Natural deep eutectic solvents and their application in natural product research and development

Dai, Y.

Chapter 8

Natural deep eutectic solvents in plants and plant cells: *in-vitro* evidence for their possible functions

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Abstract

The components of natural deep eutectic solvents (NADES) are abundant in plants. This led to our hypothesis that NADES may also exist in plant cells playing an important role in solubilizing, storing, and transporting poorly water-soluble metabolites in living cells, adjusting the water content of plants, and protecting cells when in harsh conditions. In order to test this, diverse plant materials were analyzed, including leaves, petals, plant secretions and seeds. Comparatively high amounts of ingredients of NADES are observed in plants. Dry mosses contain a higher amount of NADES components than fresh ones and the level of NADES components is higher in the outside layer (aleurone and seed cover) of barley, than in the inside (endosperm and embryo) layer. A high accumulation of sugars, sugar alcohols, amines, amino acids, organic acids, choline, and betaine dominate plant secretions such as sap and nectar. Beside their similar compositions, NADES showed similar physicochemical properties to that reported for plant cytoplasm. This implies the existence of NADES in plants. Experimentally, NADES and water can be mixed resulting in liquids with different compositions and properties. In the case of plants, NADES and water co-exist in the cells and may form ideal solvents for metabolites of diverse polarities and macromolecules in plants. Ingredients of NADES, as can be found in plants, are hygroscopic, providing evidence for the water-adjusting effect of NADES in plants. Most importantly, NADES may accumulate around the lipid bilayers, form intermolecular bonds with the polar heads of lipids, and

stabilize the membrane, as revealed in experiments with liposomes. This study gives *in vitro* evidence for the different roles NADES may play in living organisms, and opens perspectives for further exploring the existence and functions of NADES in plants cells.

Key words: natural deep eutectic solvents (NADES); metabolites; physicochemical properties; diffusion; hygroscopicity; water adjusting effect; membrane stabilizing effect; liposome

1. Introduction

Natural deep eutectic solvents (NADES) were proposed by our group with the purpose of extending the range of ionic liquids (ILs) and deep eutectic solvents (DES) and explore their applications in health related fields, (Choi *et al.*, 2011; Dai *et al.*, 2013). NADES are liquid supramolecules composed of natural compounds in certain molar ratios bonded by intermolecular interactions, particularly H-bonding (Choi *et al.*, 2011). The components of NADES are metabolites that are common in high amounts in living cells (sugars, sugar alcohols, organic acids, amino acids, amines) as well as water, that can also be an ingredient of NADES. NADES possess excellent properties as solvents (Dai *et al.*, 2013), e.g., negligible volatility, a very low melting point (they are liquid even below -20 ^{o}C), a broad polarity range and high solubilization power of a wide range of compounds, especially poorly water-soluble compounds (Choi *et al.*, 2011; Dai *et al.*, 2013). The high solubility of scarcely water-soluble metabolites and macromolecules (e.g. DNA, proteins, and cellulose) has been demonstrated as well as their suitability as (Mamajanov *et al.*, 2010; Dai *et al*., 2013) media for enzymatic reactions (Zhao *et al.*, 2011; Choi *et al*., 2011) and biotransformations (Gutiérrez *et al.*, 2010).

Drought, cold, or salinity tolerance has been found to be correlated with a high level of common cell metabolites such as sugars (sucrose, trehalose, raffinose), amino acids (proline), organic acids (ascorbic acid, abscisic acid), sugar alcohols (sorbitol, mannitol), glycine betaine, and choline (Bartels *et al*., 2005). The content of proline increased over 10 times to nearly the same molar concentrations as sucrose during the natural desiccation of the resurrection plant *Selaginella bryopteris* (Pandey *et al*., 2010). Accumulation of glucose, sucrose and amino acids in *Sporobolus staofianus* (Whittaker *et al*., 2007), and sucrose and raffinose in the resurrection plant, *Xerophyta viscosa* (Peters et al., 2007) were reported. Moreover, higher amounts of sugars (fructose, glucose, raffinose), proline, and galactinol were observed in *Arabidopsis* during cold acclimation (Kaplan *et al*., 2007). In fact, the commonly used cryoprotectants

for plants are sugars, sugar alcohols, and proline. Proline is important for the cold acclimation of plants as well as in other organisms (Kovács *et al*., 2011). All these metabolites that correlate with drought, cold, or salinity tolerance have been mentioned as compatible solutes for organisms, i.e. non-toxic molecules which do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentration under stress conditions (Bartels and Sunkar, 2005). They accumulate in the cytoplasm to a high concentration under osmotic stress (Yancey *et al*., 1982; Bartels and Sunkar, 2005). Sugars together with other compounds may form a glass-like matrix and prevent macromolecular denaturation and loss of membrane integrity during desiccation (Moore *et al*., 2009; Hoekstra *et al*., 2001). Concerning cold resistance, it was reported that a high level of proline causes the remaining unfrozen water to undergo a glass-like transition and thus prevent cryo-injury (Kovács *et al*., 2011). The so-called compatible compounds were postulated to play a major role in the above mentioned processes. In our view, these compatible substances might be part of NADES that are formed in various cellular compartments. The strong hydrogen bonding between these compounds being the crux to explain liquid crystal formation and retaining a certain amount of water as part of the NADES. The NADES components can form liquid crystals in aqueous environments and in that way lowers the sugar content in the water and thus control osmolarity. Such liquid crystals can dissolve and stabilize macromolecules and stabilize membranes. NADES thus may explain many of the questions raised above.

More detailed measurements are needed to explore and prove the existence of NADES and their functions in plants. In this study, different plant materials and secretions were analyzed to collect more evidence for the existence of NADES; the physicochemical properties of NADES were also compared with that of cytoplasm in plants. To explore the functions of NADES related to the hygroscopicity of cells, the water absorption and desorption of seeds was analyzed. The diffusion process between water and NADES was investigated to mimic the mechanism by which metabolites of different polarity are solubilized within the cells. In order to determine the effect of NADES on membranes, the behavior of liposome bilayers in NADES media was studied.

