Applications of natural deep eutectic solvents to extraction and preservation of biomolecules

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C H A P T E R 3

Natural deep eutectic solvents: from their discovery to their applications

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Abstract

It is generally thought that all enzyme-mediated reactions in biosynthetic pathways in living organisms occur in a medium that is basically composed of water or lipids. However, an obvious question arises: how can organisms, that produce a broad range of primary and secondary metabolites, control the biosynthetic process and substrate transport in an aqueous or lipid medium, considering the often-poor water and/or lipid solubility of these metabolites? Recently, it was found that mixtures of certain compounds in solid state that are abundant in all organisms, such as sugars, organic bases, amino- and organic acids become liquids when mixed in certain molar ratios. These liquids have been named natural deep eutectic solvents (NADESs). They were shown to be highly selective solvents for all kinds of medium polar compounds that are poorly soluble in water and lipids. NADESs are non-toxic, and environment-friendly. As they have virtually zero vapour-pressure, risks of flammability are low. These characteristics make them powerful green solvents. At present, NADESs are being extensively investigated as extraction solvents for various applications, with the first products already being on the market. Based on our hypothesis that NADESs are the third liquid phase in living organisms, a number of hypothetical roles of NADESs in cellular systems can be postulated, and also based on that applications can be developed. Examples are the preservation of biopolymers such as DNA, RNA and proteins, as well as a medium for enzymatic reactions. This paper reviews features of NADESs, and some recent applications of NADESs for small molecules, macromolecules and enzymatic reactions.

Keywords: Natural Deep Eutectic Solvents, DNA, RNA, Proteins, Enzyme Reaction, Preservation

Introduction

Ionic liquids (ILs) have been studied extensively as novel green solvents for various applications, for example, organic synthesis and enzymatic reactions. Several recent reviews deal with the development of these solvents and their uses. Choi et al. hypothesized that Nature already uses the principles of ionic liquids and deep eutectic solvents and introduced the concept of a new type of eutectic liquid, the Natural Deep Eutectic Solvents (NADESs). A comprehensive definition of NADESs includes not only their physicochemical features but above all involves the phenomena in living cells and organisms that can (at least, in part) be explained by their existence. NADESs constituents are abundant in all organisms, and based on their physicochemical characteristics these solvents may be formed locally under different conditions, and thus play various roles (Choi et al. 2011; Vanda et al. 2018). Particularly the modulation exerted by water on the characteristics of NADESs could be a major regulatory function in diverse metabolic processes in living organisms. In terms of applications, NADESs have several advantages over the first generation of synthetic ILs, such as their non-toxicity, lower corrosiveness, absence of environmental hazards, sustainable production, and last but not least lower costs. These characteristics are important aspects in green chemistry and key NADESs as green alternative for organic solvents.

Similarly, to ILs or DES, NADESs are formed by mixing two or more components in certain molar ratios; the difference is that these components are from natural sources such as sugars, sugar alcohols, polyalcohols, amino acids, organic acids, and organic bases. We view NADESs more as concept in which such mixtures may also contain water. The water content can be a part of the eutectic mixture at a certain molar ratio, from which the water cannot evaporate, explaining why aqueous plant extracts always contain some residual water. The other aspect is that a NADESs can be diluted with water, resulting in their increased solubilizing power of various compounds, or the activation of enzymes which are stable but not active in a NADESs, but which become active

upon dilution. NMR spectrometric studies of NADESs provide evidence that extensive hydrogen-bonding interactions are involved in this process (Choi et al. 2011; Dai et al. 2013[a]; Vanda et al. 2018). Different classes of NADESs can be distinguished on the basis of their components (Table 1). Besides their already mentioned environmental advantages, NADESs have very useful physicochemical properties as solvents, such as very low melting points, adjustable viscosity and extremely low vapor pressure with very high flash points. Additionally, they show a high solubilizing capacity of a wide polarity range of compounds and high selectivity (Dai et al. 2013[a]; Vanda et al. 2018).

Table 1. Classification of NADESs and their typical examples

Composition	Examples
Acid – base	Malic acid – choline chloride (1:1) Citric acid – betaine (1:1)
Polyalcohol – acid	Xylitol – citric acid (1:1) D-sorbitol – Malic acid (1:1)
Polyalcohol – amino acid	D-sorbitol –proline (1:1)
Polyalcohol – base	D-sorbitol – choline chloride (1:3)
Sugar – acid	Sucrose –malic acid (1:1) Fructose – citric acid (1:1)
Sugar – amino acid	Sucrose –proline (1:4) Glucose – proline (1:1)
Sugar – base	Sucrose – choline chloride (1:4)
Sugar – sugar	Glucose –fructose – sucrose (1:1:1)

Table 2. Examples of different combinations of natural deep eutectic solvents (Choi et al. 2011; Dai et al. 2013[a])

