CLUSTER AGGREGATION OF WATER-BASED DEEP EUTECTIC SOLVENTS IN WATER AND EVALUATION OF THEIR CYTOTOXICITY

Elison Lepore,[a] Gianluca Ciancaleoni,[b] Diego Romano Perinelli,[a] Giulia Bonacucina,[a] Serena Gabrielli,[c] Gaia de Simone,[d] Rosita Gabbianelli,[d] Laura Bordoni,[d] Matteo Tiecco[a]*

[a]*Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Italy.*

[b]*Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Giuseppe Moruzzi 13, I-56124 Pisa, Italy.*

[c]*Scuola di Scienze e Tecnologie, Università di Camerino, Via. S. Agostino 1, 62032, Camerino (MC), Italy.*

[d]*Unit of Molecular Biology and Nutrigenomics, Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Italy.*

* Corresponding Author: Dr Matteo Tiecco - matteo.tiecco@unicam.it

Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Italy

ABSTRACT

Deep Eutectic Solvents (DESs) are emerging in the recent literature as promising environmentally friendly liquids in different topics, finding fruitful applications as substitutes to common volatile organic solvents. These mixtures are prepared via non-solvent procedures and they have peculiar structural features and properties. They are formed via weak interactions, mainly H-bonds, between two species; the interactions between the different species as well as the ones occurring between the same species, lead to an impossible regular crystal lattice formation, therefore leading to liquid systems. The number of novel DESs mixtures present in the literature is rapidly increasing because of the wide number of H-bonds capable molecules available. One of these classes is represented by *aquo*DESs: binary mixtures in which one of the components is water, confirmed in their DESs' identity by theoretical/experimental melting curves approaches.

In this work a study of cluster formation of water-based DESs in water dilutions is presented. The three analysed mixtures are: glycolic acid/water (GA/H_2O); betaine/water (TMG/H_2O), choline chloride/water (ChCl/H_2O). Ionic conductivity, viscosity, ultrasound spectroscopy and NMR measures showed the presence of aggregates of *aquo*DESs in water dilutions until values about 50% w/w of added water. Finally, taking into account the convenient disposal as water dilution of a DES, the cytotoxicity of these dilutions was evaluated on Caco2 model cells, showing the effect to be related only to the non-water components of these liquids.

KEYWORDS

Deep eutectic solvents; Physicochemical properties; Structural properties; NMR spectroscopy; Cytotoxicity.

INTRODUCTION

The pollution prevention, the circular economy, the reduction of CO_2 emissions and a green and sustainable approach to all industrial applications are solutions urgent to be applied in order to reduce the human being impact on the environment[1–3]. Different technologies, approaches and also laws based on them are currently used and applied to face up to the environmental problems[4,5]. The green chemistry approach is nowadays developing novel technologies and solutions to face up to these matters, proposing different and relevant studies on the recycling/revaluation of waste, the reduction of subproducts and waste production, the use of safer reactants and so on[6–9]

One of the most proficient technologies that chemistry researchers are proposing for the reduction of the environmental impact of their applications is the substitution of common volatile organic solvents with more sustainable, non-vapourisable, obtained from green sources liquids[10–12]. This has a great impact because of the accumulation of the solvents in the environment as volatiles in the atmosphere as well as their accumulation in different ecosystems. It must be also considered that the solvent represents the most abundant part in any chemical application or transformation, underlining again the importance of it in any green chemistry research[13].

In this topic Deep Eutectic Solvents (DESs) are playing a prominent role. DESs are emerging in the recent literature as substitutes to commonly used VOCs (volatile organic compounds) for their green properties, together with their fascinating structural features and their catalytic properties in many different applications[14–22].

The green properties of the DESs are related to their low or absent vapour pressure; their recycle capabilities; their low or absent toxicity; their non-flammability and so on[23–25]. The most important green advantage of the DESs is represented by their synthesis that do not require any solvent neither purification procedure. They are in fact prepared by simply mixing and heating two pure substances, giving a stable liquid with no purification procedures needed with 100% yield and 100% atom economy, perfectly fulfilling the green chemistry principles[26,27]. From a structural point of view, DESs are formed via weak interactions, mainly H-bonds, between two (or more) species: namely a hydrogen bond donor (HBD) molecule and a hydrogen bond acceptor (HBA) molecule. The weak interactions occurring between HBA and HBD as well as the ones between the same HBD-HBD and HBA-HBA, lead to the impossibility of a regular crystal lattice formation, therefore leading to liquid systems even starting from high-melting points components[28,29]. Nowadays a deeper study with a comparison of the theoretical melting curves compared to the

experimental ones must be made in order to define a mixture as a DES; in particular a down-shift of the experimental melting point at the eutectic compared to the theoretical one must be observed in order to define a mixture as a DES[30–32]. This is particularly relevant in the case one of the two substances used is already a liquid: in this case simple solutions of one molecule into the other can be formed rather than DES formation thanks to the interactions occurring between the two species. Many different H-bonds capable species can be used for DESs' formation, so all their properties, especially the green ones, are related to the choice of the molecules used for their formation: many alcohols, polyols, acids, urea, sugars, metal halides and so on are used as HBDs; the most used HBA molecules are generally ammonium or phosphonium salts, choline chloride, betaine and similar species[33]. In this context, a particular relevance is played by DESs made with natural-occurring species (NADESs: Natural Deep Eutectic Solvents) thanks to the bioavailability and biodegradability of these molecules[34,35].

The catalytic properties of the DESs rely on the properties of the molecules forming them, but with peculiar, fascinating and often complex catalysis mechanisms, mainly related to the availability of the catalytic molecule present as a DES component, therefore on the association constant with the counterpart[36–40].

An interesting structural feature of the DESs is the role of water with them[41,42]. DESs are in fact reported to be hydrophilic or hydrophobic/water separable even if they are H-bonds-based mixtures[43]. In this class of liquids, widely used in extraction procedures for their efficacy, the role of the HBD was underlined as the driving force for water separation[16,44]. In the hydrophilic DESs, water plays three different roles depending on its concentration, as it can have a structural role participating directly both as HBD and HBA at low concentrations; it can form clusters of DESs in water at higher amounts, determining micro-domains of DESs and of water; it can destruct the structures of the liquids by solvating separately the two DES' components[45,46]. This normally occurs at high water contents, generally over 50 to 60% w/w depending on the nature of the formers. Recently a novel class of DESs has emerged in the literature, which is composed of binary water-based DESs systems, using the H_2O as both HBA or HBD mixing it with differently structured molecules: *aquo*DESs. To our knowledge only four *aquo*DESs mixtures are present in the literature nowadays: glycolic acid/water (GA/H_2O); betaine/water (TMG/H_2O); thymol/water (THY/H_2O, a hydrophobic mixture thanks to the HBD role of thymol) and choline chloride/water (ChCl/H_2O)[16,47–49]. This last ChCl/H_2O mixture, however, is object of controversial literature about its DES identity, because of the capability of water absorbing of choline chloride molecule

that leads to adducts with a lower melting point. The inaccuracy on the value of the fusion enthalpy of choline chloride reported in literature also render impossible a clear and defined theoretical melting curve to be compared to the experimental one[50–52].

In this work, the formation of clusters aggregation of water-based DESs in water dilutions is reported. The three *aquo*DESs mixtures GA/H$_2$O, TMG/H$_2$O and ChCl/H$_2$O were analysed in their water dilutions in all the dilution ranges from 5% to 95% w/w with different techniques: ionic conductivity, viscosity, high-resolution ultrasound spectroscopy and NMR techniques. Finally, the cytotoxicity of the water dilutions of these water-based DESs was evaluated. To this purpose, a Caco-2 monolayer, an *in vitro* model of intestinal cells commonly used for toxicity assays, was used. The results showed the presence of clusters of water-based DESs in water, a reduction of the viscosity of the liquids by increasing the dilutions, a really low toxicity that is fully dependent on the toxicity of the non-water component of the liquids, underlining the promising importance of this novel class of solvents and their water dilutions in different applications.

