin Med J* 2016, **129**: 1963–1968. https://doi.org/10.4103/0366-6999.187846.

19. Koskinen E, Junnila M, Katila T, Soini H: **A preliminary study on the use of betaine as a cryoprotective agent in deep freezing of stallion semen**. *J Vet Med Ser A* 1989, **36**:110–114. https://doi.org/10.1111/j.1439-0442.1989.tb00710.x.

20. Eroglu A: **Cryopreservation of mammalian oocytes by using sugars: intra- and extracellular raffinose with small amounts of dimethylsulfoxide yields high cryosurvival, fertilization, and development rates**. *Cryobiology* 2010, **60**:S54–S59. https://doi.org/10.1016/j.cryobiol.2009.07.001.

21. Hornberger K, Li R, Duarte ARC, Hubel A: **Natural deep eutectic systems for nature-inspired cryopreservation of cells**. *AIChE J* 2021, **67**, e17085. https://doi.org/10.1002/aic.17085.

22. Castro VIB, Craveiro R, Silva JM, Reis RL, Paiva A, Duarte C, A R: **Natural deep eutectic systems as alternative nontoxic cryoprotective agents**. *Cryobiology* 2018, **83**:15–26. https://doi.org/10.1016/j.cryobiol.2018.06.010.

23. Jesus AR, Duarte ARC, Paiva A: **Use of natural deep eutectic systems as new cryoprotectant agents in the vitrification of mammalian cells**. *Sci Rep* 2022, **12**:8095. https://doi.org/10.1038/s41598-022-12365-4.

24. Bryant SJ, Awad MN, Elbourne A, Christofferson AJ, Martin AV, Meftahi N, Drummond CJ, Greaves TL, Bryant G: **Deep eutectic solvents as cryoprotective agents for mammalian cells**. *J Mater Chem B* 2022, **10**:4546–4560. https://doi.org/10.1039/D2TB00573E.

25. de Carvalho AC: **Stem cells and degenerative diseases**. *BMC Proc* 2014, **8**:O19. https://doi.org/10.1186/1753-6561-8-S4-O19. O19.

26. Bekkem A, Selby G, Chakrabarty JH: **Retrospective analysis of intravenous DMSO toxicity in transplant patients**. *Biol Blood Marrow Transplant* 2013, **19**:S313. https://doi.org/10.1016/j.bbmt.2012.11.466.

27. Nanoparticles Niemeyer CM: **Proteins, and nucleic acids: biotechnology meets materials science**. *Angew Chem Int Ed* 2001, **40**:4128–4158. https://doi.org/10.1002/1521-3773(20011119)40:22<4128::AID-ANIE4128>3.0.CO;2-S.

28. Dimitrov DS: **Therapeutic proteins**. *Methods Mol Biol* 2012, **899**: 1–26. https://doi.org/10.1007/978-1-61779-921-1_1.

29. Wang W: **Instability, stabilization, and formulation of liquid protein pharmaceuticals**. *Int J Pharm* 1999, **185**:129–188. https://doi.org/10.1016/s0378-5173(99)00152-0.

30. Capelle MAH, Gurny R, Arvinte T: **High throughput screening of protein formulation stability: practical considerations**. *Eur J Pharm Biopharm* 2007, **65**:131–148. https://doi.org/10.1016/j.ejpb.2006.09.009.

31. Chi EY, Krishnan S, Randolph TW, Carpenter JF: **Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation**. *Pharm Res (N Y)* 2003, **20**:1325–1336. https://doi.org/10.1023/A:1025771421906.

32. Bhatnagar BS, Bogner RH, Pikal MJ: **Protein stability during freezing: separation of stresses and mechanisms of protein stabilization**. *Pharmaceut Dev Technol* 2007, **12**:505–523. https://doi.org/10.1080/10837450701481157.

33. Pal S, Roy R, Paul S: **Potential of a natural deep eutectic solvent, glyceline, in the thermal stability of the trp-cage mini-protein**. *J Phys Chem B* 2020, **124**:7598–7610. https://doi.org/10.1021/acs.jpcb.0c03501.

34. Xin R, Qi S, Zeng C, Khan FI, Yang B, Wang Y: **A functional natural deep eutectic solvent based on trehalose: structural and physicochemical properties**. *Food Chem* 2017, **217**:560–567. https://doi.org/10.1016/j.foodchem.2016.09.012.

35. Harifi-Mood AR, Ghobadi R, Divsalar A: **The effect of deep eutectic solvents on catalytic function and structure of bovine liver catalase**. *Int J Biol Macromol* 2017, **95**:115–120. https://doi.org/10.1016/j.ijbiomac.2016.11.043.

36. Sequeira RA, Bhatt J, Prasad K: **Recent trends in processing of proteins and DNA in alternative solvents: a sustainable approach**. *Sustain. Chem.* 2020, **1**:116–137. https://doi.org/10.3390/suschem1020010.

