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Preface: Natural deep eutectic solvents: A third liquid phase in living organisms? Discovery, theory, biology, and applications

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1. Discovery

About 20 years ago the concept of metabolomics was introduced with the aim of qualitative and quantitative chemical analysis of all compounds present in an organism or any biological material. This approach should ideally give a total picture of all small molecules in an organism, and complement the data obtained by proteomics, transcriptomics, and genomics. The methods applied are mass spectroscopy (MS) and nuclear magnetic resonance-spectroscopy (NMR), either stand alone or coupled with a chromatographic system (LC-MS, GC-MS, and LC-NMR) (Schripsema & Verpoorte, 1991; Verpoorte, Choi, & Kim, 2007; Wolfender, Rudaz, Choi, & Kim, 2013). These methods are capable of analyzing complex mixtures, and in combination with various chemometric methods new insights can be obtained from the large data sets generated in various experiments. The omics were the beginning of a paradigm shift of doing research, moving from a hypothesis-based strategy to a systems biology approach. The latter is based on as many observations as possible and analyzing the huge data sets by chemometric methods, e.g., to identify which genes, proteins, and compounds correlate with the resistance of a plant against pests and diseases (Kim, Choi, & Verpoorte, 2010; Leiss, Choi, Verpoorte, & Klinkhamer, 2011; Mouden, Klinkhamer, Choi, & Leiss, 2017).

A major advantage of ¹HNMR-based metabolomics is that the integral of a proton signal is proportional to the molar concentration of the molecule it is part of and can be directly compared with internal standard. This advantage makes the quantitation of NMR metabolomics data much easier as all

the compounds can be compared with each other. In our research using NMR-based metabolomics, we have built up a library with more than 25,000 plant extracts and including also some other organisms like marine organisms, microorganisms, fish, and mammalian cells. Also the spectra of some 2000 pure plant compounds are in our database. In all the spectra of biological materials we find that sugars, some amino acids, and organic bases and acids are present in relatively high levels compared to other common primary and secondary metabolites. Even, some of these abundant compounds keep their molar ratio constant to certain other compounds. This raised the question about the role of these compounds for cells and organisms. That was the point that we asked ourselves if the common acids like malic acid and citric acid might be forming ionic liquids (ILs) with the common bases like choline and betaine. The first experiment was made with malic acid and choline chloride, and indeed these two solid compounds did form an IL. A whole series of ILs was then made from compounds we identified by the NMR-based metabolomics in the biological materials. We then came across the work of [Abbott, Boothby, Capper, Davies, and Rasheed \(2004\)](#) who described a series of ILs as deep eutectic solvents. We continued making other combinations of common primary metabolites found in the biological samples, such as sugars, sugar alcohols, amino acids, organic bases, and organic acids. Many of these mixtures of solids indeed showed a lowering of the melting point to a temperature below room temperature ([Choi et al., 2011](#); [Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013](#)). Because these liquids turned out to be good solvents for various poorly water-soluble natural products, we named these mixtures “natural deep eutectic solvents” (NADES). The NMR-based metabolomics showed that in all organisms certain ingredients for NADES are present as major compounds, often keeping a constant molar ratio to some others. This brought us to the hypothesis that “Everywhere in living systems Natural Deep Eutectic Solvents (NADES) occur and form a third liquid phase of intermediate polarity” ([Choi et al., 2011](#)). This hypothesis can explain many biological phenomena, for example, biosynthesis, including transport and storage of nonwater-soluble compounds, like terpenoids and polysaccharides ([Chapters 4, 5, 8](#)); how seeds can survive long periods of drought and cold ([Solberg, Brodal, et al., 2020](#); [Solberg, Yndgaard, et al., 2020](#); [Chapter 15](#)); how resurrection plants may survive in the desert ([Chen, Jung, Giarola, & Bartels, 2020](#); [Chapter 9](#)); and how cacti might be able to catch water from the atmosphere ([Chapter 7](#)).



2. Basics

The past decade showed an explosion in the number of studies on NADES, particularly for the various potential applications, such as a green extraction solvent and as solvent for formulations of poorly water-soluble compounds in cosmetics and medicines (Chapters 11–14). On the theoretical aspects new insights have been reported (Chapters 1–6). These applications concern hydrophilic NADES that are miscible with water. Hydrophobic eutectic mixtures are already known since long (Vanstone, 1909), e.g., camphor–borneol that occurred in old pharmacopeias. In the past years these were revisited (Chapter 2; Martins et al., 2019; Van Osch, Dietz, Warrag, & Kroon, 2020), which raised interest in further studies.