Component 1	Component 2	Component 3	Mole ratio
Choline chloride	Lactic acid		1:1
Choline chloride	Malonic acid		1:1
Choline chloride	Maleic acid		1:1, 2:1,
choline chloride	DL-malic acid		1:1, 1.5:1
Choline chloride	Citric acid		1:1, 2:1,
Choline chloride	D-mannose		5:2
Choline chloride	D-(+)-galactose		5:2
Choline chloride	Sucrose		4:1, 1:1
Choline chloride	Proline	DL-malic acid	1:1:1
Choline chloride	Xylitol	DL-malic acid	1:1:1
Betaine	Sucrose		2:1
Betaine	D-(+)-trehalose		4:1
Betaine	D-(+)-glucose	Proline	1:1:1
Betaine	DL-malic acid	D-(+)-glucose	1:1:1
Betaine	DL-malic acid	Proline	1:1:1
DL-malic acid	Sucrose		1:1
DL-malic acid	D-mannose		1:1
DL-malic acid	D-sorbitol		1:1
DL-malic acid	D-(+)-glucose	D-(-)-fructose	1:1:1
DL-malic acid	D-(+)-glucose	Glycerol	1:1:1
DL-malic acid	Sucrose	Glycerol	1:1:2
D/L-proline	D-sorbitol		1:1
D/L-proline	Lactic acid		1:1
D/L-proline	DL-malic acid		1:1
D-proline	D-(+)-glucose		5:3
L-proline	D-(+)-glucose		5:3
D-(+)-glucose	DL-malic acid		1:1
D-(+)-glucose	Citric acid		1:1
D-(+)-glucose	L-(+)-tartaric acid		1:1
D-(+)-glucose	D-(-)-fructose	Sucrose	1:1:1
β-alanine	Citric acid		1:1

Initially, some 50 combinations of NADESs were proposed (Choi et al. 2011; Dai et al. 2013[a]) but since then many other different NADESs have been reported, all based on combinations that can fit into the categories mentioned in Table 1. They are mixtures of two or more compounds such as sugars,

polyalcohols, amino acids, organic acids, choline chloride, and betaine (Choi et al. 2011; Dai et al. 2013[a]; Vanda et al. 2018). Some of these NADESs candidates are shown in Table 2. With the rapidly increasing interest in research associated to green technology, new candidates for NADESs are continually reported (e.g. Paiva et al. 2014; Wikene et al. 2015; Bakirtzi et al. 2016; Espino et al. 2016; Wikene et al. 2017; Huang et al. 2018).

Due to their non-toxic nature, the interest in applications of NADESs for food, agrochemicals, cosmetics and pharmaceuticals has grown exponentially since the concept was introduced (Choi et al. 2011) and patented in 2011 (Van Spronsen et al. 2016). Examples of NADESs applications are the extraction of active compounds from medicinal plants (Dai et al. 2013[b]; Dai et al. 2016), the solubilization of pharmaceuticals (Rozema et al. 2015; Wikene et al. 2015; Bakirtzi et al. 2016; Shamseddin et al. 2017; Wikene et al. 2017), the production of plant extracts as cosmetic ingredients (Jeong et al. 2017), agrochemical applications (Huang et al. 2018; Zahrina et al. 2018), and as food flavoring additives (Gonzales et al. 2018).

Besides their use as extraction solvents, many other types of applications are expected to be developed. For example, NADESs could be excellent solvents for enzymatic and synthetic reactions involving poorly water- or lipid-soluble compounds. Several studies have already shown very promising results. These applications were actually predictable considering our hypothesis of NADESs as a third liquid phase in organisms. Numerous primary and secondary metabolites are biosynthesized in nature, most of which are poorly water soluble. For example, the biosynthesis of taxol (paclitaxel) encompasses a large number of steps in which non-water soluble intermediates have to move from one enzyme to another, something which is difficult to explain in an aqueous medium. However, knowing that enzymes are quite well soluble in various NADESs (Choi et al. 2011) and assuming a NADESs environment e.g. in vesicles or metabolons, non-water soluble compounds could be processed in a multienzymes system. The biosynthesis of various non-water soluble

metabolites including macromolecules like cellulose and lignin in plants, could be easily accounted for in such a system. Learning from Nature is the way to go, and some interesting results have already been reported, such as the use of NADESs as media for enzymatic reactions (Zhao et al. 2011; Paiva et al. 2014; Yang et al. 2017; Khodaverdian et al. 2018), for the solubilisation of biomacromolecules (Kumar et al. 2016; Lores et al. 2017), and for the extraction and preservation of DNA and RNA (Mamajanov et al. 2010; Mondal et al. 2013).

In this paper, after a short general overview of NADESs as an extraction solvent, we will focus particularly on a review of the applications of NADESs involving enzymatic reactions, macromolecules and as a preservative.

Natural deep eutectic solvents is a concept based on how physicochemical features might be used for biological functions

Since the introduction of the concept of NADESs, there has been a discussion concerning their definition and various proposals for the nomenclature of deep eutectic solvents such as low-melting mixtures (Ruß et al. 2012), low transition temperature mixtures (Francisco et al. 2012), and bio-ILs (Fukaya et al. 2007) have been put forward. These names, however, are based on strict physicochemical features, whereas NADESs is a concept that goes beyond this, as it explains various functions of mixtures of NADESs components in nature. In this context, the effect of water in NADESs is of great interest.

When the concept of NADESs was introduced, they were hypothesized to constitute a third liquid phase in living organisms as an alternative to water and lipids, and thought to be vital for various biological functions (Choi et al. 2011). This alternative liquid phase could explain, for example, the biosynthesis of non-water-soluble compounds. The NMR analysis of extracts obtained from plant material using a comprehensive extraction method, (Yuliana et al. 2011) showed clearly that the compounds present in a plant represent three groups that

can be categorized as hydrophilic, medium hydrophilic and lipophilic. This would support a model in which three different liquids or solvents are present in cells.

NADESs have been proposed as solvents for the storage or solubilization of poorly water-soluble compounds (most secondary metabolites) (Dai et al. 2013[b]; Bakirtzi et al. 2016; Faggian et al. 2016; Jeong et al. 2017; Sut et al. 2017); as media for enzymatic reactions in biosynthesis (Zhao et al. 2011; Yang et al. 2017; Khodaverdian et al. 2018); to enable the transport of metabolites (Markham et al. 2000; Beaudoin and Facchini, 2014); for the preservation of biomacromolecules such as DNA, RNA or proteins (particularly for survival during extreme conditions, such as resurrection plants, cacti and lichens) (Hoekstra et al. 2001; Mamajanov et al. 2010; Mondal et al. 2013) and as media in exudates, latex or saps (Gowda et al. 1983).