2. Experimental

2.1 Plant materials

A dry Mexican moss (*Selaginella pallescen*) was grown with roots in water, harvested after two days, and dried in a freeze-drier. *Arabidopsis* plants were provided by Dr. Jieun Shin (Plant Developmental Biology Department, Max Planck Institute, Germany). These plants were grown under long-day conditions for one month, after which the water feeding was stopped during two weeks,

and re-initiated for one week. The control and dried plants were then collected. Barley seeds (*Hordeum distichum* L.) were obtained from Prof. Dr. Bert van Duijn from Leiden University, The Netherlands. The barley seeds were allowed to absorb water between two layers of paper (Whatman) for one day at ambient conditions. Then, the seeds were cut into two parts of which the outside (aleurone and seed cover) and inside parts (endosperm and embryo) were separated (Schuurink *et al*., 1992). The dry flower buds of *Sophora japonica* were brought from the Kyung Dong Traditional Medicine Market (Seoul, Korea Korea). C*atharanthus roseus* with purple petals, belonging to the Pacifica Orchid Halo Varitey, was bought from a flower shop in Leiden. Saps of *Cleome hasselorana* and *Drosera* from different species (*Drocera adelae*, *D. capensis*, *D. mucipula*, *D. glabripes*, *D. glabripes*) were collected into microtubes for sample preparation from the Hortus Botanicus garden in Leiden, The Netherlands.

2.2 Chemicals and reagents

Sucrose, glucose, fructose, choline chloride, 1,2-propanediol, proline, and 5-doxyl stearic acid (5-DS) were purchased from Sigma (St. Louis, MO, USA). 1,2-dioleoyl-3 trimethylammonium-propane (chloride salt), soybean L-α phosphatidylcholine, and cholesterol were purchased from Avanti Polar lipids, Inc. (Alabaster, Alabama, USA).

2.3 Sample preparation

*2.3.1 Preparation of plant material***.** All the above collected materials (mosses, *Arabidopsis* plants and barley seeds) were ground to powder with liquid nitrogen, freeze dried, and extracted with 1 mL of 50% CH_3OH-d_4 in buffer (90 mM KH_2PO_4 in deuterium oxide) containing 0.05% TMSP (trimethylsilyl propionic acid sodium salt, w/v). The mixture was vortexed at room temperature for 30 s, ultrasonicated for 20 min, and centrifuged at 30,000 rpm at 4 ^oC for 20 min. An aliquot of 700 l of the supernatant was transferred to a NMR tube for NMR analysis. Nectar and sap were added to 1 mL phosphate buffer (KH_2PO_4, pH 6) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France), vortexed at room temperature for 30 s, centrifuged at 30,000 rpm at 4 ^oC for 20 min and the later steps are the same as above. For HR-MAS NMR analysis, the dry flower buds (50 mg) of *Sophora japonica* and the fresh petals (80 mg) of *C. roseus* with one drop of buffer containing 0.1% TSP (pH 7.0) were packed into a rotors equipped with close fitting and caps.

2.3.2 Preparation of NADES. Sucrose-choline chloride-water (1:4, molar ratio) (SuCH), glucose-choline chloride-water (1:2.5) (GCH), 1,2-propanediol-choline chloride-water (1:1:1) (PCH), Sucrose-malic acid-water (1:1) (SMH), Sucrose-proline-water (1:1) (SPH), and sucrose-fructose-glucose-water (1:1:1) (SFG) were prepared using the stirring method (Dai *et al*., 2013).

2.3.3 Preparation of liposomes. Liposomes were prepared by the film hydration-extrusion method employing a laboratory LiposoFast-Pneumatic extruder from (Avestin Inc., Ottawa, Canada). To prepare a 2 mL dispersion of liposomes, 7.8 mmol 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt), 3.9 mmol soybean L-*α*-phosphatidylcholine, and 3.9 mmol cholesterol were dissolved in 2 mL chloroform to form a lipid film using a rotary evaporator. The film was hydrated with 2 mL aqueous solution (PBS phosphate buffer, PBS phosphate buffer (pH 7.0) with 35.6% (w/w) SFG, PBS phosphate buffer with 19.6% (w/w) SMH, or PBS phosphate buffer with 25.2% (w/w) SPH) and the obtained suspension was extruded successively through filters with a pore size of 200 and 100 nm. The PBS phosphate buffer contains 0.81% sodium chloride, 7.1% disodium hydrogen phosphate, 0.02 % potassium dihydrogen phosphate and 0.019% potassium chloride with phosphate sodium water.

2.3.4 Liposomes Labeling. Membrane spin probe 5-doxyl stearic acid (5-DS) (150 μg) from a stock solution in ethanol at -20 ℃ was put into a microtube, the ethanol was evaporated by blowing nitrogen gas over the solution and then 0.133 mL prepared liposome suspension (5 mg/ml, 0.0078 M) (5-DS:lipid=1:100 (molar ratio)) was added and mixed. 5-doxyl stearic acid can be easily incorporated into liposome membranes due to its high lipid/water partition coefficient. Then the final suspension was filled in to a 50 μl capillary for electron paramagnetic resonance (EPR) measurement.

2.4 Diffusion experiments

Sucrose-choline chloride-water (10 mL), or PCH (10 mL) was placed in the lower part of a tube and then 10 mL water was gently added above the NADES. A natural dye, carthamin, was added in either the water or NADES part for observation. A clear interface was observed between NADES and water in each tube. The position of the interface was marked and photos were taken every 24 hours. On the 10th day, a third layer was formed between water and SuCH layers with an interface between water and the intermediate layer (the initial position, line 1 Fig. 3) and an interface between the NADES and the intermediate layer (line 2). Samples were collected from the upper and lower part of each layer into a microtube, weighed, and freeze dried for 3 days. Then the samples were weighed and 100 mg of the sample was transferred into another microtube, dissolved with 1 mL phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide and analyzed by ^1H NMR. The above experiments were performed in triplicate.

2.5 Hygroscopicity test

Glucose-choline chloride-water (GCH) was used for the hygroscopicity test at 25 ^oC. Initially the sample was dried in the Q5000 apparatus (TA-Instruments, New Castle, USA) at 60 ^oC. Then humidity levels of 0, 20, 40, 60, and 80% (and

then decreasing again from 80% to 0 with the same step) were programmed with 720 min for each step. Intact barley seeds (single piece) were used for the hygroscopicity test at 25 ^{o}C. During the first 20 hours the sample was dried in the Q5000 apparatus at 60 ^{o}C. After that a relative humidity of 80 % was applied for 84 hours. The relative amount of absorbed moisture M_t is defined as

$$M_t = \frac{m_t}{m_{dry}} = \frac{\text{mass of absorbed water}}{\text{mass of dry sample}} \qquad \text{Eq.1}$$

2.6 Instruments and measurements

*2.6.1 NMR spectroscopy***.** ^{1}H NMR spectra were recorded at 25 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) equipped with a TCI cryoprobe and Z-gradient system. All the parameters were the same as those described in our previous reports (Dai *et al*., 2013). ^{1}H NMR and 2D NOESY spectra of flower materials were recorded at 25 ^{o}C on a Bruker 400 MHz HR-MAS NMR spectrometer at a frequency of 3000 Hz.