37. Klibanov AM: **Why are enzymes less active in organic solvents than in water?** *Trends Biotechnol* 1997, **15**:97–101. https://doi.org/10.1016/S0167-7799(97)01013-5.

38. Esquembre R, Sanz JM, Wall JG, del Monte F, Mateo CR, Ferrer ML: **Thermal unfolding and refolding of lysozyme in deep eutectic solvents and their aqueous dilutions**. *Phys Chem Chem Phys* 2013, **15**:11248–11256. https://doi.org/10.1039/C3CP44299C.

39. Sanchez-Fernandez A, Edler KJ, Arnold T, Alba Venero D, Jackson AJ: **Protein conformation in pure and hydrated deep eutectic solvents**. *Phys Chem Chem Phys* 2017, **19**:8667–8670. https://doi.org/10.1039/C7CP00459A.

40. * Hossain SS, Paul S, Samanta A: **Structural stability and conformational dynamics of cytochrome c in hydrated deep eutectic solvents**. *J Phys Chem B* 2021, **125**:5757–5765. https://doi.org/10.1021/acs.jpcb.1c01975.

The authors showed that the structure of proteins, in particular case cytochrome C, is highly affefed by the strength of the interactions between the components of NADES and the interaction between the components and the water molecules. Furthermore, the authors also showed that viscosity is important for the stability of proteins

41. Mondal D, Sharma M, Mukesh C, Gupta V, Prasad K: **Improved solubility of DNA in recyclable and reusable bio-based deep eutectic solvents with long-term structural and chemical stability**. *Chem Commun* 2013, **49**:9606–9608. https://doi.org/10.1039/C3CC45849K.

42. Bhatt J, Mondal D, Bhojani G, Chatterjee S, Prasad K: **Preparation of bio-deep eutectic solvent triggered cephalopod shaped silver chloride-DNA hybrid material having antibacterial and bactericidal activity**. *Mater Sci Eng C* 2015, **56**: 125–131. https://doi.org/10.1016/j.msec.2015.06.007.

43. * Mondal D, Mahto A, Veerababu P, Bhatt J, Prasad K, Nataraj SK: **Deep eutectic solvents as a new class of draw agent to enrich low abundance DNA and proteins using forward osmosis**. *RSC Adv* 2015, **5**:89539–89544. https://doi.org/10.1039/C5RA20735E.

In this study the authors have showed that it is possible to simply, safely and efficiently use choline-based deep eutectic systems (DES) as draw agents for the enrichment of biomolecules such as proteins (e.g. BSA) and DNA. This was the first study with DES for this methodology.

44. Offit, P. A. Global Immunization: Worldwide Disease Incidence https://www.chop.edu/centers-programs/vaccine-education-center/global-immunization/diseases-and-vaccines-world-view.

45. UNICEF. Ultra-low temperature (ULT) storage and transport for vaccines an overview of options and challenges.

46. Elgar, K.; Lopert, R.; Carey, E.; M, W. Coronavirus (COVID-19) vaccines for developing countries: an equal shot at recovery https://www.oecd.org/coronavirus/policy-responses/coronavirus-covid-19-vaccines-for-developing-countries-an-equal-shot-at-recovery-6b0771e6/.

47. Jennings GT, Bachmann MF: **The coming of age of virus-like particle vaccines**. *Biol Chem* 2008, **389**:521–536. https://doi.org/10.1515/bc.2008.064.

48. Quan FS, Steinhauer D, Huang C, Ross TM, Compans RW, Kang S-M: **A bivalent influenza VLP vaccine confers complete inhibition of virus replication in lungs**. *Vaccine* 2008, **26**: 3352–3361. https://doi.org/10.1016/j.vaccine.2008.03.055.

49. Almeida JS, Capela EV, Loureiro AM, Tavares APM, Freire MG: **An overview on the recent advances in alternative solvents as stabilizers of proteins and enzymes**. *ChemEngineering* 2022. https://doi.org/10.3390/chemengineering6040051.

50. Marchel M, Niewisiewicz J, Coroadinha AS, Marrucho IM: **Purification of virus-like particles using aqueous biphasic systems composed of natural deep eutectic solvents**. *Separ Purif Technol* 2020, **252**, 117480. https://doi.org/10.1016/j.seppur.2020.117480.

51. * Correia R, Meneses L, Richheimer C, Alves PM, Carrondo MJT, Duarte ARC, Paiva A, Roldão A: **Improved storage of influenza HA-VLPs using a trehalose-glycerol natural deep eutectic solvent system**. *Vaccine* 2021, **39**:3279–3286. https://doi.org/10.1016/j.vaccine.2021.04.033.

Using an eutectic mixture composed of trehalose and glycerol the authors were able to significantly improve the stability of HA-VLPs, highlighting the potential of these systems for the VLP-based production of vaccines.