EXPERIMENTAL

DESs and water dilutions preparation

Glycolic acid (GA), trimethylglycine (or betaine, TMG), choline chloride (ChCl) (all purities >99%) were purchased from Sigma-Aldrich and Merck suppliers; they were dried under vacuum with P$_2$O$_5$ prior DESs' preparation. *aquo*DESs were prepared according to the literature by mixing the components with deionized water in small screw-sealed vials with low headspace at the proper molar ratios; the samples where then heated at 130 °C with a heating gun with heavy shaking in order to prevent evaporation of the components[48]. The mixtures were then left cooling to room temperature. The molar ratios of the *aquo*DESs are: GA/H$_2$O 1/4, TMG/H$_2$O 1/5 and ChCl/H$_2$O 1/4. The water dilutions of the DESs were prepared by adding the specific amounts of water to the mixtures in a range from 5% to 90% w/w and they were left stirring at room temperature overnight prior to any measure. The starting water content of the DESs components was determined via titration with a Karl Fischer (Metrohm 684 KF Coulometer), via dilutions of the samples in anhydrous methanol, showing values spanning from 0.6 to 1.1% w/w.

Ionic conductivity and viscosity measures

The ionic conductivity measures were performed with a COND 60 VioLab instrument (XS instruments) with the temperature controlled with an external thermostated bath (25.0 ± 0.1 °C). The viscosities of the samples were measured with a rotational rheometer (Kinexus Lab+, Malvern) using a C40/4 cone-plate geometry. The analyses were conducted at 25.0 ± 0.1 °C in the shear rate range of 1-100 s^{-1}. The reported viscosity values were the mean of the viscosities determined in the shear rate range applied.

High-resolution ultrasound spectroscopy

Ultrasound attenuation was measured using a high-resolution ultrasound spectrometer (HR-US 102, Ultrasonic Scientific, Ireland) using distilled water as a reference. The analyses were performed at 25.0 ± 0.1 °C and the temperature was controlled through a HAAKE C25P water bath.

Each sample was left at the tested temperature for at least 20 min to allow equilibration before the acquisition. Ultrasound attenuation was measured at a frequency of 5.2 MHz, preliminarily determined by a larger amplitude frequency scan. Attenuation was recorded as an absolute value and the reported values are the mean of the collected points over a measurement time of 300 s.

NMR measures

NMR spectra of pure and diluted DESs were recorded at 298 K on a Jeol JNM-ECZ500R instrument equipped with a Royal HFX Broadband probe. An internal capillary containing C_6D_6 or $(CD_3)_2CO$ was used for external reference and technical operations (locking, shimming). ^{1}H diffusion NMR measurements were performed by using the double-stimulated echo sequence with longitudinal eddy current delay without spinning. The dependence of the resonance intensity of the observed spin echo (I) on a constant waiting time and a varied gradient strength G is described using the following equation (1):

$$ln\left(\frac{I}{I_0}\right) = (\gamma\delta)^2 D_t \left(\Delta - \frac{\delta}{3}\right) G^2 \qquad\qquad \text{(Eq. 1)}$$

where I_0 the intensity of the spin echo in the absence of gradient, D_t the self-diffusion coefficient, Δ the delay between the midpoints of the gradients (0.1 s), δ the length of the gradient pulse (2 ms), and γ the magnetogyric ratio.

The shape of the gradients was rectangular, and their strength G was varied during the experiments. The self-diffusion coefficient, D_t, was estimated by evaluating the proportionality constant for a

sample of HDO (5%) in D_2O (known diffusion coefficients in the range 274–318 K) under the exact same conditions as those for the sample of interest.

D_t has been successively treated through a modified version of Stokes-Einstein equation (Eq. 2):

$$D_t = \frac{k_B T}{c \pi \eta r_H} \tag{Eq. 2}$$

where k_B is the Boltzmann constant, c is a numerical factor, η is the viscosity of the liquid and r_H the average hydrodynamic radius of all the species contributing to the NMR peak. The c factor is equal to 6 when the diffusing species is much larger than the solvent, as in the original study, and lower than 6 when the diffusing species has a comparable size than the solvent. As proposed in the literature, c can be estimated using the empirical Chen and Chen equation (Eq.3):

$$c = \frac{6}{1 + 0.695 \left(\frac{r_{solv}}{r_H} \right)^{2.234}} \tag{Eq. 3}$$

Cell culture and cytotoxicity assay

The cytotoxicity of Deep Eutectic Solvents (glycolic acid/water (GA/H_2O), betaine/water (TMG/H_2O), and choline chloride/water (ChCl/H_2O)) and non-water components of the liquids (GA, TMG and ChCl) was tested on Caco-2 cells (ATCC, Rockville, MD), an immortalized cell line of human colorectal adenocarcinoma cells, primarily used as a model of the intestinal epithelial barrier (PMID: 29787057). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM 1X) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% L-glutamine, 1% Non-Essential Amino Acids (NEAA), and 1% penicillin/streptomycin. Cells were maintained in T75 culture flasks at 37°C in a humidified atmosphere with 5% CO_2. For the cytotoxicity assay, cells in the exponential growth phase (~90% confluency) with fresh medium changes every two days, were used.

The cytotoxicity was assessed using the 3-(4,5-Di-2-yl)-2,5-ditetrazolium bromide (MTT) assay. This colorimetric assay relies on the quantification of a dark-coloured formazan compound generated through the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by metabolically active cells. Cells with reduced viability exhibit decreased metabolic activity and consequently reduce less MTT. The extent of reduction in formazan production in cells exposed to a specific molecule serves as an indicator of the molecule's

cytotoxicity[53]. Caco-2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µl of culture media. After overnight incubation, cells were treated with the selected DESs or their non-water components separately. Before treatments, DESs or their non-water components were diluted in DMEM at different concentrations. DESs were maintained under agitation by a rotating mixer (Continental Instrument LLC, Texas, USA) for 24h. Both DESs non-water components have been sterilized with a 0.22 µm syringe filter. For TMG/H_2O, a range of concentrations varying from 20 % to 1 % v/v for the DES and its single compounds (TMG and water), were tested. A range of concentrations varying from 0.5 % to 0.015625 % v/v of aquoDESs dilutions for GA/H_2O and ChCl/H_2O, and their single compounds (GA, ChCl and water), were tested.

After 24 h of exposure, the cells were treated with a 5 mg/ml MTT solution. To dissolve the formazan crystals, the medium was substituted with 100 µL of dimethyl sulfoxide (DMSO) after four hours of incubation. The absorbance was measured at 550 nm using a spectrometer (FLUOstar Omega multidetection microplate reader, BMG LABTECH's, Ortenberg, Germany). Results were expressed as percentage of cell viability (%) relative to control (cells grown in culture medium only). Experiments were performed with six biological replicates and 5 technical replicates for each condition.

Data were expressed as the mean ± standard deviation (SD). Results from each test (GA/H_2O, TMG/H_2O, ChCl/H_2O and the individual compounds) were analysed by Kruskall-Wallis test followed by Bonferroni's correction for multiple testing. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Ionic conductivity measures

Ionic conductivity is a simple and cheap technique that can show relevant structural information on different systems and can easily underline their structural changes[54–57]. The measures of the ionic conductivities of water dilutions of DESs can show the water concentrations in which the systems collapse to start to give water dilutions of the single components, usually reported in the literature at about 50% w/w of added water[46]. In Figure 1 the conductivity measures of the water dilutions of the three analyzed aquoDESs (TMG/H_2O, GA/H_2O, ChCl/H_2O) are reported, in the dilution range from 5% to 90% w/w at the temperature of 25 °C.
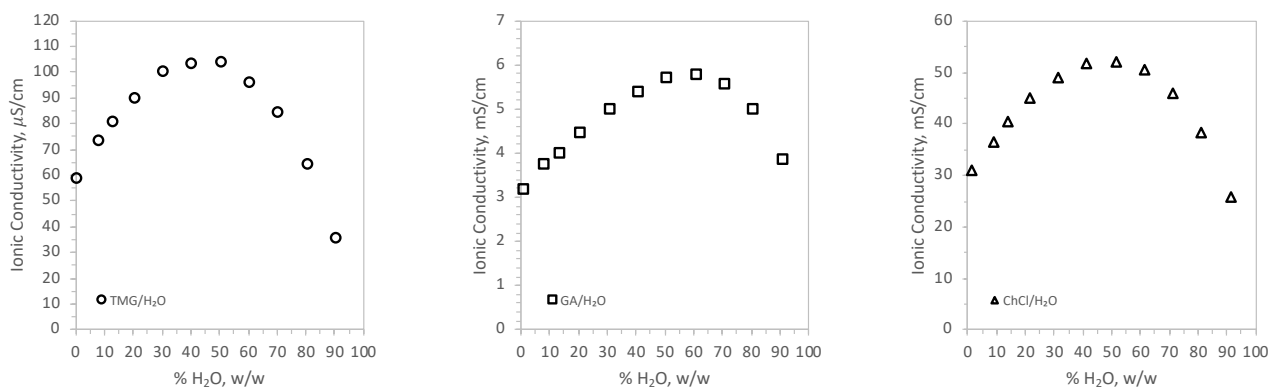
Figure 1: Ionic Conductivities at 25.0 ± 0.1 °C of water dilutions from 5% to 90% w/w of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H₂O; center, squares = glycolic acid/water GA/H₂O; left, triangles = Choline chloride/water ChCl/H₂O.