All these processes are very difficult to explain considering that they all require a liquid medium and the only available "liquids" are water and lipids, the conventional natural solvents thought to be present in an organism, neither of which can readily dissolve most secondary metabolites. Thus, it was proposed that certain ionic liquids or deep eutectic solvents composed of ingredients found in Nature, i.e. NADESs, could play a role in those processes. Candidates for NADESs ingredients in various organisms were all obtained from the in-house ^{1}H NMR based metabolomics data library. The spectra of a great number of samples showed the high abundance of sugars, sugar alcohols, organic acids (e.g. malic, citric, succinic and lactic acid), bases (e.g. choline and betaine) and amino acids (e.g. proline, alanine). These were often present in certain molar ratios as could be calculated directly from the quantitation of their NMR signals (Choi et al. 2011). The first examples of NADESs (sugar and malic acid based) are shown in Figure 1 (Choi et al. 2011). Since this first discovery, the presence of NADESs ingredients in specific plant tissues and saps has been described, for example in the nectar of flowers, maple syrup, and the barley seed aleurone.

According to our hypothesis, NADESs could play a major role in the survival of plants. For example, under extreme drought conditions, when resurrection plants dehydrate, the formation of a NADESs can keep the last available water as part of the NADESs. In this liquid, enzymes remain dissolved and stabilized but are inactive, however when water becomes available, the NADESs will mix with water and at a certain water percentage the enzymes will become active again. The difference with the formation of a glass from sugar is that these do not retain water and eventually crystallize (Hoekstra et al. 2001; Choi et al. 2011). This idea is supported when considering the cryoprotectants added to plant cells to protect them during cryopreservation are all typical NADESs constituents (Mustafa et al. 2011). In fact, various studies on cold resistance of plants report compounds which are now known to be able to be part of NADESs, e.g. proline, sugars, betaine, linolenic acid, and palmitic acid (Beck et al. 2007; Xie et al. 2013; Chen et al. 2014).

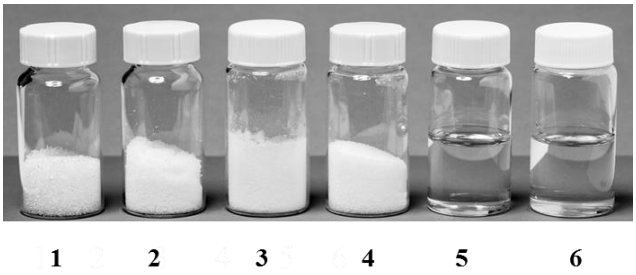

Figure 1. Typical natural eutectic solvents (NADESs) 1: sucrose, 2: fructose, 3: glucose, 4: malic acid, 5: sucrose-fructose-glucose (1:1:1, mol/mol), 6: sucrose-malic acid (1:1, mol/mol). Remade with permission from Choi et al. (2011).

Assuming that solid state biosynthesis does not occur in Nature, there must be a medium in which the enzyme-mediated reactions that produce water-insoluble small molecules and polymers such as cellulose, amylose, and lignin can proceed. In the case of macromolecules, the hydrophilic intermediates and enzymes must be in the same phase that dissolves both the precursors and the

resulting polymer. Several NADESs have indeed been found to dissolve starch and the increasing levels of water may result in the precipitation of the polysaccharide (Choi et al. 2011).

As mentioned above, cells also contain compounds like flavonoids which are not very soluble in water, yet are present in relatively high amounts. It is not only their biosynthesis that is inexplicable given their low solubility, but also their storage in very high levels in certain tissues or cells in apparently solution. In some cases, compounds are synthesized in specialized cells or tissues and then transported to another cellular compartment or other cells. Vesicles seem to be involved in the transport and storage of secondary metabolites. For example, alkaloids synthesized in sieve elements are transported to laticifers for storage in large cytoplasmic phospholipid bilayer vesicles containing a fluid which contains several common NADESs ingredients (Wink, 1993; Carqueijeiro et al. 2013; Beaudoin and Facchini 2014). Anthocyanoplasts (ACPs) and anthocyanin vacuolar inclusions (AVIs) as described by Markham (2000) might very well consist of NADESs dissolved flavonoids and anthocyanins in a phospholipids-based vesicle, providing an explanation for the presence of these compounds in cells in concentrations that are way above their solubility in water. Further supporting evidence for a biological function of NADESs is the chemical composition of the plant vacuolar content. The most abundant compounds in plant vacuoles aside from water are sucrose and malic acid (Yamaki 1984). Considering one of the roles of vacuoles, i.e., the storage of toxic defense compounds, it is likely that those with low water solubility would precipitate. Thus, NADESs constituents could play a role in increasing their solubility, either forming a layer on the inside vacuolar membrane, or alternatively, forming a complex with the metabolite that increases its water solubility, i.e., a similar principle to ion-pairing. The endoplasmic reticulum (ER) could be a NADES in which enzymes and medium polar substrates are dissolved with the ER absorbing these from the aqueous phase of the cytoplasm (Choi et al. 2011).

In connection with a possible role in plant defense, a high concentration of a soluble compound could be an advantage. For example, the solubility of rutin, the most common flavonoid in Nature (accounting, for example, for 30% of the dry-weight of *Sophora japonica* flowers) is 50 to 100 times higher in NADESs than in water (Choi et al. 2011). Moreover, it has been shown that NADESs increase the bioavailability of bioactive compounds such as antioxidants (Faggian et al. 2016) and berberine, a quaternary alkaloid (Sut et al. 2017). A similar effect could be important in plant defense against pests and diseases.