2.6.2. Size and zeta potential measurements. The size and zeta potential of liposomes were measured on a Zeta sizer Nano-ZS 4800 (Malvern instruments Ltd., Malvern, UK). The average dynamic size was obtained with dynamic light scattering with the method of cumulants. *Zeta* potential measurements were carried out on a laser Doppler electrophoresis with a dip cell. Each measurement was repeated at least three times.

2.6.3 Electron paramagnetic resonance (EPR) spectroscopy. To monitor the structural interactions between NADES and liposomes, EPR was applied with the spin-labeled derivative of the stearic acids, 5-DS as an indicator. ESR spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker EMXplus, Rheinstetten, Germany). Microwave power was 10 mW, the modulation amplitude was 5 G and the scan range was 200 G. To increase signal/noise ratio, 24 scans were accumulated.

3. Results and discussion

3.1 Different compositions of NADES in plants

3.1.1 Plants in harsh conditions. Our former experiments showed that a comparatively high amount of the components of NADES were observed in desert plants of the *Selaginela* species. In this experiment ^{1}H NMR spectroscopy showed that a high amount of sugar (glucose), organic acids (succinic acid, caffeic acid, tartatic acid, acetate, *γ*-aminobutyric acid), and amino acids (alanine, threonine, arginine) were found in dry moss (*S. pallescen*) if compared with fresh moss. The level of the primary metabolites increased in the case of water shortage even for plants, such as *Arabidopsis* which showed

increased levels of sugar (sucrose), amino acids (proline, alanine, arginine), organic acids (succinic acid, fumaric acid, malic acid), and an amine (choline) in water depleted conditions if compared with normal growing conditions. So, a relatively high amount of components of NADES exists in plants under dry conditions. However, to find direct evidence of the existence of NADES in plants, in-situ analysis of plant materials would be required instead of the indirect evidence from the contents of extracts.

3.1.2 Petals containing pigments. In cells, NADES may dissolve and preserve the compounds responsible for color that are not soluble or stable in water since NADES showed a high solubilizing and stabilizing ability for phenolic compounds (Dai *et al*., chapter 4). The dry flower buds of *Sophora japonica* contain up to 40% rutin, which is not very soluble in water (Paniwnyk *et al*., 2001). The concentration of anthocyanins in vacuolar inclusions has been reported to be higher than that expected according to their solubility in water (Markham *et al*., 2000). To get an insight into what is present in the flower, flower petals were directly measured using HR-MAS NMR. HR-MAS NMR spectroscopy is a rapid, non-destructive method and tissue can be analyzed directly without extraction. We expected to observe the components of NADES, the interactions between their components, and even the interaction between NADES and the phenolics. The ^{1}H NMR spectrum of petals is dominated by sugars and organic acids (Fig. 1). However, no signal of color compounds was observed, although a high amount of rutin was detected in HPLC-DAD with the same material.

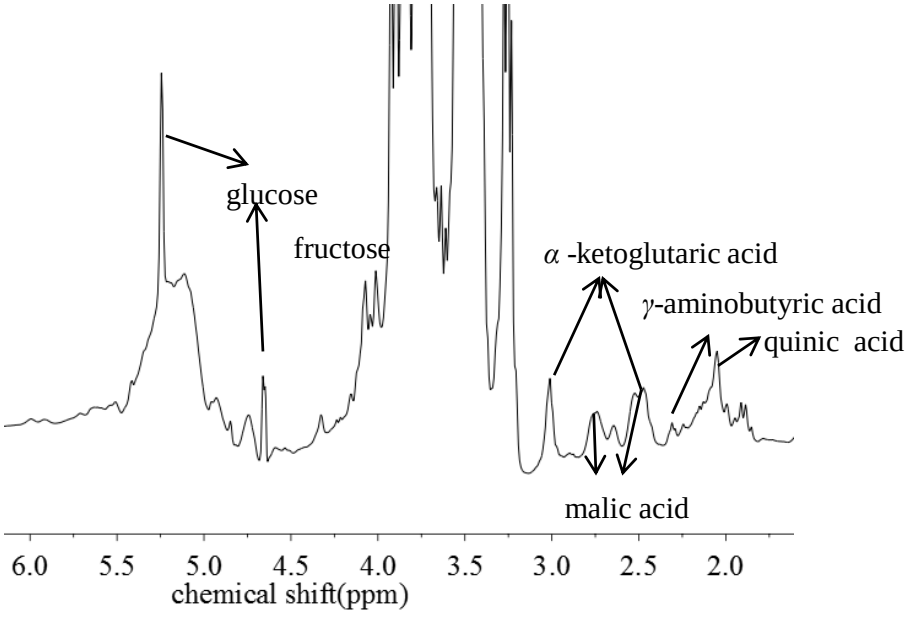

Fig. 1 ^{1}H NMR spectrum of the red petals of *Catharanthus roseus*.

It is difficult to detect NADES in plants. On one hand, the dynamic changes of NADES in cells and the presence of different potential ingredients of NADES in cells or cellular compartments, such as vacuoles, vesicles, or plastids hinder their in-situ detection. A high amount of NADES may be concentrated in anthocyanoplasts and anthocyanic vacuoles with the function of dissolving high concentrations of anthocyanins. In the analysis of the whole plant, we detected an average, diluted profile of all cell types present, and thus of all possible NADES mixtures. In the previous chapter the highest solubility may occur in NADES that contain some amount of water. This may also be the case in plant cells. One the other hand, although rutin accounts for 40% of the weight of dry flower buds of *Sophora japonica*, it was still not detected in HR-MAS HNMR. This might be due to the attachment of rutin to the cellular structures or the broadening of signal resulting from its interactions with macromolecules. This requires further study, probably with mass spectroscopy.

3.1.3 Plants secretions and seeds. Various secretions, such as maple syrup, nectar, and mucilage are present extracellularly on the plant surface. For example, glucose: sucrose: fructose with a molar ratio around 1:1:1 was detected in the nectar of *Cleome hasselorana*, and honey is composed of glucose and fructose (1:1, molar ratio) (Fig. 1 in chapter 2). Obvious signals of *myo*-inositol and betaine were observed in the ^{1}H NMR of *Drosera* extracts from five different species (Fig. 2).