Coherently with what reported in the literature on differently structured DESs[41,42], the ionic conductivity profiles of the three *aquo*DESs water dilutions have bell-shapes, with an increase of the overall values until 40%-60% w/w dilutions and then a decrease. At water percentages (w/w) below the change in the slope, the presence of two domains are reported: a DES domain and a water domain[42,58]. The increase of the ionic conductivity values observed at higher water amounts is due to the enlargement of the water domain dimensions, thereby affecting the amount of conducting molecules present in them. The DESs themselves, as it can be clearly observed from the values at 0% of added water, showed lower ionic conductivity values because of the aggregation, therefore of the lower mobility, of the species. The supramolecular structures of the *aquo*DESs start to collapse into single solvated species in water progressively by increasing the water contents to values over about 50% w/w. Above this point, the increase of the water content determines a dilution of the species leading to a decrease in the observed ionic conductivity values. Interestingly, the values at which the conductivity profiles change in their trends correlate with the maximum solubility of the DESs' forming molecules: about 60% w/w of water for GA/H₂O DES (GA maximum solubility in water = 7.894 mol/L); 45% w/w of added water for TMG/H₂O DES (TMG maximum solubility in water = 5.216 mol/L); about 40% w/w of added water for ChCl/H₂O DES (ChCl maximum solubility in water = 4.655 mol/L)[59]. This suggested that the systems can disrupt to give single species in water solution at concentration values dependent on their maximum water solubility.

The initial part of the conductivity profiles at low water content is slightly different from the ones reported in literature for other DESs systems[45,46]. It is commonly reported a first change in the slope of the curves under 15-20% w/w of water, where a first region with a lower slope is observed. This is reported to be due to the structural role of water at low concentrations that can participate in the H-bonds network with the other two components. This phenomenon occurs, for example also

with hydrophobic/water separable mixtures[44,60]. In the case of *aquo*DESs the curves are straight lines in this region. Moreover, the DES system formed by the two non-water components of these systems (glycolic acid/betaine 2/1 molar ratio) showed this inflection point, and an increase in the number of experiments in this region of the curves did not underline any discontinuity (see supporting Figure S1 and S2). This behavior of the aquoDESs dilutions was more deeply analyzed in this work with NMR techniques (see further).

The overall ionic conductivity values observed were strictly related to the nature of the components of the DESs: the presence of ionic species in ChCl/H_2O DES determined its highest values in the set (in the order of magnitude of tens of mS/cm); the GA/H_2O DES showed intermediate values, which derived from acid/base equilibriums leading to ionic species in water (units of mS/cm); the zwitterionic betaine can undergo only to slight protonation in water leading to ionic species, therefore the conductivity values of TMG/H_2O DES showed the lowest values in the set in the order of magnitude of tens of μS/cm.

Viscosity measures

The viscosity is a relevant physical property for the characterization of DESs, especially when the effect of water dilutions on them is studied because the added water reduces significantly the viscosity of the liquids, facilitating their applications[41,45,61]. Indeed, the decrease of the measured viscosity of DESs as a function of water dilutions has been reported to be affected by the aggregation state of the molecules and their interaction with water[41]. Figure 2 shows the viscosity of the water dilutions of the three analyzed aquoDESs (TMG/H_2O, GA/H_2O, ChCl/H_2O) in the dilution range from 5% to 90% w/w at the temperature of 25 °C.
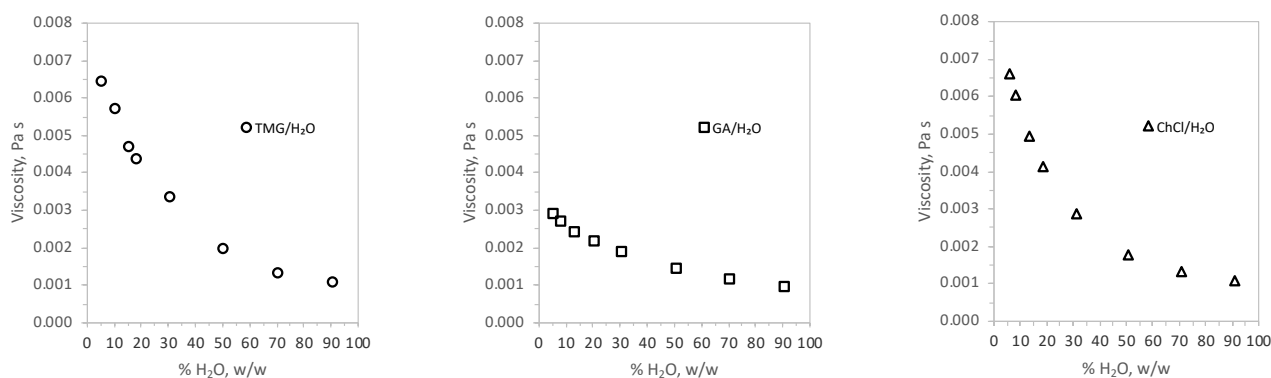

Figure 2 Viscosity (mPa s) at 25.0 ± 0.1 °C of water dilutions from 5% to 90% w/w of the aquoDESs: left, circles = trimethylglycine/water TMG/H_2O; center, squares = glycolic acid/water GA/H_2O; right, triangles = choline chloride/water ChCl/H_2O.

The measured viscosity values were very low, in the range of 0.001-0.007 Pa s, and close to that of pure water. As expected, and similarly to what was reported in the literature[45,61], the measured viscosity decreased after water dilutions with an exponential trend. Some differences between the samples were observed only at a water content below 50% w/w after dilution. Specifically, a slightly higher viscosity was calculated for TMG/H_2O and ChCl/H_2O with respect to GA/H_2O. However, above a water content of 50% w/w, the viscosity values measured for all three samples are similar, suggesting that the addition of water led to DESs' disruption. The really low values of viscosity of the samples observed could promote the use of these solvents in applications where low-viscous liquids are needed, for example supercritical CO_2 extractions.