NADESs in extraction and solubilization of non-water-soluble metabolites

Since the introduction of the NADESs concept, a rapidly increasing number of applications for the extraction or solubilization of non-water-soluble metabolites have been published, and reviewed (Dai et al. 2013[a]; Dai et al. 2013[c]; Rozema et al. 2015; Dai et al. 2016; Espino et al. 2016; Huang et al. 2018; Vanda et al. 2018).

Many secondary metabolites such as taxol, ginkgolides and rutin have been found to be much more soluble in NADESs than water (Choi et al. 2011; Dai et al. 2013[a]; Faggian et al. 2016). Quercetin, a ubiquitous flavonoid in Nature, showed an enhanced solubility of up to 380% in a NADESs as compared to a buffer (Gomez et al. 2016).

As a basis for the patent of Van Spronsen et al. (2016), several applications of NADESs for extraction, including phenolic compounds from *Carthamus tinctorius* L. (Dai et al. 2013[b]), vanillin from *Vanilla planifolia* pod (Gonzales et al. 2018), and anthocyanins from *Catharanthus roseus* (Dai et al. 2016) were reported. Later, the study of the selective extraction of ginkgolides, phenolics, and ginkgolic acids from *Ginkgo biloba* as well as ginsenosides from *Panax ginseng* with NADESs from their respective plant materials was performed using a newly developed high-performance thin layer chromatography-based metabolomics method for their analysis. This method

showed to be a promising tool for the study of NADESs extracts (Liu et al. 2018).

Recently, Nam et al. (2015) published the results of a comprehensive study of the extraction of flavonoids from the traditional Chinese medicinal plant, *Styphnolobium japonicum* (L.) Schott. Quercetin, kaempferol, and isorhamnetin glycosides were extracted by ultrasound assisted extraction (UAE) using a L-proline-glycerol NADESs, giving higher yields than conventional organic solvent extraction and solid phase extraction (SPE).

Wei et al. (2015) compared the extraction of phenolics from *Cajanus cajan* leaves, with fourteen NADESs combinations. The results indicated that a combination of choline chloride-maltose (1:2, mol/mol) provided the highest extraction of both polar and weakly-polar compounds compared to conventional solvents, additionally exhibiting high linearity and recovery.

Lipophilic compounds such as plant volatiles or flavors have also been successfully extracted with NADESs (Gonzales et al. 2018; Křížek et al. 2018). For example, the extraction of cannabinoids from *Cannabis sativa* was reported by Křížek et al. (2018). Using the hydrophobic NADESs composed of menthol-acetic acid (1:1), they achieved a highly efficient extraction of tetrahydrocannabinol, tetrahydrocannabinolic acid, cannabidiol, and cannabidiolic acid with yields ranging from 118.6% to 132.6% times those obtained with organic solvents. NADESs have also been applied for the extraction of flavors. For example, the solubility in NADESs of vanillin, the most common flavor compound in the world, was tested and the combinations that proved to be most promising were then used for the extraction of the whole flavor from vanilla pods. The efficiency of the extraction with some of these NADESs proved to be higher than that obtained with conventional organic solvents or water extraction. Furthermore, the NADESs extracts proved to be suitable for flavoring, and could be marketable (Gonzales et al. 2018).

The successful application of NADESs to the extraction of natural products illustrates the potential of NADESs to replace toxic organic solvents in

large scale industrial extraction. However, their successful commercial application requires the solution of some significant drawbacks. Firstly, the vapor pressure of NADESs, which is virtually zero, means that the classical evaporation of the extraction solvent required to recover a compound(s) of interest is not feasible. On the other hand, their non-volatility can be an advantage if using the NADESs extract as such, e.g., as a food additive or in cosmetic formulations. In the cosmetic industry, NADESs have been applied to extract epigallocatechin-3-gallate (EGCG), a potent antioxidant, from green tea (*Camellia sinensis*) using ultrasound-assisted extraction, and a NADESs composed of betaine-glycerol-glucose (4:20:1) was selected, among other, for its non-toxicity and low-cost. The extraction yield was significantly higher than in classical extraction methods, with the additional advantage of an increased stability of EGCG over at least a three-week storage period (Jeong et al. 2017).

A very different application of NADESs was the removal of cadmium (Cd) from rice flour using choline chloride-tartaric acid-water (1:1:2) and choline chloride-xylitol-water (5:2:5). The removal of 51 - 96% of the Cd was achieved and the addition of 1% (w/w) of a surfactant saponin aqueous solution to the mixture increased this to 99% of the Cd without affecting the chemical composition of the flour (Huang et al. 2018).

The NADESs composed of choline chloride, betaine, glycerol, sucrose, malic acid, glucose, and urea were employed in food-grade synthesis of Maillard-type taste enhancers to reduce the use of salt, sugar, and monosodium glutamate in food. Kranz and Hofmann (2018) demonstrated that NADESs had the ability to increase the yield of the taste enhancers 1-deoxy-D-fructosyl-*N*-β-alanyl-l-histidine (49%), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene) amino propionic acid (54%) and N^2-(1-carboxyethyl) guanosine 5′-monophosphate (22%), as compared to those obtained with an aqueous buffered solution, at temperatures between 80–100 °C within a two-hour reaction period. Hence NADESs may open a new direction in culinary chemistry. It might be of

interest to study the commonly applied glazing in cooking with the NADESs concept in mind.