The hydrophilic NADES which are discussed in this book can be divided into five classes (Scheme 1). For finding the optimal solvent for a given compound, two approaches are used, one is modeling and the other is testing. Both are needed, but optimization for a single compound requires addressing both high selectivity and high yield, so sometimes a compromise is needed. The extraction of a plant material in which several compounds are important for the quality also might need a compromise as the most suited solvent. Measuring the solubility of a pure compound for finding the optimal extraction solvent does not necessarily result in the solvent that gives the highest yield. Apparently, liberating the desired molecules from the plant

	Sugars e.g. sucrose, fructose, glucose, trehalose Polyalcohols e.g. inositol, mannitol, glycerol	Organic acids e.g. malate, citrate, succinate, lactate, phytate, malonate	Organic bases, e.g. Choline, betaine	Amino acids, e.g. proline, alanine
Ionic liquids: acid and base		X	X	
Neutral compounds only	X			
Neutral compounds with base	X		X	
Neutral compounds with acid	X	X		
Neutral compounds with amino acids	X			X

Scheme 1 Classes of Natural Deep Eutectic Solvents.

matrix is also an important parameter to be considered (Gonzalez, Mustafa, Wilson, Verpoorte, & Choi, 2018). Concerning the modeling, the major problem is that there is an almost infinite number of combinations possible to make NADES, when considering the number of ingredients for making a NADES consisting of two or three components and having the water content as a further variable. In Chapter 1, some examples of a rational approach for optimization of extractions are discussed. Obviously, the field of molecular modeling for selecting the best solvent for a certain application should be developed further.

The NADES are more like a concept for a dynamic multiphase system in cells, than a well-defined static eutectic mixture. The NADES are miscible with water, so in nature they may occur with different percentages of water, which, e.g., makes a difference for the activity of enzymes and for the solubility of various precursors and products (Chapters 5, 6). The biological challenge is to prove the *in situ* presence of NADES in living cells and living organisms, which means that one should identify and quantify the NADES molecules through time and in space. In a static system, one can measure all kind of parameters, but in a dynamic system that is complicated. Understanding such dynamic systems is the other physicochemical challenge for the coming years.



3. Biology

Through the systems biology approach the various omics methods generate a lot of information on the DNA, RNA, proteins, metabolites, and various high-molecular-weight macromolecules such as starch, cellulose, and lignin. In fact, the omics allow the identification, characterization, and quantitation of all molecules in a cell. However, so far the efforts failed to make a synthetic cell out of these components. In fact, it is like a four-dimensional jigsaw puzzle (three dimensions of space and one dimension of time), we have all the pieces, but we have not been able yet to put all pieces into one model, though we know the cell morphology very well from direct observations. Cells are highly organized with a number of cell organelles with different functions. Compartments are shaped by cell membranes, which consist of small lipid molecules that may arrange themselves in liposomes in an aqueous environment. In searching the literature, one will find all kinds of transient cellular processes involving membranes. A large number of keywords is needed to find these, e.g., formation of vesicles, ER bodies,

exosomes, ectosomes, extracellular vesicles, particles without membranes, metabolons, lipid droplets, vacuolar droplets, cytoplasmic droplets, fibrils, anthocyanic vascular inclusions, biocondensates, nanobubbles, lipid rafts, phase separation, exclusion zones, and heterogeneous water structure. Obviously, many dynamic processes occur in the life cycle of a cell. In the past years, there have been quite some discussions about these particles and various processes leading to the different structures mentioned. NADES may play a role in these processes. Some of the research on NADES include various types of liposomes, i.e., working with in vitro models, (e.g., see [Chapters 2, 3, 6–8](#)). In vivo research is done with different plant species and different types of plant cells (e.g., [Ballesteros & Walters, 2019; Chapters 6, 8,10](#)). But it is obvious that particularly in this field NADES might play an essential role. Because of the strong hydrogen bond donor and hydrogen bond acceptor character of the NADES components, they should be able to interact with the polar head groups of the membranes as well as with macromolecules such as proteins and the cell wall's cellulose and lignin. Considering that in cells several metabolites are present that are candidates for the formation of a NADES, different NADES may be formed as a third liquid phase in different cellular compartments under different conditions. In case of the vacuolar membrane one may think of a sugar–malic acid based NADES on the inside vacuolar membrane, whereas the outside carries a positively charged NADES, like choline chloride–sugar.