Ultrasound spectroscopy analyses

High-resolution ultrasound spectroscopy analyses were carried out to confirm further the results obtained from conductivity measurements regarding the formation of water-based DESs domains in water. The measured ultrasound parameter was absolute attenuation, which is representative of the amplitude fluctuation of the ultrasound wave throughout the materials. These fluctuations are consequent to the energy loss of the ultrasound wave itself, due to the interaction with any discontinuity inside the material. Indeed, only for an ideal isotropic material, the energy of the ultrasound wave is conserved and its amplitude remains constant. Therefore, any heterogeneity in the sample can contribute in producing a variation of ultrasound attenuation[62,63]. High-resolution ultrasound spectroscopy has been extensively used for the characterization of colloidal systems, biocatalyst and crystallization processes; however, to our knowledge it has never been applied for the characterization of DESs. In this case, changes in attenuation can be attributed to structural relaxation in the solvent originating from the molecular transformations (e.g. structural rearrangements, cluster formation). The oscillation of the amplitude in the ultrasonic wave and the energy loss can be due to scattering phenomena, which represents one of the six different mechanisms of interaction of the ultrasound wave with a material (i.d. scattering, viscous, thermal, intrinsic, structural, and electrokinetic mechanisms)[64]. The variations of ultrasound attenuation for the water dilutions of the aqueous DESs are reported in Figure 3.
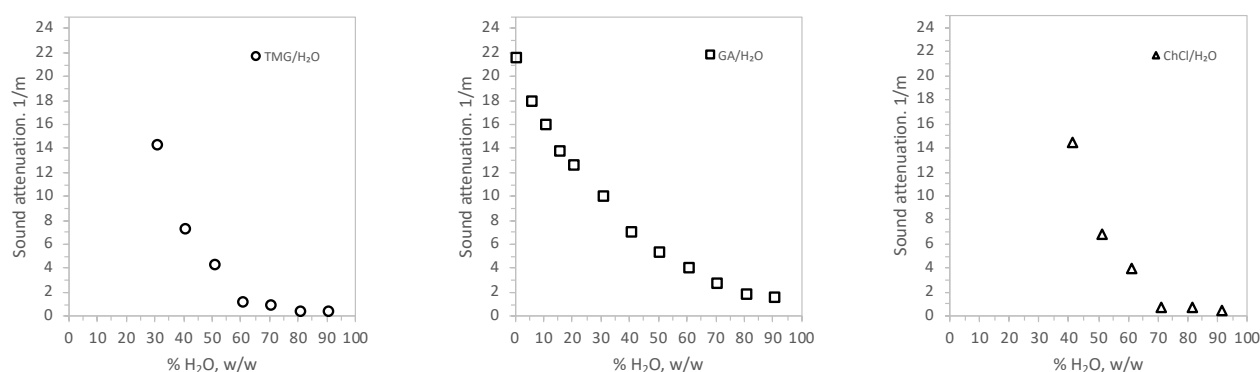
Figure 3 Ultrasound attenuation (1/m) at 25.0 ± 0.1 °C of water dilutions from 5% to 90% w/w of the aquoDESs: left, circles = trimethylglycine/water TMG/H_2O; center, squares = glycolic acid/water GA/H_2O; right, triangles = choline chloride/water ChCl/H_2O.

The measured attenuation values increased moving from high to lower water contents, as a function of DESs concentration. However, the increase of attenuation is not linear but it follows a typical trend. Specifically, for TMG/H_2O and ChCl/H_2O a slope change can be recognized in the attenuation profiles at a water content of 60% w/w and 70% w/w, respectively, corresponding to the starting of DESs aggregation as evidenced by ionic conductivity measurements. GA/H_2O showed a slightly different profile, since a clear change in the slope of the attenuation trend was not observed, but a progressive increase in the curves was observed by increasing the GA content. This difference can be related to the behavior of glycolic acid, which can undergo also to self-aggregation or also more complex structures in water as also reported in literature[65,66]. TMG/H_2O and ChCl/H_2O DESs at the lower water contents (below 30 and 40% w/w, respectively) showed measurements of sound attenuation with too high errors, due to poor accuracy in the resonance frequency used for the analysis.

NMR experiments

The three DESs have been studied by NMR spectroscopy firstly without any dilution, with the specific aim to preserve their structure, using a sealed capillary containing a deuterated solvent (generally benzene-d_6 or acetone-d_6) as an external reference. The proton spectra of the three systems are in the Supporting Information (Figures S3, S4 and S5). It is interesting to note that in the case of DESs GA/H_2O and TMG/H_2O the NMR signal from water and those from the -OH groups are fused together, likely because a fast proton exchange between the different sites. ChCl/H_2O DES is an exception and in this case two different peaks are present, at 4.383 and 3.479 ppm. The former peak integral is less than 1, but the latter one exceeds its natural value (8) and the sum of the two integral

values accounts for all the -OH protons of the liquids. This can be explained assuming that the two sites are in chemical exchange, but with a rate that is not fast enough to collapse them in the same peak, as for the previous cases. The same phenomenon has been recently observed for other DESs containing ChCl and water[67]. Increasing the amount of water up to 26% w/w, the two -OH peaks overlap and coalesce, as the exchange process becomes faster than NMR temporal resolution.

To obtain structural information, diffusional NMR techniques have been used on pure and diluted DESs. The diffusion coefficients D_t, the average hydrodynamic radii r_H and the volumes V_H of the aggregates as a function of the percentage of added water are reported in Table 1.

Table 1: Diffusion coefficients ($10^{10} D_t$, m^2 s^{-1}), average hydrodynamic radii (r_H, Å) and volumes (V_H) as a function of the percentage of added water (%H_2O, w/w).

%H_2O	D_t	r_H	V_H
	TMG/H_2O		
0	0.912	5.49	698.7
6.5	1.15	5.27	613.7
12.2	1.48	4.85	476.8
20.7	1.94	4.68	430.4
29.0	2.48	4.59	405.7
46.0	3.70	4.30	333.1
	GA/H_2O		
0	3.37	4.99	519.4
7.9	3.48	4.64	419.3
9.9	3.57	4.61	411.5
16.3	3.92	4.35	343.9
32.2	4.79	4.20	310.7
47.4	5.98	4.15	299.5
	ChCl/H_2O		
0	1.10	4.95	506.9
12.6	2.05	4.29	331.8
26.8	3.17	4.06	280.5
46.0	5.11	3.91	250.8

In all the cases the dilution causes a monotonical increasing of D_t, which reflects in a decreasing of the average hydrodynamic radius. This demonstrates the presence of large aggregates in the pure DESs, whose size decreases when the water content increases. At high dilution, the sensitivity of the technique decreases.

From the value of r_H and considering only spherical aggregates, hydrodynamic volumes (V_H) can be derived. The conversion of V_H to an aggregation number (how many molecules form the aggregate) is not straightforward, as an aggregate contains an unknown number of the DES component (or components, in case of ChCl cation and anion can diffuse independently) and H_2O, which have different volumes. However, it is plausible these aggregates are water/counterpart rather than non-water components only aggregates because the values of their concentrations are much higher that their maximum water solubility.

Despite this, it is interesting to compare V_H values observed with the van der Waals volume of the species involved:

- for TMG/H_2O, V_H goes from 698.7 to 230.9 Å^3 (%H_2O = 71.2% w/w) which can be compared with the volume of a single TMG molecule, 145 Å^3;

- for GA/H_2O, V_H goes from 519.4 to 262.9 Å^3 (%H_2O = 64.4% w/w), which can be compared with the volume of a single GA molecule, 86 Å^3;

- for ChCl/H_2O, V_H goes from 860.3 to 184.7 Å^3 (%H_2O = 79.6% w/w), which can be compared with the volume of a single ChCl molecule, 185 Å^3.

For a clearer visualization of the results, Figure 4 shows the same data of Table 1 reporting the trend of D_t and r_H with the degree of dilution.
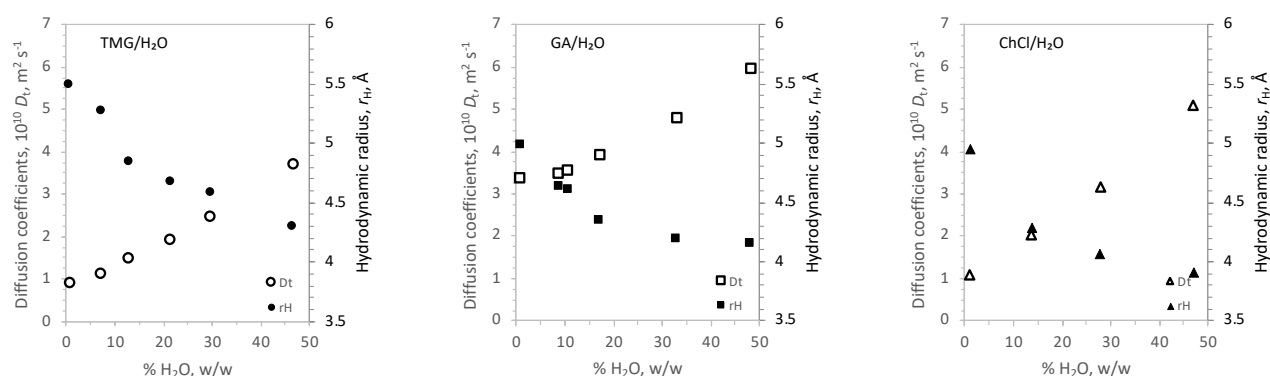

Figure 4 NMR Diffusion Coefficients D_t (empty symbols) and average hydrodynamic radii r_H (filled symbols) at 25.0 ± 0.1 °C of water dilutions from 0% to 50% w/w of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H_2O; center, squares = glycolic acid/water GA/H_2O; right, triangles = choline chloride/water ChCl/H_2O.