Reading all the application papers, it is remarkable that most studies follow a trial-and-error approach when developing an application. Clearly there is still a lack of a theoretical background that would allow the prediction of which NADESs is the best for a certain application. In fact, we have observed that the solubility of a compound is not always the same as its extractability from plant material. For example, in the extraction of vanillin from vanilla pods the NADESs that showed highest yields of vanillin did not fully coincide with those that showed the highest solubility of vanillin (Gonzalez et al. 2018). It thus appears that the matrix plays a role. It is indisputable that hydrogen-bonding is the key factor in the formation of NADESs, and possibly also in its dissolving power of several compounds. Also, the role of water is interesting (Dai et al. 2015), as the addition of even small amounts produces a substantial decrease in the viscosity of the extract, but also affects the solubility of compounds in an unpredictable manner. The percentage of water in a NADESs is thus an important variable that needs to be optimized for any application.

NADESs in extraction and solubilization of macromolecules

Among the challenges faced when developing novel sustainable industrial processes, the solubilization of macromolecules is a priority. Macromolecules, such as DNA, RNA, proteins, polysaccharides and lignin, have diverse basic functions in living organisms. They differ clearly in their solubility, and particularly most polysaccharides and lignin are known to be very poorly water soluble. However, in nature these polymers must have been biosynthesized in a solution. When the NADESs concept was introduced, their possible functions in plant physiology resulting from their solubilization of macromolecules were considered. For example, in the dormancy period of seeds, NADESs could be the storage media that prevents the denaturation of proteins, DNA or RNA until germination (Choi et al. 2011).

Certain points in the physiology of the biosynthesis of polysaccharides are still unclear, particularly the chain-elongation to obtain polysaccharides must occur in a dissolved state of substrate and product before crystallization or precipitation of the end product. A NADES might be the solvent that contains the enzymes and precursors needed for the elongation. Changes in the NADESs composition, e.g. change in water content, could end the biosynthesis.

Natural polymers

The use of ILs and DES for the dissolution of natural polymers such as polysaccharides and lignin has been studied extensively. Lignin and cellulose are a source of renewable organic polymers (Financie et al. 2016). Particularly cellulose has been the subject of many studies as it is the most abundant polymer on earth. Its application, e.g. for bioethanol production, as an industrial commodity is hampered by its insolubility in water and in most other common industrial solvents.

Solubilization in ILS and DES has thus been the subject of a number of studies. It was found that cellulose could be dissolved in the synthetic IL, 1-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazolium chloride without any derivatization. Cellulose then could be precipitated by the addition of water, ethanol, or acetone (Zhu et al. 2006). Another abundant natural polymer, lignin, is presently extracted industrially from lignocellulose in an environmentally unfriendly complex process. With the purpose of improving this process, 1-ethyl-3-methylimidazolium acetate was applied as a pretreatment solvent to provide suitable conditions for lignin degradation. The cellulose of the pretreated wood flour becomes far less crystalline without undergoing solubilization and enhanced lignin extraction (Lee et al. 2009). Another example is the pretreatment of oil palm frond biomass with a hydrophilic IL, 1-ethyl-3-methylimidazolium diethyl phosphate, which is known to solubilize lignocellulosic biomass, which resulted in an increase in the following enzymatic delignification of the biomass. The treatment lowers the lignin

content from 24.0 % to 8.5 % (w/w) (Financie et al. 2016). A series of choline based ILs was applied in pretreatment of grass lignocelluloses and eucalyptus. The treatment resulted in a significant increase in glucose yield. Choline argininate showed excellent recyclability with a total recovery as high as 75% after reuse for 8 cycles. Furthermore, rice straw pretreated by the recycled ILs remained highly digestible, and 63-75% of good glucose yields were achieved after its enzymatic hydrolysis (An et al. 2015).

The NADESs combinations, lactic acid-choline chloride and lactic acid-betaine were shown to extract lignin of high purity (>90%) and a high yield, achieving a 60 ± 5% (w/w) separation of the biomass. The addition of 5.0% (v/v) water significantly enhanced total lignin extraction as shown by HPLC and FTIR (Kumar et al. 2016).

DNA and RNA

Several NADESs have been tested to dissolve nucleosides. The formation of at least 4 different secondary structures of nucleic acids was reported by Mamajanov et al. (2010) in a water-free DES consisting of the choline chloride-urea mixture (1:2, mol/mol). Studying the nucleic acids revealed sequence-related differences in the stability and type of secondary structure formed. These findings seem to open the way to applications of catalytic nucleic acid activities and of enzyme–nucleic acid complexes. Zhang et al. (2017) optimized the extraction of RNA in an aqueous biphase system in combination with a DES consisting of PEG and a quaternary ammonium salt. High recoveries could be obtained when a low concentration of low molecular weight PEG was used in combination with longer alkyl chain (C4) quaternary amines, and more hydrophobic inorganic salts. Mondal et al. (2013) demonstrated the high solubility and stability of DNA in the NADESs composed of choline chloride with glycerol and ethylene glycol.

A number of experiments have been performed by our group, the results of some of which will be summarized here. The stability of DNA and RNA was

tested by storing dead fruit flies (*Drosophila melanogaster*) in five different NADESs which were selected on the basis of major ingredients in cells (see Table 3.).

Table 3. Stability of DNA and RNA in dead fruit flies (*Drosophila melanogaster*), intact or as dried powder in NADESs and controls, stored for 12 months in ambient temperature, in the light, PCR analysis after 12 months

Solvent	Preserved	
	Whole fly	Dried powdered fly
N1 (sucrose-choline chloride-water, 1:4:4, mol/mol)	+	+
N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol)	+	+
20% water	+	+
30% water	+	+
40% water	+	+
N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water, 1:2:3:5, mol/mol)	-	-
N4 (malic acid-choline chloride-water, 1:1:5, mol/mol)	-	-
N5 (sucrose-betaine-water, 1:2:5, mol/mol)	+	
Control 1 (70% ethanol)	+	+
Control 2 (water)	-	-

From the results it is clear that NADESs N1 and N2 are preserving the DNA and RNA in the original sample at least over a period of one year stored in the laboratory in the light, without any precautions to avoid decomposition. The major advantage of the storage in NADESs would be the absence of evaporation of the preserving solvent, as could happen with ethanol for example. The DNA and RNA dissolved in NADESs could be used as such for PCR analysis.