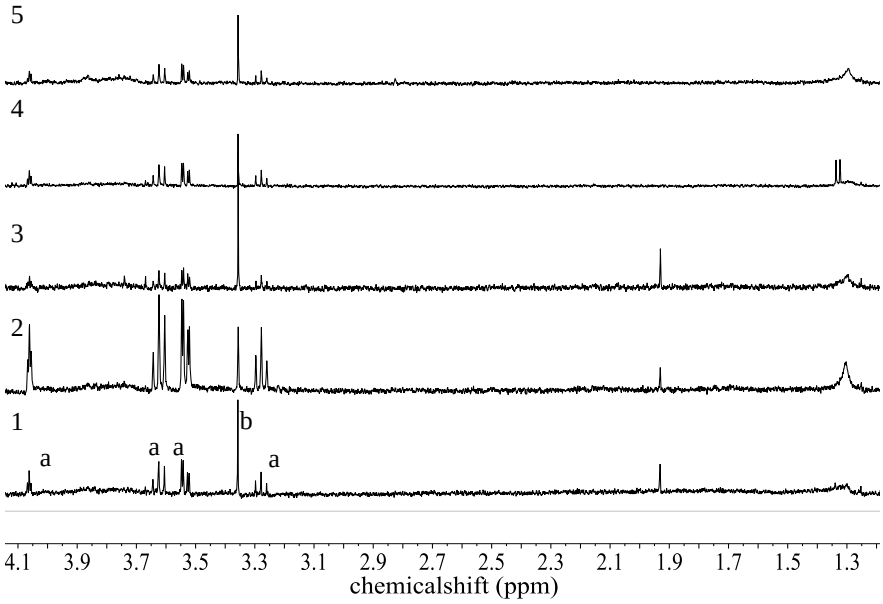

Fig. 2 ^{1}H NMR of sap from different *Drocera* species (**1**, *Drocera capensis*; **2**, *D. binata* ; **3**, *D. capensis*; **4**, *D. glabripes*; **5**, *D. adelae*) with *myo*-inositol (**a**) and betaine (**b**) in all samples.

A seed, as a temporary static system, is an interesting model to study the presence of NADES. In our view NADES could be the explanation for the cold and drought resistance of seeds and the relative ease to revive when under the right conditions for germination. NADES could keep essential enzymes dissolved and upon addition of water be activated (Choi *et al*., 2011). The ^{1}H NMR of extracts of the outside and inside parts of barley seeds revealed higher amounts of sugars (sucrose), organic acids (acetic acid, succinic acid), amino acids (threonine, alanine), alcohols (isobutanol, ethanol), and amines (choline, betaine) in the outside layer (aleurone and seed cover) of a barley seed than its inside part. During the dormant period, enzymes stored in the outside layer of the seed must survive in extremely dry conditions (Schuurink *et al*., 1992). The presence of typical NADES components in the outside layer of the seed implies that the NADES could be the solvents that protect the enzymes.

3.2 The physicochemical properties of NADES

Experiments to determine the physicochemical properties of NADES showed that the density of NADES is in the range of 1.08 -1.36 g/cm^3 (Dai *et al*., 2013) and their viscosity between 37 and 720 mm^2/s, which is much higher than water (1 mm^2/s). The viscosity increases dramatically at low temperature and with low water contents. The NADES have a T_g far below minus 50 ^{o}C, giving them a stable liquid status over a wide temperature range.

Several studies have reported the physicochemical properties of cytoplasm in cells before the intracellular molecular glass formation or glass in plant anhydrobiosis, which is in fact similar to the NADES. The cytoplasmic glass in dry cells results from a complex of sugars and other cytoplasmic components such as organic acids, amino acids, and salts (Buitink and Leprince, 2004). The viscosity of cytoplasm increases dramatically during drying-out conditions and eventually transforms into a glass state. In general, the glass has the following physical properties: high density, temperature dependent mobility and slow mobility in solid state, high viscosity, and low T_g (Dijksterhuis *et al*., 2007; Buitink and Leprince, 2008), which are typical of the physical properties of NADES (Dai *et al*., 2013). The consistency in physicochemical properties as well as in the composition of cellular cytoplasm may be explained by the existence of NADES in plant cells. The major difference is that a glass does not have an orderly structure like the NADES where a liquid crystal-like structure strongly retains water in a certain molar ratio to the NADES components.

3.3 The diffusion between NADES and water

The diffusion process between water and NADES was studied. All tested NADES are hydrophilic and miscible with water since the individual

components are polar compounds. However, the high viscosity may slow down the diffusion process. We selected SuCH from among all our different NADES combinations as it has the highest viscosity and PCH as the lowest viscosity NADES. A natural dye, carthamin, was added in either the water or NADES part for observation.The diffusion test was performed with the NADES as the lower layer, since it is denser than water. With an equal diffusion rate of the constituents in both layers one would not expect any change, except that at a certain point the interface should disappear. If the diffusion ratio were not equal, then the interface would move up (water diffuses faster into lower NADES layer, than NADES into the water layer) or down (NADES components have faster diffusion in upper layer, than water in the lower layer).

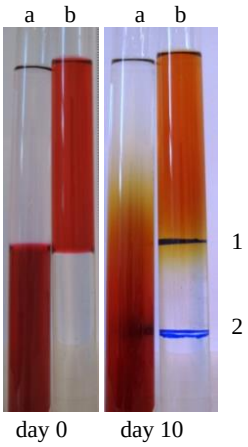

Fig.3 The picture of diffusion tests between sucrose-choline chloride-water (1:4:4) and water on day 0 and 10, and 3 layers were observed with two interfaces (line 1 and 2) labeled in blue on day 10.

Interestingly, diffusion between NADES and water behaved in an unexpected manner as three phases were formed (two interfaces marked with blue color), instead of one (Fig. 3), both in SuCH and PCH. Interface 1 is obvious in the tube b which is indicated by the position of the dye and also the chemical composition of the phases (Table 1). In tube a, the different chemical composition of the different layers is revealed by the interface 1 (line 1) between the water layer and intermediate layer, this interface is at the same height as in tube b, although the position of the dye is different in the tube a. Apparently the mass transfer of the dye through interface 1 is difficult. Interface 2 is clear in both tubes and moved down in the same rate and reached the same height over a 10-day period in both tubes. The separation into three phases is not affected by the added dye.The red dye was found to diffuse from the NADES to the intermediate layer, where a gradient in the color was observed

with the lowest concentration in the upper part. In the case of the dye in the water layer the diffusion of the dye seemed to be impeded at the interface between the upper water layer and the intermediate layer, although its solubility is higher in NADES than in water (Dai *et al*., 2013). The diffusion process between the NADES and water was monitored by recording the position of the interface with the initial interface as reference, measuring the water content in the different layers as well as the ratio of the two NADES components.