As it can be seen in Figure 4, in all the cases a discontinuity of r_H is present around 10-15% of added water, indicating some kind of change in the structuration of the solvent. These changes at low dilutions were not evidenced by conductometric curves but are reported in the literature for the structural role of the added water. In this case the water itself is a DESs component, therefore the behavior could be slightly different or peculiar. Further deeper and more focused studies are needed to elucidate this behavior. Anyway, the aggregation remains significant up to 50% of added water, in agreement with conductivity measurements and with the literature[41,46].

Cytotoxicity of *aquo*DESs dilutions

The disposal of the DESs via their dilution with water and subsequently with water treatment plants emerged in recent studies as an environmentally feasible solution[68]. Therefore, the study of the toxicity of water dilutions of DESs has a prominent importance for their use.

In this work, the suitability of *aquo*DESs dilutions as biocompatible solvents was evaluated by studying their cytotoxicity on the human Caco-2 cell line. The experiments were conducted with the DESs' dilutions as well as with water solutions of the single components (GA, TMG and ChCl) at the same concentrations as in the corresponding DESs dilution. This was made considering that in the range of the tested concentrations (all abundantly under 50% w/w) the DESs' molecules are disaggregated to give solutions of the single components.

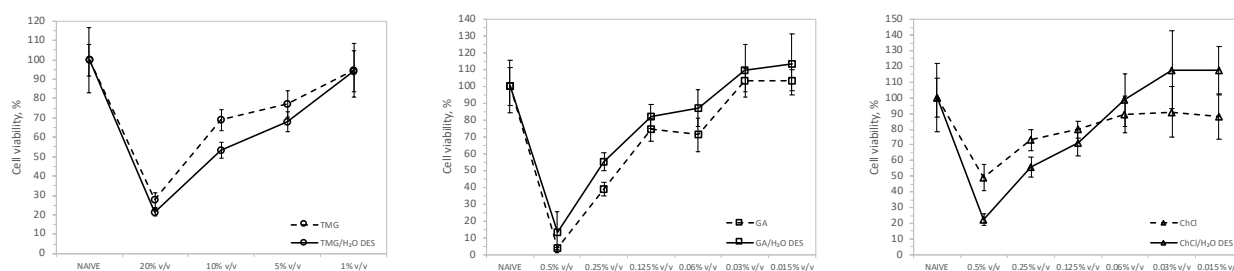The results of the DESs as well as the ones of the DESs' forming molecules are shown in Figure 5.

Figure 5: Cytotoxicity on Caco2 cells of *aquo*DESs water dilutions (lines) and DESs' forming molecules (dashed lines): left, circles = TMG/H$_2$O DES and TMG; center, squares = GA/H$_2$O DES and GA; right, triangles = ChCl/H$_2$O DES and ChCl.

ChCl/H$_2$O and GA/H$_2$O DESs did not show any toxic effect when tested at concentrations lower than 0.125% v/v. TMG/H$_2$O did not show any toxicity at concentrations lower than 1% v/v. The cytotoxicity values observed for *aquo*DESs dilutions did not exhibit significant differences compared

to the cytotoxicity of the non-water components of the liquids (Figure 5). This confirms the hypothesis that the toxicity of the DESs can be attributed to the toxicity of the non-water component of the liquids present in a dissociated state when tested separately. The concentrations at which cytotoxic effects were observed suggest a high level of biocompatibility, especially for the TMG-based liquid dilutions. Trimethylglycine (betaine) is a molecule already recognized as safe for food applications and as a functional ingredient[69,70], therefore a liquid formed by it and by water clearly showed low cytotoxicity. Cytotoxicity studies on Caco2 cells of other Choline chloride-based DESs (ChCl/Glycerol) on Caco2 cells reported in the literature showed results similar to the ones of the ChCl/H_2O liquid dilutions studied in this work, with values of about 0.1% v/v of dilution of the DES giving good survival percentage of the cells[71]. This suggests the effect of the toxicity to be related to the choline chloride molecule considering the very low toxicity of glycerol molecule[72]. The glycolic acid-based liquid dilutions showed similar, but still good, values of cytotoxicity, and this correlates with the glycolic acid properties as it is more suitable for dermatological applications[73].

CONCLUSIONS

The DESs formed by water as a component of binary mixtures, i.d. *aquo*DESs betaine/water, glycolic acid/water and choline chloride/water, behave in water dilutions forming aggregates; these clusters decrease in dimensions by increasing the water amounts. The DESs structures are preserved until over about 50% w/w of added water, then leading to solutions of the components at higher dilutions. All the samples showed a reduction of the viscosity by increasing the water amounts, especially in this first region in which the DESs' structure in preserved, leading to values comparable to other common organic solvents or water. At low water concentrations, the water added seemed to have the same structural behaviour observed with other DESs in the literature, but further deeper studies are needed to elucidate this phenomenon that is not observed in conductometric curves.

These behaviours of the DESs in water dilutions are already reported in literature, but this is a peculiar case because these aggregates are clusters of water-based aggregates in water. So, this work underlines again the DESs' identity of the GA/H_2O and TMG/H_2O *aquo*DESs, even though it was already demonstrated with the theoretical/experimental melting curves comparison. The choline chloride/water DES showed the same behaviour of GA/H_2O and TMG/H_2O *aquo*DESs, so these data seem to underline the DES' identity even for this controversial mixture.

Taking into account the disposal in water dilution of a DES as a relevant and convenient way for their discarding, the cytotoxicity on Caco2 cells was evaluated for all the three *aquo*DESs water dilutions: these data underlined the toxicity of these liquids to be related to the non-water component. This could "split in a half" their toxicity matters as one of the two components is water. Trimethylglycine/water DES showed the best results in terms of a having a low toxicity, related to the betaine itself, which is an edible molecule that is found in many different food matrixes.

AUTHOR CONTRIBUTION

Elison Lepore: Investigation, Validation, Methodology; **Gianluca Ciancaleoni:** Investigation, Data curation, Writing - original draft; Methodology **Diego Romano Perinelli:** Investigation, Methodology, Visualization, Writing - original draft; **Giulia Bonacucina:** Resources, Validation, Writing - review & editing; **Serena Gabrielli:** Validation, Visualization, Methodology, Writing - review & editing; **Gaia de Simone:** Investigation, Validation, Methodology; **Rosita Gabbianelli:** Resources, Writing - review & editing; **Laura Bordoni:** Validation, Methodology, Writing - original draft; **Matteo Tiecco:** Conceptualization, Methodology, Data curation, Supervision, Formal analysis, Project administration, Writing - original draft, Funding acquisition, Software, Validation, Visualization.

AKNOWLEDGEMENTS

REFERENCES

[1]   F. Perera, K. Nadeau, Climate Change, Fossil-Fuel Pollution, and Children's Health, N. Engl. J. Med. 386 (2022) 2303–2314. doi:10.1056/NEJMra2117706.

[2]   X.Y.D. Soo, J.J.C. Lee, W.-Y. Wu, L. Tao, C. Wang, Q. Zhu, J. Bu, Advancements in CO_2 capture by absorption and adsorption: A comprehensive review, J. CO_2 Util. 81 (2024) 102727.

[3]   J. Liao, X. Liu, X. Zhou, N.R. Tursunova, Analyzing the role of renewable energy transition and industrialization on ecological sustainability: can green innovation matter in OECD countries, Renew. Energy. 204 (2023) 141–151.