Most conventional DNA isolation protocols involve its dissolution in a buffer and storage at -20 or -80°C for preservation. Therefore, the NADESs were further tested for the stabilizing effect on DNA and RNA extracts. After

isolation of DNA using a conventional extraction, the extract was dissolved in the NADESs and left for three days in the light at room temperature. The DNA did not show any degradation in N1, N2 and N5.

In the case of long-term storage of RNA, the N5 solution of RNA proved to be the most stable after six months of storage in the light at room temperature. These preliminary results revealed the great potential of NADESs as a storage media of DNA and RNA samples. Further experiments on the stability under diverse conditions are needed to determine the long-term stability and validate NADESs as a storage media for DNA and RNA in different concentrations, i.e., in biological samples or DNA and RNA extracts.

Proteins

NADESs have been employed for solubilizing or stabilizing proteins. For example, the determination of gluten, which is a water-insoluble allergenic protein from wheat, barley, and oats, was achieved by extraction with dilute fructose-citric acid followed by analysis with ELISA. Apart from solubilizing the gluten, NADESs prevented its oxidation (Lores et al. 2017).

Li et al. (2016) used a betaine-urea NADESs in combination with an aqueous two-phase system for the extraction of proteins from calf blood sample. Betaine-urea exhibited the most suitable extractive properties with a yield of 99.8% under optimum conditions. Analysis with UV–Vis, FT-IR spectrometry and circular dichroism (CD) confirmed that the conformation of the proteins was unaltered during the extraction.

The preservation of proteins in ILs, DES and NADESs has been reported, as well as the use of these media for enzymatic reactions. In the case of plant enzymes, it was shown that laccase was easily solubilized in NADESs but was inactive. However, laccase was activated upon the addition of water (50% w/w), indirectly providing evidence, for example, of its possible role in the preservation of enzymes in NADESs in seeds of resurrection plants (Choi et al. 2011). Carnivorous plants obtain their nutrients from the digestion of trapped

insects and small animals. Proteolytic enzymes digest the prey and the resulting nutrients are absorbed by the plant. For example, *Drosera* species produce proteases in the glandular hairs of their tentacles to digest their prey and a sticky mucilage to capture them. This viscous liquid contains sugars and organic acids as their main components (Gowda et al. 1983). Analysis of the exudate of seven species of *Drosera* sp. using ^{1}H NMR spectroscopy, showed that the exudates were composed of acetoacetic acid, acetic acid, citric acid, ethanol, lactic acid, myo-inositol, glucose, sucrose, and malic acid. All these compounds are candidate constituents of NADESs that together with the enzymes attach to the prey and dissolve it, while simultaneously stabilizing the enzymes that have to be preserved for many days in the light and at high temperatures.

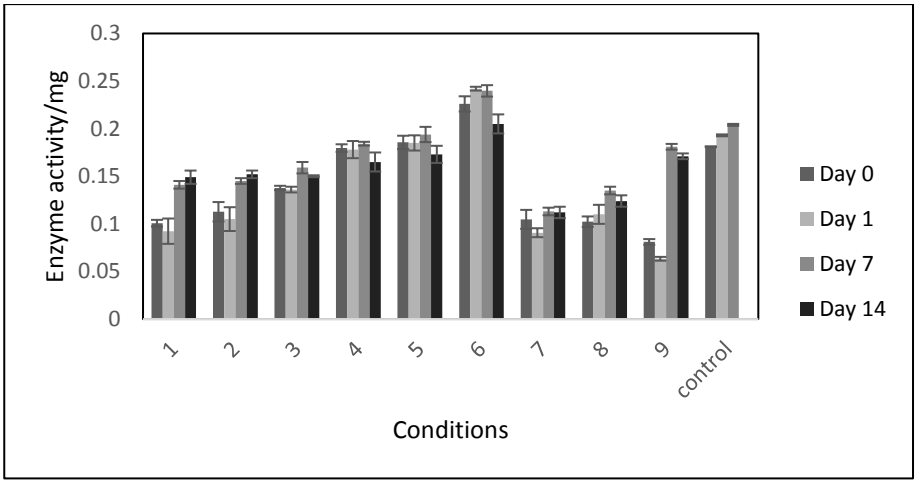

Figure 2. The effect of water on the preservation of protease activity in three NADESs and a water control. 1-3: (mannose-glucose-choline chloride, 1:2:1, mol/mol), modified with 30%, 40%, 50% water (w/w), 4-6: (fructose-glucose-sucrose, 1:1:1, mol/mol), modified with 30%, 40%, 50% water (w/w), 7-9: (sucrose-betaine, 1:2, mol/mol) modified with 30%, 40%, 50% water (w/w).

The stability of a protease from *Streptomyces griseus* has been tested in several NADESs: mannose-glucose-choline chloride, 1:2:1, mol/mol, fructose-sucrose-glucose, 1:1:1, mol/mol, sucrose-betaine, 1:2 mol/mol) with different concentrations of water (30%, 40%, and 50%, w/w). The protease activity did

not show any significant change after two weeks of storage at room temperature. Interestingly there were significant differences in activity for the enzyme in different NADESs (Figure 2).