The interface (line 1 in Fig. 3) between water and intermediate layer remained more or less in the same position. The interface between SuCH and the intermediate layer (line 2 in Fig. 3) moved down in a gradually decreasing rate from 1.1 cm/day to around 0.2 cm/day in 10 days with 10 mL of each water and SuCH phase at the start of the experiment. In the other experiment the interfaces between PCH and the intermediate layer disappeared in two days. So, the dye diffusion between PCH and water was much faster than that between SuCH and water. Faster diffusion was also observed when the starting phase of SuCH was diluted with 10% (v/v) water. Thus, the high viscosity of NADES decreases the diffusion between NADES and water. Clearly, viscosity plays an important role in the phase behavior between NADES and water.

Table 1 The water weight percentage and molecular ratio of components of sucrose-choline chloride in two parts of three different layers (upper, middle and lower) after 10 days' diffusion between water and sucrose-choline chloride-water (1:4:4) (n=3).

		up1	up2	mid 1	mid 2	low	low
water w%	1	90±2.2	60.1±3.3	50.0±4.5	18.0±3.7	9.0±2.8	6.4±1.5
	2	91.9±1.7	64.2±5.7	42.1±4.5	15.3±2.7	6.4±1.0	6.3±0.9
	3	90	62	49	15	7.9	7.2
molar ratio (choline: sucrose)	1	14.5	3.2	2.8	3.1	3.5	3.7
	2	16.6	3.0	3.0	3.5	3.5	3.9
	3	10.3	3.0	2.7	3.1	3.5	3.7

1: carthamin in the sucrose-choline chloride layer; 2: carthamin in the water layer; 3 sucrose-choline chloride.

The composition of the organic components and the water content are different in the different layers. At day 10, the water content in the water layer showed a gradient from about 90% to 60% (up to down), in the middle layer 50% to 20% and more or less unchanged in the lower phase, showing that instead of complete mixing, a new metastable NADES was formed as an intermediate phase (Table 1). The ratio of the two components in NADES is

different in the layers. The molar ratio of choline chloride to sucrose in SuCH is 4 when prepared. During the diffusion process, the ratio was around 15 in the upper layer, 3 in the middle layer, and remained at the same value of 4 in the lower layer. Apparently, in the interface of SuCH and water a new phase is formed from which choline can move into the water layer whereas the sugar is kept in the middle layer by the hydrogen bonding.

This mixing behavior indicates the complexity of the physicochemical properties of the cellular contents. A change in pH, concentrations, temperature etc. may cause the formation of separate phases, experiencing a dynamic system like the endoplasmic reticulum e.g. creating the conditions for the biosynthesis of poorly water soluble compounds in a cell by creating temporarily a third phase in which enzymes are dissolved and where poorly water soluble substrates are concentrated.

3.4 Adjustment of the water content in cells and plants (the hygroscopicity of GCH and barley seeds)

The NADES in plants may play a role in absorbing water from the surroundings. Hygroscopicity measurements of GCH showed that (Fig. 4a) the water percentage in GCH increases gradually with an increasing humidity level in the surroundings from 0% to 80% and decreases when the humidity level in surroundings went down. The water percentage in GCH reached equilibrium in about 10 hours for a certain condition (Fig. 4b). So, the NADES may have the function of adjusting the water level in a plant through interaction with water vapor in the air.

To further explore the water adjustment effect of NADES in plants, the hygroscopicity level of a barley seed was tested at a moisture level of 80% at 25 ^{o}C. The relative amount of absorbed water in Fig. 5 shows a combined water absorption and desorption curve. As can be seen, most water was absorbed during the first 48 hours and the equilibrium water content for 80% external relative humidity was about 9.1 wt%. When the humidity level decreased to zero, the amount of absorbed water in the barley seed returned to the initial level within the same time interval. The observation fits our hypothesis of NADES being involved in water loss and uptake, e.g. several NADES are clearly hygroscopic and at the same time are able to strongly retain certain minimum amounts of water.

Apparently, plants can lose water in relative dry conditions and absorb water in relative humid conditions. The hygroscopicity may explain how mosses can live on rocks, without roots, and how Cactaceae can possibly live without any water absorbed by the roots. Cacti open their stomata during the night when in deserts the relative humidity may increase considerably as temperature drops. A hygroscopic NADES in the cacti could then absorb water diluting the NADES, activating the enzymes of photosynthesis that subsequently produce sugars

again during the day thus restoring a hygroscopic NADES for nightly water harvesting.