[4]   European Commission, Circular economy action plan, Eur. Comm. (2020) 28. doi:10.2775/855540.

[5]   W. Wang, M.A. Rehman, S. Fahad, The dynamic influence of renewable energy, trade openness, and industrialization on the sustainable environment in G-7 economies, Renew. Energy. 198 (2022) 484–491.

[6]   V.K. Ahluwalia, Green chemistry: Environmentally benign reactions, Springer Nature, 2021.

[7]   F. Cardellini, L. Brinchi, R. Germani, M. Tiecco, Convenient Esterification of Carboxylic Acids by SN2 Reaction Promoted by a Protic Ionic Liquid System Formed in Situ, in Solvent-Free Conditions, Synth. Commun. 44 (2014) 3248–3256. doi:10.1080/00397911.2014.933353.

[8]   S.R. Cicco, G.M. Farinola, C. Martinelli, F. Naso, M. Tiecco, Pd-promoted homocoupling reactions of unsaturated silanes in aqueous micelles, European J. Org. Chem. (2010) 2275–2279. doi:10.1002/ejoc.201000021.

[9]   D. Pan, F. Su, H. Liu, C. Liu, A. Umar, L. Castañeda, H. Algadi, C. Wang, Z. Guo, Research progress on catalytic pyrolysis and reuse of waste plastics and petroleum sludge, ES Mater. Manuf. 11 (2021) 3–15.

[10]   V. Hessel, N.N. Tran, M.R. Asrami, Q.D. Tran, N.V.D. Long, M. Escribà-Gelonch, J.O. Tejada, S. Linke, K. Sundmacher, Sustainability of green solvents–review and perspective, Green Chem. 24 (2022) 410–437.

[11]   M. Rollo, F. Raffi, E. Rossi, M. Tiecco, E. Martinelli, G. Ciancaleoni, Depolymerization of polyethylene terephthalate (PET) under mild conditions by Lewis/Brønsted acidic deep eutectic solvents, Chem. Eng. J. 456 (2023) 141092. doi:https://doi.org/10.1016/j.cej.2022.141092.

[12]   N. Winterton, The green solvent: A critical perspective, Clean Technol. Environ. Policy. 23 (2021) 2499–2522.

[13]   M. Yang, F. Li, C. Huang, L. Tong, X. Dai, H. Xiao, VOC characteristics and their source apportionment in a coastal industrial area in the Yangtze River Delta, China, J. Environ. Sci. 127 (2023) 483–494. doi:https://doi.org/10.1016/j.jes.2022.05.041.

[14]   A. Paiva, R. Craveiro, I. Aroso, M. Martins, R.L. Reis, A.R.C. Duarte, Natural deep eutectic solvents–solvents for the 21st century, ACS Sustain. Chem. Eng. 2 (2014) 1063–1071.

doi:https://doi.org/10.1021/sc500096j.

[15] D.A. Alonso, A. Baeza, R. Chinchilla, G. Guillena, I.M. Pastor, D.J. Ramón, Deep eutectic solvents: The organic reaction medium of the century, European J. Org. Chem. 2016 (2016) 612–632. doi:http://dx.doi.org/10.1002/ejoc.201501197.

[16] M. Tiecco, A. Grillo, E. Mosconi, W. Kaiser, T. Del Giacco, R. Germani, Advances in the development of novel green liquids: thymol/water, thymol/urea and thymol/phenylacetic acid as innovative hydrophobic natural deep eutectic solvents, J. Mol. Liq. 364 (2022) 120043. doi:https://doi.org/10.1016/j.molliq.2022.120043.

[17] A. Isci, M. Kaltschmitt, Recovery and recycling of deep eutectic solvents in biomass conversions: A review, Biomass Convers. Biorefinery. 12 (2022) 197–226.

[18] M.S. Rahman, R. Roy, B. Jadhav, M.N. Hossain, M.A. Halim, D.E. Raynie, Formulation, structure, and applications of therapeutic and amino acid-based deep eutectic solvents: An overview, J. Mol. Liq. 321 (2021) 114745. doi:https://doi.org/10.1016/j.molliq.2020.114745.

[19] B.B. Hansen, S. Spittle, B. Chen, D. Poe, Y. Zhang, J.M. Klein, A. Horton, L. Adhikari, T. Zelovich, B.W. Doherty, B. Gurkan, E.J. Maginn, A. Ragauskas, M. Dadmun, T.A. Zawodzinski, G.A. Baker, M.E. Tuckerman, R.F. Savinell, J.R. Sangoro, Deep Eutectic Solvents: A Review of Fundamentals and Applications, Chem. Rev. 121 (2021) 1232–1285. doi:10.1021/acs.chemrev.0c00385.

[20] J. González-Rivera, C. Pelosi, E. Pulidori, C. Duce, M.R. Tiné, G. Ciancaleoni, L. Bernazzani, Guidelines for a correct evaluation of Deep Eutectic Solvents thermal stability, Curr. Res. Green Sustain. Chem. 5 (2022) 100333. doi:https://doi.org/10.1016/j.crgsc.2022.100333.

[21] T. El Achkar, H. Greige-Gerges, S. Fourmentin, Basics and properties of deep eutectic solvents: a review, Environ. Chem. Lett. 19 (2021) 3397–3408.

[22] A. Shishov, A. Pochivalov, L. Nugbienyo, V. Andruch, A. Bulatov, Deep eutectic solvents are not only effective extractants, TrAC Trends Anal. Chem. 129 (2020) 115956. doi:https://doi.org/10.1016/j.trac.2020.115956.

[23] F. Cardellini, R. Germani, G. Cardinali, L. Corte, L. Roscini, N. Spreti, M. Tiecco, Room temperature deep eutectic solvents of (1S)-(+)-10-camphorsulfonic acid and sulfobetaines: Hydrogen bond-based mixtures with low ionicity and structure-dependent toxicity, RSC Adv. 5 (2015) 31772–31786. doi:10.1039/C5RA03932K.

[24] M. Marchel, H. Cieśliński, G. Boczkaj, Deep eutectic solvents microbial toxicity: Current state of art and critical evaluation of testing methods, J. Hazard. Mater. 425 (2022) 127963.

[25] J. Torregrosa-Crespo, X. Marset, G. Guillena, D.J. Ramón, R.M. Martínez-Espinosa, New guidelines for testing "Deep eutectic solvents" toxicity and their effects on the environment and living beings, Sci. Total Environ. 704 (2020) 135382.

[26] T.-L. Chen, H. Kim, S.-Y. Pan, P.-C. Tseng, Y.-P. Lin, P.-C. Chiang, Implementation of green chemistry principles in circular economy system towards sustainable development goals: Challenges and perspectives, Sci. Total Environ. 716 (2020) 136998.

[27] M. Zhang, R. Tian, H. Han, K. Wu, B. Wang, Y. Liu, Y. Zhu, H. Lu, B. Liang, Preparation strategy and stability of deep eutectic solvents: A case study based on choline chloride-carboxylic acid, J. Clean. Prod. 345 (2022) 131028.

[28] M. Fronduti, T. Del Giacco, E. Rossi, M. Tiecco, R. Germani, Insights into the structural features of deep eutectic solvents: the eutectic point as an unicum in their physical properties and the surface tension as a method for its determination, J. Mol. Liq. 379 (2023) 121679.

[29] C.F. Araujo, J.A.P. Coutinho, M.M. Nolasco, S.F. Parker, P.J.A. Ribeiro-Claro, S. Rudić, B.I.G. Soares, P.D. Vaz, Inelastic neutron scattering study of reline: shedding light on the hydrogen bonding network of deep eutectic solvents, Phys. Chem. Chem. Phys. 19 (2017) 17998–18009. doi:DOI: 10.1039/C7CP01286A.

[30] D.O. Abranches, J.A.P. Coutinho, Type V deep eutectic solvents: Design and applications, Curr. Opin. Green Sustain. Chem. 35 (2022) 100612. doi:https://doi.org/10.1016/j.cogsc.2022.100612.