NADESs applications in enzyme reactions

Many secondary metabolites are poorly soluble in water and this also applies to their biosynthetic precursors. Many biosynthetic pathways involve different cellular compartments and even different cells. The intra- and intercellular transport of intermediates or precursors is difficult to explain for non-water soluble compounds. Solubilization in NADESs of poorly water-soluble compound metabolons in a cytosolic pathway could provide an explanation. A metabolon is thought to be an aggregate of enzymes that can catalyze different steps in a biosynthetic pathway (Jørgensen et al. 2005). Our hypothesis proposes NADESs to be third liquid phases in cells. Furthermore, NADESs might be part of a metabolon as a factor that binds the enzymes together and form an intracellular liquid phase as a compartment where both enzymes and precursors are dissolved. In this case, poorly water-soluble compounds would be absorbed by such a metabolon. To prove the *in-situ* presence of such a third liquid phase in cells is difficult, as it might be a dynamic metastable system based on strong hydrogen bond formation in which a NADES is present in the ER, vesicles or cell membranes. This liquid phase would be in dynamic equilibrium with the water phase. The ^{1}HNMR spectra of NADESs with increasing amounts of water added, show that the signals related to hydrogen bond formation are still involved in such binding.

Based on this hypothesis NADESs would be of interest as media for (bio)synthetic reactions. Indeed, several studies already reported the occurrence of enzymatic reactions in NADESs (Table 4) which supports the hypothesis. Choi et al. (2011) showed that laccase is stabilized by dissolving in certain NADESs and that after adding water to more than 50% the enzyme becomes active. This supports the possible role of NADESs as a storage media for

proteins in nature.

Another clear support for the hypothesis is the study by Milano *et al.* that provides information about the application of choline chloride -based NADESs to photosynthetic enzymes known as reaction centers (RC). This complex membrane protein extracted from *Rhodobacter sphaeroides* bacteria was used as a model system. The results of ATR-FTIR spectroscopy showed that a complex membrane protein such as RC can work properly in a eutectic mixture (Milano et al. 2017).

Table 4. Application of NADESs in enzymatic reactions

NADESs (mole ratio)	Enzyme	Substrate	Reference
Malic acid-choline chloride (1:1)	Laccase		Choi et al. (2011)
Lactic acid-choline chloride (5:1)	Cellulase	Lignocellulose	Kumar et al. (2016)
Choline chloride–tartaric acid (1:1) Choline chloride–sorbitol (5:2) Choline chloride–lactose (4:1)	Whole cell *Lysinibacillus fusiformis*	Isoeugenol	Yang et al. (2017)
Choline chloride based NADES	Reaction centers from *Rhodobacter sphaeroides*	Quinone	Milano et al. (2017)
Glycerol-choline chloride (2:1) Glycerol-betaine (2:1)	Chondroitinases ABCI	Chondroitin-4-sulfate	Daneshjou et al. (2017)
Mannose-glucose-choline chloride (1:2:1) Fructose:sucrose:glucose (1:1:1) Sucrose-betaine (1:2)	Protease	BSA	-
Mannose-glucose-choline chloride (1:2:1) Fructose:sucrose:glucose (1:1:1) Sucrose-betaine (1:2)	Bromelain	BSA	-

The stability of enzymes can be improved with NADESs as reported by Daneshjou et al. (2017). This group demonstrated the capacity of NADESs composed of glycerol-choline chloride (2:1 mol/mol) and glycerol-betaine (2:1, mol/mol) to stabilize the chondroitinase ABCI, a clinical enzyme for treating spinal lesions. The stability of this enzyme was higher in NADESs than an aqueous buffer at −20.4 and 37 °C exhibiting activity levels of approximately 82% of the initial activity after 120 min. After 15 days, the enzyme still retained about 80% of its initial activity, but in absence of NADESs (aqueous buffer), total activity was lost after five days. Yang *et al.* demonstrated the catalytic power of NADESs as cosolvents in whole-cell biocatalysis of the reaction of isoeugenol to vanillin in *Lysinibacillus fusiformis.* NADESs enhanced the cellular membrane permeability of the bacteria (Yang et al. 2017).

Pharmaceutical applications

One of the major problems in drug development in the pharmaceutical field is the solubilization of drugs in appropriate doses in safe, non-toxic, compatible media. Considering that NADES are able to solubilize poorly water-soluble compounds (Choi et al. 2011) the idea of developing novel formulations using NADESs as solubility-enhancing excipients seems very promising.

A study by Liu et al. (2018) demonstrated the use of NADESs composed of mannose-dimethylurea-water to solubilize lipophilic molecules and load these into a hydrogel made of sodium alginate. The spontaneous separation of the hydrogel and the NADESs allowed the loaded hydrogels to be administered successfully and the lipophilic metabolites released from the hydrogel in the GI tract were readily absorbed. The delivery mechanism developed in this study could well be present in Nature, as a way of potentially improving the bioavailability of lipophilic metabolites, for example, those involved in the resistance of plants against pests and diseases.