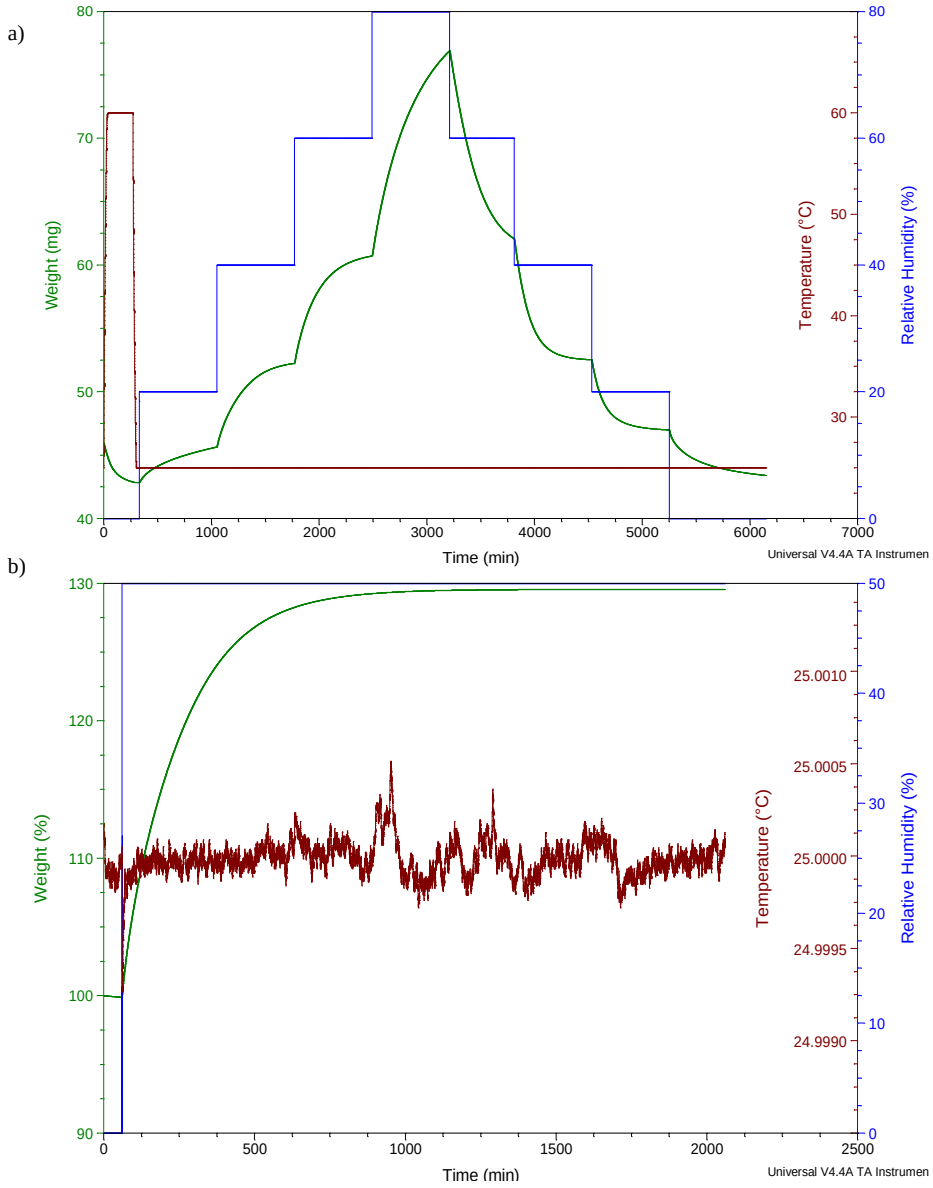

Fig. 4 Hygroscopicity test of glucose:choline chloride:water (1:2.5:2.5) at 25 ^{o}C with relative humidity steps (**a**) and 50% relative humidity level **(b)**. The green line indicates the weight of glucose:choline chloride:water and the blue line refers to the relative humidity of test conditions.

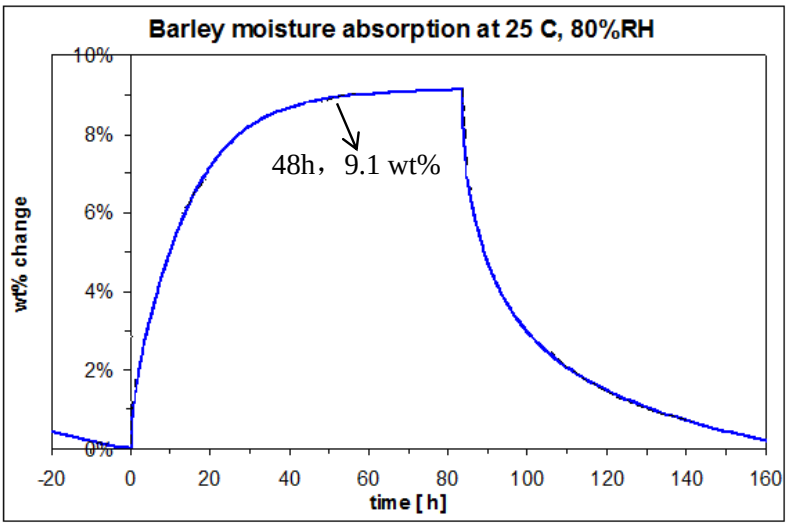

Fig. 5 The hygroscopicity test of barley seeds at 25 ^{o}C with 80% relative humidity level.

3.5 The interaction between NADES and liposomes: the membrane stabilizing effect

3.5.1 The effect of NADES on the size, zeta potential and stability of liposome. The size (hydrodynamic diameter) of liposomes was increased by 30 nm in a buffer with 35.6% sucrose:fructose:glucose (SFG), increased by 600 nm in buffer with 25.2% sucrose:proline (SPH), and decreased by 40 nm in buffer with 19.56% sucrose:malic acid (SMH), compared with phosphate buffer (Table 2). It means that NADES may form layers around liposomes, which increase the size of liposomes in the case of SFGH, SPH and decreases the size in the case of SMH. The zeta potential decreased in buffer with SFG, SPH and increased in buffer with SMH, compared with phosphate buffer. The zeta potential is widely used for quantifying the electrical charge at the double layer. The different zeta potentials confirm the interactions between NADES and liposomes and also imply that the interactions between liposomes and NADES are different. For SFGH, the sugar components may hydrogenbond to the head group of the lipids and form a film around the liposome through a hydrogen bonding network, increasing the size of liposome (Crowe et al., 1992; Christensen et al., 2007). For SMH, the negatively charged acid group of the tested malic acid may have ionic interactions with the positively charged head group of the lipids and this interaction may shrink the size of liposome due to the attraction effect. The size of liposomes in SPH increased a lot but they were physically unstable probably due to the zwitterionic form of proline. The preparation of liposomes with sucrose: choline chloride: water (SCH) in buffer (pH 7.0) was also attempted

but the hydration of the lipid film failed and the lipid precipitated in the buffer containing SuCH. This might be because of the repulsive forces between choline and the head group of the lipid (1,2-dioleoyl-3-trimethylammonium-propane). In all, the different behavior of liposomes in buffers containing NADES with neutral (SFG), acid (SMH), basic (SCH) and zwitterionic (SPH) components indicates the existence of interactions between liposomes and the molecules of NADES components.

The stability of liposomes in different media was investigated with the size and zeta potential as parameters. The size, polydispersity index, and zeta potential of liposomes were practically stable for one month at 4 ℃ in buffer and buffer with SFG or SMH, but unstable in SPH. This indicates that liposomes are stable for shape, homogeneity, and interaction between NADES and liposomes for at least one month in buffer, buffer with SFG or SMH. However, after five months at 4 ℃, the liposomes had precipitated in both buffer and SPH, while they were still in suspension in SFG and SMH. This proves the stabilizing effect of some NADES on liposomes.

Table 2. The size (hydrodynamic diameter, nm) and *zeta* potential (ZP) of liposome in three media (n=3): phosphate buffer (pH=7.0), buffer with 19.56% (w/w) sucrose:malic acid:water (SMH), buffer with 35.6% (w/w) sucrose: fructose: glucose-water (SFG) and buffer with 25.2% (w/w) sucrose-proline-water (SPH).