[31] N. Schaeffer, D.O. Abranches, L.P. Silva, M.A.R. Martins, P.J. Carvalho, O. Russina, A. Triolo, L. Paccou, Y. Guinet, A. Hedoux, Non-Ideality in Thymol+ Menthol Type V Deep Eutectic Solvents, ACS Sustain. Chem. Eng. 9 (2021) 2203–2211.

[32] D.O. Abranches, J.A.P. Coutinho, Everything You Wanted to Know about Deep Eutectic Solvents but Were Afraid to Be Told, Annu. Rev. Chem. Biomol. Eng. 14 (2023). doi:https://doi.org/10.1146/annurev-chembioeng-101121-085323.

[33] K.A. Omar, R. Sadeghi, Database of deep eutectic solvents and their physical properties: A review, J. Mol. Liq. (2023) 121899.

[34] A.P. Santana, J.A. Mora-Vargas, T.G.S. Guimaraes, C.D.B. Amaral, A. Oliveira, M.H. Gonzalez, Sustainable synthesis of natural deep eutectic solvents (NADES) by different methods, J. Mol. Liq. 293 (2019) 111452.

[35] Y. Liu, J.B. Friesen, J.B. McAlpine, D.C. Lankin, S.-N. Chen, G.F. Pauli, Natural deep eutectic

solvents: properties, applications, and perspectives, J. Nat. Prod. 81 (2018) 679–690.

[36] M. Tiecco, D.A. Alonso, D.R. Ñíguez, G. Ciancaleoni, G. Guillena, D.J. Ramón, A.A. Bonillo, R. Germani, Assessment of the organocatalytic activity of chiral l-Proline-based Deep Eutectic Solvents based on their structural features, J. Mol. Liq. 313 (2020) 113573. doi:10.1016/j.molliq.2020.113573.

[37] S.V. Giofrè, M. Tiecco, A. Ferlazzo, R. Romeo, G. Ciancaleoni, R. Germani, D. Iannazzo, Base-free Copper-Catalyzed Azide–Alkyne Click Cycloadditions (CuAAc) in Natural Deep Eutectic Solvents as Green and Catalytic Reaction Media, European J. Org. Chem. (2021). doi:https://doi.org/10.1002/ejoc.202100698.

[38] S. Khandelwal, Y.K. Tailor, M. Kumar, Deep eutectic solvents (DESs) as eco-friendly and sustainable solvent/catalyst systems in organic transformations, J. Mol. Liq. 215 (2016) 345–386.

[39] A.E. Ünlü, A. Arıkaya, S. Takaç, Use of deep eutectic solvents as catalyst: A mini-review, Green Process. Synth. 8 (2019) 355–372.

[40] T. Palomba, G. Ciancaleoni, T. Del Giacco, R. Germani, F. Ianni, M. Tiecco, Deep Eutectic Solvents formed by chiral components as chiral reaction media and studies of their structural properties, J. Mol. Liq. 262 (2018) 285–294. doi:10.1016/j.molliq.2018.04.096.

[41] F. Gabriele, M. Chiarini, R. Germani, M. Tiecco, N. Spreti, Effect of water addition on choline chloride/glycol deep eutectic solvents: Characterization of their structural and physicochemical properties, J. Mol. Liq. 291 (2019) 111301. doi:https://doi.org/10.1016/j.molliq.2019.111301.

[42] C. Ma, A. Laaksonen, C. Liu, X. Lu, X. Ji, The peculiar effect of water on ionic liquids and deep eutectic solvents, Chem. Soc. Rev. (2018). doi:https://doi.org/10.1039/C8CS00325D.

[43] D.J.G.P. van Osch, L.F. Zubeir, A. van den Bruinhorst, M.A.A. Rocha, M.C. Kroon, Hydrophobic deep eutectic solvents as water-immiscible extractants, Green Chem. 17 (2015) 4518–4521. doi:10.1039/C5GC01451D.

[44] M. Tiecco, F. Cappellini, F. Nicoletti, T. Del Giacco, R. Germani, P. Di Profio, Role of the hydrogen bond donor component for a proper development of novel hydrophobic deep eutectic solvents, J. Mol. Liq. 281 (2019) 423–430. doi:10.1016/j.molliq.2019.02.107.

[45] Y. Dai, G.-J. Witkamp, R. Verpoorte, Y.H. Choi, Tailoring properties of natural deep eutectic solvents with water to facilitate their applications, Food Chem. 187 (2015) 14–19. doi:https://doi.org/10.1016/j.foodchem.2015.03.123.

[46] O.S. Hammond, D.T. Bowron, K.J. Edler, The effect of water upon deep eutectic solvent nanostructure: An unusual transition from ionic mixture to aqueous solution, Angew. Chemie Int. Ed. 56 (2017) 9782–9785. doi:https://doi.org/10.1002/anie.201702486.

[47] M.S. Rahman, D.E. Raynie, Thermal behavior, solvatochromic parameters, and metal halide solvation of the novel water-based deep eutectic solvents, J. Mol. Liq. 324 (2021) 114779.

[48] E. Picciolini, G. Pastore, T. Del Giacco, G. Ciancaleoni, M. Tiecco, R. Germani, aquo-DESs: Water-based binary natural deep eutectic solvents, J. Mol. Liq. 383 (2023) 122057.

[49] M. Sajjadur Rahman, J. Kyeremateng, M. Saha, S. Asare, N. Uddin, M.A. Halim, D.E. Raynie, Evaluation of the experimental and computed properties of choline chloride-water formulated deep eutectic solvents, J. Mol. Liq. 350 (2022) 118520. doi:https://doi.org/10.1016/j.molliq.2022.118520.

[50] E. Mangiacapre, F. Castiglione, M. D'Aristotile, V. Di Lisio, A. Triolo, O. Russina, Choline chloride-water mixtures as new generation of green solvents: A comprehensive physico-chemical study, J. Mol. Liq. 383 (2023) 122120. doi:https://doi.org/10.1016/j.molliq.2023.122120.

[51] A. van den Bruinhorst, J. Avila, M. Rosenthal, A. Pellegrino, M. Burghammer, M. Costa Gomes, Defying decomposition: the curious case of choline chloride, Nat. Commun. 14 (2023) 6684.

[52] A.I.M.C. Lobo Ferreira, S.M. Vilas-Boas, R.M.A. Silva, M.A.R. Martins, D.O. Abranches, P.C.R. Soares-Santos, F.A. Almeida Paz, O. Ferreira, S.P. Pinho, L.M.N.B.F. Santos, J.A.P. Coutinho, Extensive characterization of choline chloride and its solid–liquid equilibrium with water, Phys. Chem. Chem. Phys. 24 (2022) 14886–14897. doi:10.1039/D2CP00377E.

[53] J. Hansen, P. Bross, A cellular viability assay to monitor drug toxicity, Protein Misfolding Cell. Stress Dis. Aging Concepts Protoc. (2010) 303–311.

[54] P. Di Profio, R. Germani, L. Goracci, R. Grilli, G. Savelli, M. Tiecco, Interaction between DNA and cationic amphiphiles: a multi-technique study, Langmuir. 26 (2010) 7885–7892. doi:10.1021/la9047825.

[55] C. Comminges, R. Barhdadi, M. Laurent, M. Troupel, Determination of Viscosity, Ionic Conductivity, and Diffusion Coefficients in Some Binary Systems: Ionic Liquids + Molecular Solvents, J. Chem. Eng. Data. 51 (2006) 680–685. doi:10.1021/je0504515.

[56] A. Suzuki, H. Sato, R. Kikuchi, Ionic conductivity in binary systems, Phys. Rev. B. 29 (1984) 3550–3566. doi:10.1103/PhysRevB.29.3550.

[57] N.M. Sammes, G.A. Tompsett, H. Näfe, F. Aldinger, Bismuth based oxide electrolytes—structure and ionic conductivity, J. Eur. Ceram. Soc. 19 (1999) 1801–1826. doi:https://doi.org/10.1016/S0955-2219(99)00009-6.

[58] M. Tiecco, I.D. Guida, P.L. Gentili, R. Germani, C. Bonaccorso, A. Cesaretti, Probing the Structural Features and the Micro-heterogeneity of Various Deep Eutectic Solvents and their Water Dilutions by the Photophysical Behaviour of Two Fluorophores, ChemRxiv. (2020). doi:10.26434/chemrxiv.13395683.v1.