Table 5. Application of NADESs in Pharmaceuticals

NADESs (mole ratio)	Target compounds	Result	Reference
Proline-glutamic acid (2:1) Proline-choline chloride (1:1)	Rutin	Improved solubility and bioavailability	Faggian et al. (2016)
Proline-malic acid (1:2) Proline-urea (2:1) Lactic acid-proline-malic acid-water (0.3:1:0.2:0.5)	Berberine	Improved solubility and bioavailability	Sut et al. (2017)
Mannose-dimethylurea-water (2:5:5)	Fruit extract of *Schisandra chinensis*	Improved bioavailability of gomisin A, gomisin J, and angeloylgomisin H	Liu et al. (2018)
Poly-ϵ-caprolactone polymeric blend (SPCL 30:70)	Menthol-ibuprofen (3:1)	Faster release profile	Aroso et al. (2015)
Choline chloride – oxalic acid (1:1)	Grape skin phenolic compounds	Antioxidant, antitumor	Bubalo et al. (2016)
1,2-propanediol–choline chloride – water (1:1:1)	Salsalate	Inducer brown adipose tissue (BAT)	Rozema et al. (2015)
Choline chloride–malic acid (1:1)	Grape skin extract	Antioxidant, antitumor,	Radošević et al. (2016)

A study conducted by Aroso et al. (2015) showed that bioactive or pharmaceutical ingredients themselves can form a NADESs. A starch- poly-ϵ-caprolactone polymeric blend (SPCL 30:70) with menthol-ibuprofen (3:1) in different ratios (10 and 20 %, w/w), with supercritical fluid sintering at 20 MPa and 50 ^{o}C gave a matrix that showed a faster release profile of the drug, mostly by diffusion. Another study showed that the solubility and bioavailability of both rutin and berberine were increased significantly when these compounds were administered in a NADESs, as well as their levels and permanence in plasma (Faggian et al. 2016; Sut et al. 2017), thus achieving the same

pharmacological effect with a lower dose as a result of the synergy with the NADESs.

Applications of NADESs in diverse fields have been reviewed by our group (Vanda et al. 2018) and some of the most representative applications are summarized in Table 4- 5.

Toxicity of NADESs

The assessment of toxicity is a must when developing applications for human uses. Many NADESs have already been tested for their cytotoxicity. Mbous et al. (2017) compared the cytotoxic profiles of choline chloride-glucose (2:1, mol/mol) and choline chloride-fructose (2:1, mol/mol) and the DES, *N*,*N*-diethyl ethanol ammonium chloride-triethyleneglycol (1:3, mol/mol). The cytotoxicity was tested on HelaS3, PC3, A375, AGS, MCF-7, and WRL-68 hepatic cell lines. The EC_{50} of the tested NADESs ranged from 98 to 516 mM, while the toxic profile of DES was clearly higher (34-120 mM). A glucose-based NADESs was found to be less toxic than a fructose-based NADESs due to their different metabolic pathways. Cytotoxicity of choline chloride-based NADESs was also assessed by Hayyan et al. (2013). Their antimicrobial effect was assessed using two Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* and their cytotoxicity was evaluated with the Brine shrimp (*Artemisia salina*) test. No growth inhibition of the bacteria was observed, showing that the tested NADESs had neither toxic nor beneficial effects on these bacteria. However, they exhibited a toxic effect on brine shrimp larva. The authors considered that the observed effect should be further studied for its potential in chemotherapy. Another experiment conducted by Paiva et al. (2014) on cytotoxicity of several NADESs suggested that while choline-based NADESs are non-cytotoxic, the presence of tartaric acid affected the metabolic activity of L929 fibroblast-like cells when NADESs was administered at a concentration of 25 mg/mL.

Perspective of NADESs

In the past years, NADESs have become a hot topic in cosmetics as their use as extraction solvents opens the options to an impressive array of novel types of extracts with improved bioactivities and interesting properties for their formulation in products for skin care. It is anticipated that many new applications of NADESs in cosmetics will be developed in the near future.

Another area of application is food, particularly in flavors and fragrances, given their generally poor water solubility. NADESs may be used to obtain flavorings and aromatic extracts to be used as food additives. A major advantage for both cosmetic and food applications is the increased stability of the compounds present in the NADESs extracts that could eventually avoid the need of preservatives.

Pharmaceutical applications are not yet common, though the increase in bioavailability of drugs in NADESs has been proven and merits further research. The increased solubility of drugs in NADESs can allow the extension of the range of dose-response curves for bioassays. This could also provide a basis for the development of novel formulations including NADESs as vehicles of poorly water-soluble drugs. All these applications are directed at small molecules with interesting properties that are difficult to solubilize.

The hypothesis that NADESs are important in a variety of processes in cells and organisms is a source of inspiration to explore possible applications. Among these, their great solubilization power of macromolecules appears to be one of their most attractive features. In the first place, NADESs could play a role in nature as media for various biosynthetic reaction steps that involve water insoluble precursors. This implies that NADESs could be applied in processes involving bioconversions with diverse enzymes. As mentioned, the feasibility of several enzymatic reactions in synthetic ILs has been proved. In our experience, NADESs has shown the capacity of stabilizing enzymes that can be activated by the addition of water (e.g. 50%). Further studies in this direction

would be of interest as with much higher levels of precursors in solution the enzyme kinetics would be different.

The dissolution of DNA and RNA in NADESs has been described (Choi et al. 2011), though the stability of the resulting solutions differs considerably according to the NADESs. In some cases, stability is affected negatively while in others the stability is enhanced. Thus, further research is needed to better understand the possible relationship between RNA and DNA and NADESs in the cells. At present one may predict that the use of NADESs to store DNA and RNA samples at room temperature could be feasible. In terms of biological applications, cryopreservation seems an interesting area for NADESs, as commonly used cryoprotectants (sugars like trehalose, sugar alcohols like mannitol and proline) can actually constitute a NADESs.

Regarding the hypothesis that proposes NADESs as a third media in living cells, the evidence is so far only indirect, and its *in-situ* demonstration is a major challenge, the more so as one may expect the presence of different NADESs in different tissues and in different cellular compartments.

A major aspect of NADESs is their physical chemistry, and to date, in-depth studies on the interactions between NADESs and solutes are lacking. As a result, most investigations related to applications are based on a trial and error approach. Based on sound predictions of their behavior obtained from the physicochemical data provided by such studies, it should be possible to build models simulating the role of NADESs in biological systems and select appropriate combinations according to the application.

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