	Size (d,nm)			**ZP**	
	average	**PDI**[a]	**RSD%**	**average**	**RSD%**
buffer+SMH	117.2	0.124	0.60	40.5	0.65
buffer+SFG	192.2	0.175	4.85	20.4	12.70
buffer+SPH	790	0.369	25.90	15.2	34.30
buffer	162.5	0.092	1.07	27.4	1.03

[a] PDI: polydispersity index, the width of molecular size distribution.

3.5.2 The effect of NADES on the membrane dynamics of liposomes. The effect of NADES on membrane dynamics was studied with the EPR spin probe technique. Spin-labeled 5-doxylstearic acid (5-DS) was used to probe the membrane interface in liposomes. In the 5-DS molecule the nitroxide doxyl group (a stable radical) is attached in a rigid, stereospecific manner to stearic acid at the 5^{th} carbon from the COOH group. In phospholipid membranes the COOH group of spin-labeled stearic acid is inserted between the polar heads. Therefore, the nitroxide (doxyl group) of 5-DS resides in a polar area of the bilayer, which is called bilayer surface or bilayer interface. The EPR spectral shape of 5-DS depends on the motion and angular orientation of the nitroxide group with respect to the membrane lipid-water interface (Marsh, 1981). This spin label allows the probing of the motional freedom in membranes at the lipid-water interface of the bilayer.

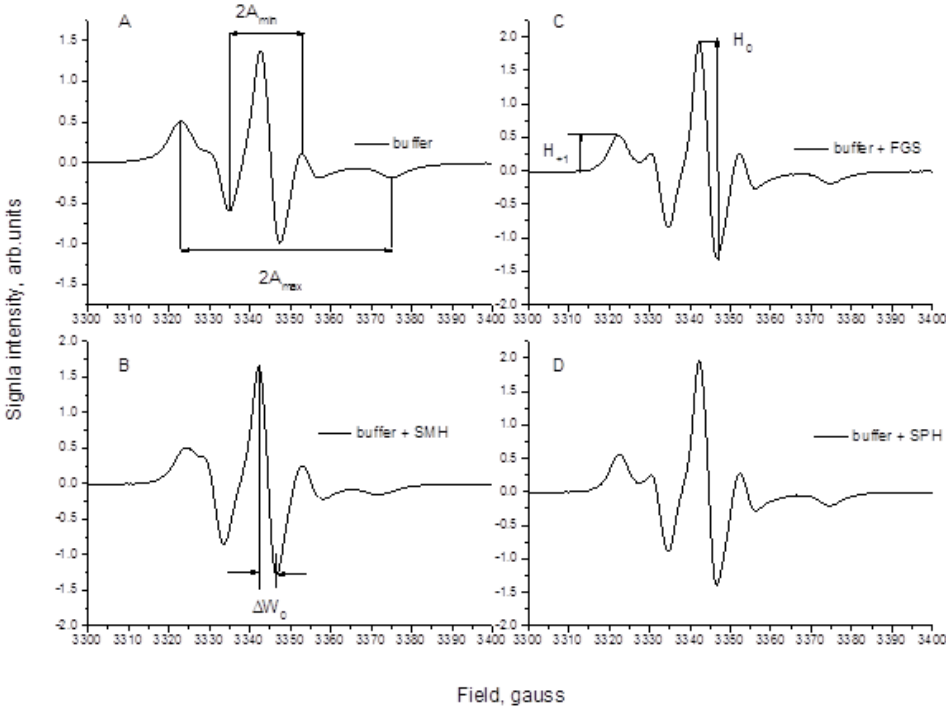

Fig. 6 Electron paramagnetic resonance spectra of 5-doxyl stearic acid in liposomes in PBS phosphate buffer (pH=7.0) (A); in PBS phosphate buffer with 19.6% (w/w) sucrose:malic acid:water (SMH) (B); in PBS phosphate buffer with 35.6% (w/w) sucrose:fructose:glucose (SFG) (C); and in PBS phosphate buffer with 25.2% (w/w) sucrose:proline:water (SPH) (D).

The anisotropic character of the spectral shape of 5-DS in liposomes in normal buffer (Fig. 6A) results from the restricted angular freedom of the radical group of 5-DS in the bilayer interface. The spectral parameters A_{max} and A_{min} indicate the outer and inner hyperfine splitting in an experimental spectrum (as shown in Fig. 6A). The membrane-order parameter S_{zz} can be calculated as the ratio between the observed hyperfine anisotropy (A_{max} - A_{min}) to the maximum theoretically obtainable value, which corresponds to the completely rigid orientation of 5-DS (Knowles *et al*., 1976). With membrane fluidization outer splitting (A_{max}) decreases and inner splitting (A_{min}) increases, so that the order parameter decreases. Later on, some corrections have been proposed to account for differences in polarity and for the range of membrane-order parameter. Here the order parameter is calculated according to the formula proposed by Marsh and Schorn (1998). This formula takes into account the

principle splitting values for 5-DS and is corrected for polarity of the spin label environment. S_{zz} = 1/7 (A_{max}+2A_{min}) – sqrt{ [1/7(A_{max}+2A_{min})]^2 -0.46(A_{max}-A_{min})+0.6}

The EPR spectra of the 5-DS incorporated into the liposome in three media were compared: phosphate buffer, phosphate buffer with SFG (35.6%) and phosphate buffer with SMH (19.56%). The data on 2A_{max} and S_{zz} are presented in Table 3. Outer splitting 2A_{max} considerably decreases in the presence of 19.6% SMH and decreases insignificantly in the presence of SFG and SPH. The order parameter S_{zz} of the EPR spectra of 5-DS in liposomes in the presence of 19.6% SMH is considerably reduced (Table 3). In the presence of SFG and SPH the order parameter slightly increases.

The decrease of outer splitting 2A_{max} and order parameter S_{zz} in the presence of SMH results from the fluidization of the membrane interface. This data is also supported by a decrease in the width of the central line (Table 3). The results are in accordance with a considerable decrease of liposome size from 162.5 to 117.2 nm (Table 2). Obviously, the decrease of liposome size inevitably causes the fluidization of the liposome bilayer outer surface due to an increased bilayer curvature and the spacing between lipids. The effect of bilayer curvature on phospholipid behavior recently has been demonstrated by molecular dynamics simulations (Risselada and Marrink, 2009). The size of liposomes in the presence of SFG and SPH considerably increases (Table 3). However, this causes only a slight ordering of the membrane interface as determined by order parameter (Table 3). If the ordering/immobilization increases slightly in the presence of SFG and SPH, an increase of the outer splitting 2A_{max} would also be expected. However, this was not the case. The slight decrease of the 2A_{max} for these cases could be explained by the decrease of the polarity in the vicinity of spin label moiety.