4. Applications

The real in situ presence of a NADES in cells has not been demonstrated yet. Most studies concern the interactions of proteins with membranes, not considering any effect of the various ions and molecules present in cells as major components. At least it is clear that proteins could be dissolved and stored in pure NADES for prolonged periods, and by dilution with water, enzyme activities are restored ([Choi et al., 2011](#)). The much better solubility of various medium polar compounds in partly diluted NADES compared to pure water, or to pure NADES, improves the substrate availability for enzyme reactions and thus the production ([Chapters 4, 5](#)).

Though there is still much work to do to understand the NADES and their biological roles, since 2012 there has been an exponential growth of

the number of papers on applications of NADES. Most papers concern the use of NADES to extract various natural products from different materials. Particularly phenolic compounds have been subject of these studies. It was shown that NADES stabilizes such compounds (e.g., Dai, Verpoorte, & Choi, 2014). From the various studies it becomes clear that NADES are rather selective solvents; thus, for extracting highest amounts of certain compounds, one needs to develop a specific extraction protocol. The NADES have a number of advantages, a major one is their status as stable natural solvents. Moreover, they are nontoxic, which makes them suitable for various applications in agriculture, food, cosmetics, and medicines. However, their extremely low volatility is a disadvantage if one wants to extract a single compound, as a further step will be needed to isolate the desired compound from the NADES, e.g., by adding water as an antisolvent. But, on the other hand, this can be an advantage for the use of the extract itself, as the NADES extracts can be added to food, or be formulated in cosmetics or medicinal preparations (Chapters 11–14). The NADES can also be used to dissolve poorly water soluble medicines, including biologicals. Such NADES solutions, e.g., biologicals or small molecules, are more stable than solutions in alcohol, and can also be applied as such as they are nontoxic. The dissolved medicine may have a higher bioavailability (Chapters 11, 14). The NADES itself may be used as medicine, e.g., for wound healing (Chapters 11, 14). In line with the potential biological role of NADES in plant, cold resistance is the application of NADES in cryopreservation (Chapter 15).



5. Perspectives

The hypotheses on the potential roles of the NADES are based on a concept of a dynamic liquid system, rather than seeing NADES as a completely defined, static, *in vitro* deep eutectic mixture of two or three pure common primary metabolites. That two or more natural compounds can give a NADES shows that these compounds are eager to form hydrogen bonds. That characteristic seems to be used by Nature in many different ways. First of all, of course, the NADES itself, but also in interaction with other molecules, and in particular the small molecules that act as hydrogen bond acceptors (HBA) and donors (HBD) can be seen as important versatile building blocks in the organization of the cellular infrastructure. They could function as a reversible cement or glue that can easily be reallocated as part of a response to always changing conditions. The fact that NADES ingredients

can be found in all organisms and that under various stress conditions the levels of some common NADES components increase shows that they play an important role in the cellular processes. Most evidence for the various hypothetical roles of NADES is still mostly indirect, i.e., the presence of the compounds was shown, but clear evidence for their direct interaction forming a NADES is still missing.

The quote “Learning from Nature, learning from our ancestors,” applies very well to NADES. For example, the nectar–honey connection has a number of biological implications, and a number of applications were discovered by our ancestors ([Chapter 14](#)). Applications in cosmetics have already been developed. Pharmaceutical formulations for poorly water-soluble drugs and biologicals seem to be an interesting field for applications. NADES may also be useful for coatings of fruits to increase their shelf life. Seed and bulb coatings with NADES may improve their quality in terms of viability and protect them from microbial infections.

Understanding the NADES and their interaction with membranes, macromolecules such as proteins, lignin, polysaccharides, metabolites, and various ions will be instrumental for finding novel applications. The tools that will be developed for the basic physicochemical research can be applied in studying the possible biological roles of the NADES in, e.g., drought and cold resistance. The viability of seeds, spores, resurrection plants, and lichen would be interesting models for studying for possible biological roles of NADES. Obviously, much more research is needed to get insight in the various mentioned biological aspects of the NADES. Particularly, multidisciplinary collaborations are needed. Advanced microscopic methods are required to enable in situ chemical analysis to show the co-occurrence of the NADES ingredients in cells. Physical and chemical modeling of interactions between NADES, proteins, and lipids (membranes) requires both experimental and computational approaches. Chemistry and biochemistry are needed to identify and quantify potential NADES-forming molecules. In a systems biology approach, these disciplines may shape a model for the cellular fluid dynamics in which liposomes and an aqueous phase interact with the various hydrogen bond donors and acceptors. Such a model should explain the dynamic compartmentalized structures of living organisms and cells that are able to respond to various cues from the environment. For understanding the biological role of NADES, the major challenge will be studies on nanoscale of the dynamics of the metabolome, ionome, lipidome, proteome, and transcriptome in living cells.

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