Table 3. Parameters of electron paramagnetic resonance spectra of 5-doxyl stearic acid in liposomes in the presence of three different natural different deep eutectic solvents (buffer with 19.56% (w/w) sucrose:malic acid (SMH), buffer with 35.6% (w/w) sucrose:fructose:glucose (SFG), and buffer with 25.2% (w/w) sucrose:proline:water (SPH), compared with phosphate buffer (pH=7.0)). The parameters include membrane-order parameter (S_{zz}), the outer hyperfine splitting (2A_{max}), the width of the central line (ΔW_0) and the ratio between the heights of the central (H_0) and low-field (H_{+1}) lines (H_0/H_{+1}).

media composition	**2A_{max},**	**S_{zz},**	**ΔW_0**	**H_0/H_{+1}**
buffer (pH=7.0)	52.08	0.617	4.6919	2.71391
buffer + 35.6% (w/w) SFG	51.99	0.629	4.30102	3.67734
buffer + 19.6% (w/w) SMH	47.45	0.507	4.30103	3.26143
buffer + 25.2% (w/w) SPH	51.87	0.622	4.30102	3.55936

The width of the central line decreases in the presence of all NADES (Table 3, Fig. 6) while the ratio between the heights of the central (H_0) and low-field (H_{+1}) lines (as indicated in Fig. 5C) increases in the presence of all NADES (Table 3, Fig. 7). There are many factorsthat determine the line width in the EPR spectra. Line broadening can be paramagnetic and motional. Line narrowing can only be explained in the case of SMH by fluidization of the membrane interface, because in this case the order parameter of the spectrum also decreases. In the case of SFG and SPH the situation is more complicated. Both spectra are identical, thus the mechanism of line narrowing for these two compounds must be the same. In these samples, line narrowing occurs without the decrease of the order parameter. The order parameter relates to the average angle of the departure of the molecular axis of the acyl chain from the normal to the bilayer surface (angular orientation or angular freedom). When the system is disordered, this angle increases and the spin label moiety experiences more freedom to move. This results in line narrowing. If order parameter does not change, this can only mean that the rate of rotation around the molecular axis of the acyl chain increases without system disordering. Usually such effect is observed in the membrane interface at higher temperature. The fact that the same phenomenon is observed in case of NADES at room temperature, is very intriguing.

If line narrowing originates from a decreased probability of interaction between the spin label moieties at the liposomal membrane (paramagnetic broadening due to dipole-dipole interaction or exchange), then such an effect may result from decreased membrane lateral diffusion (Sachse *et al*., 1987**)**. The restriction of lateral diffusion of lipids within bilayers in the presence of NADES would be one other possible mechanism for their stabilizing effect. All the parameters in EPR show an unexpected behavior of 5-DS which may probably indicate the establishment of a NADES layer around the liposomes. Generally label experiments are in aqueous surroundings, with the -COOH group of 5-DS at the polar heads of the lipid bilayer and the doxyl group in a polar area of the bilayer. If NADES builds up a shell around the liposomes, the label will be in NADES, a less polar but viscous environment, in which the 5-DS might bind more loosely with the polar heads of the bilayer as it dissolves better in the NADES than in water. In other words, the 5-DS will have a different behavior in a water-lipid system than in a water-NADES-lipid system due to a different interface at the bilayer. In the spectra of 5DS in liposomes in the presence of FGS and SPH there is a clear indication of the presence of another fraction of 5-DS, which demonstrates liquid-type behavior. This may be caused by the deeper localization of the spin-label moiety in the bilayer. Such vertical excursion can result from the lost interactions of 5-DS with the bilayer interface due to protonation at low pH (Fig. 7).

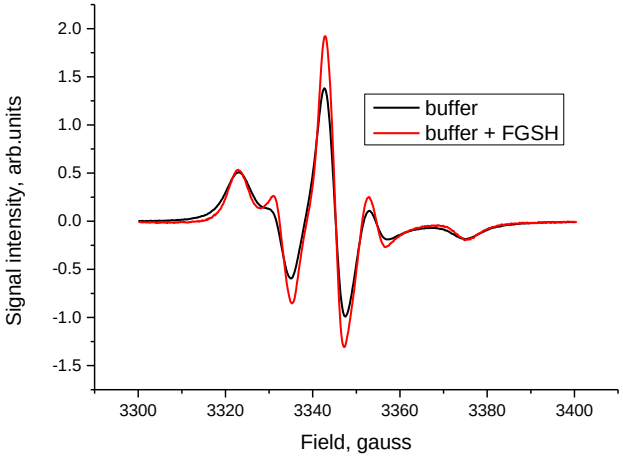

Fig. 7 Comparison of the spectral shape of 5-doxyl stearic acid in liposomes in buffer (black lines) and in buffer with SFG (red lines).

4. Conclusion

This study provides in-vitro evidence for the existence of NADES in biological systems and some of their possible functions. NADES show a similar chemical composition and physicochemical properties to cytoplasm in plants. NADES may be involved in controlling the water level in plants and even trap water from humid air. The phase separation of NADES and water is a dynamic process in which even three phases may be formed different with different solubilization properties. This may explain the phenomenon that metabolites of quite diverse polarities can be produced and stored in a plant. Furthermore, NADES may exist around cell membranes and play a role in stabilizing the lipid membrane through intermolecular interactions. The attached NADES would be the medium where biosynthetic enzymes may function in an environment in which non-water soluble intermediates are dissolved and may be transferred from one enzyme to the next in the sequence of the biosynthesis pathway. This could be a metastable NADES phase that interacts with the water phase again, and may form vesicles as vehicles for transport of poorly water-soluble compounds, explaining biosynthesis and translocation of poorly water-soluble metabolites.

NADES might be involved in the biosynthesis and storage of various non water-soluble metabolites (e.g. secondary metabolites and macromolecules) in cells and play an important role in protecting organisms from extreme conditions. To fully address this hypothesis, further experiments are required, and these may revolutionize our views on cell physiology, as well as the physiology of whole organisms. When plants are submitted to harsh conditions, such as drought, excessivesalinity or very low temperatures, NADES may protect the integrity of the cell membrane as well as the macromolecules. The biosynthesis of poorly water-soluble compounds could then occur in the NADES media in which both substrates and enzymes are dissolved. Thus, the characteristics of the enzyme might be quite different to those displayed when disolved in water, among other things because of the much higher concentration of substrates.

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