[59] S.H. Yalkowsky, Y. He, P. Jain, Handbook of aqueous solubility data, CRC press, 2016.

[60] H. Kivela, M. Salomaki, P. Vainikka, E. Makila, F. Poletti, S. Ruggeri, F. Terzi, J. Lukkari, Effect of water on a hydrophobic deep eutectic solvent, J. Phys. Chem. B. 126 (2022) 513–527.

[61] G. Gygli, X. Xu, J. Pleiss, Meta-analysis of viscosity of aqueous deep eutectic solvents and their components, Sci. Rep. 10 (2020) 21395.

[62] V. Buckin, High-resolution ultrasonic spectroscopy, J. Sensors Sens. Syst. 7 (2018) 207–217.

[63] G. Bonacucina, D.R. Perinelli, M. Cespi, L. Casettari, R. Cossi, P. Blasi, G.F. Palmieri, Acoustic spectroscopy: a powerful analytical method for the pharmaceutical field?, Int. J. Pharm. 503 (2016) 174–195.

[64] T.A. Litovitz, C.M. Davis, Physical acoustics, Vol. II, Part A., edited by WP Mason, (1964).

[65] M. Lackinger, W.M. Heckl, Carboxylic acids: versatile building blocks and mediators for two-dimensional supramolecular self-assembly, Langmuir. 25 (2009) 11307–11321.

[66] A.K. Roy, J.R. Hart, A.J. Thakkar, Clusters of glycolic acid and 16 water molecules, Chem. Phys. Lett. 434 (2007) 176–181. doi:https://doi.org/10.1016/j.cplett.2006.12.010.

[67] C. Pelosi, J. Gonzalez-Rivera, M.R. Tiné, G. Ciancaleoni, L. Bernazzani, C. Duce, Evaluation of physicochemical properties of Type-II deep eutectic solvents/Water mixtures based on choline chloride and calcium/magnesium chloride hexahydrate, J. Mol. Liq. 392 (2023) 123459. doi:https://doi.org/10.1016/j.molliq.2023.123459.

[68] P.D. de María, S. Kara, On the fate of deep eutectic solvents after their use as reaction media: the CO 2 production during downstream and ultimate disposal, RSC Sustain. 2 (2024) 608–615.

[69] D. Dobrijević, K. Pastor, N. Nastić, F. Özogul, J. Krulj, B. Kokić, E. Bartkiene, J.M. Rocha, J. Kojić, Betaine as a functional ingredient: metabolism, health-promoting attributes, food sources, applications and analysis methods, Molecules. 28 (2023) 4824.

[70] M. Lever, S. Slow, The clinical significance of betaine, an osmolyte with a key role in methyl

group metabolism, Clin. Biochem. 43 (2010) 732–744.

[71] K. Saiswani, A. Narvekar, D. Jahagirdar, R. Jain, P. Dandekar, Choline chloride: glycerol deep eutectic solvents assist in the permeation of daptomycin across Caco-2 cells mimicking intestinal bilayer, J. Mol. Liq. 383 (2023) 122051.

[72] E.P. on F.A. and N.S. added to F. (ANS), A. Mortensen, F. Aguilar, R. Crebelli, A. Di Domenico, B. Dusemund, M.J. Frutos, P. Galtier, D. Gott, U. Gundert-Remy, Re-evaluation of glycerol (E 422) as a food additive, EFSA J. 15 (2017) e04720.

[73] J. Sharad, Glycolic acid peel therapy–a current review, Clin. Cosmet. Investig. Dermatol. (2013) 281–288.

SUPPORTING INFORMATION

CLUSTER AGGREGATION OF WATER-BASED DEEP EUTECTIC SOLVENTS IN WATER AND EVALUATION OF THEIR CYTOTOXICITY

Elison Lepore,[a] Gianluca Ciancaleoni,[b] Diego Romano Perinelli,[a] Giulia Bonacucina,[a] Serena Gabrielli,[c] Gaia de Simone,[d] Rosita Gabbianelli,[d] Laura Bordoni,[d] Matteo Tiecco[a]*

[a]*Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Italy.*
[b]*Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Giuseppe Moruzzi 13, I-56124 Pisa, Italy.*
[c]*Scuola di Scienze e Tecnologie, Università di Camerino, Via. S. Agostino 1, 62032, Camerino (MC), Italy.*
[d]*Unit of Molecular Biology and Nutrigenomics, Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Italy.*

* Corresponding Author: Dr Matteo Tiecco - matteo.tiecco@unicam.it

Scuola di Scienze del Farmaco e Prodotti della Salute, Università di Camerino, via Madonna delle Carceri, 62032 Camerino (MC), Ital
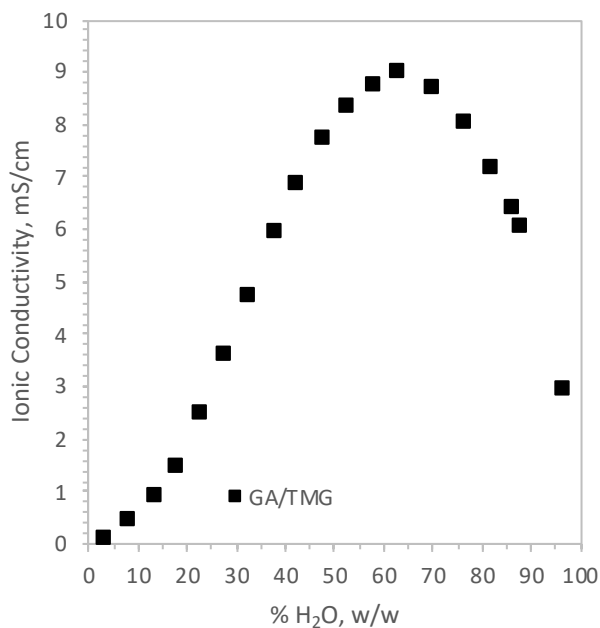
Figure S1: Ionic conductivity profile of water dilutions of glycolic acid/trimethylglycine (2/1 molar ratio) DES from 0% w/w to 95% w/w of added water at 25.0 ± 0.1 °C.
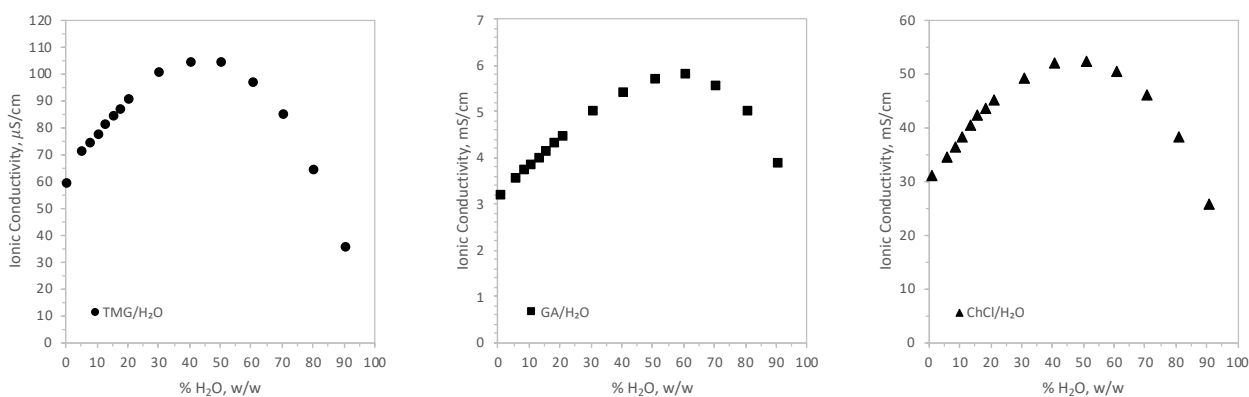

Figure S2: Ionic Conductivities at 25.0 ± 0.1 °C of water dilutions from 5% to 90% w/w (with increased reads at low percentages) of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H_2O; center, squares = glycolic acid/water GA/H_2O; left, triangles = Choline chloride/water ChCl/H_2O.
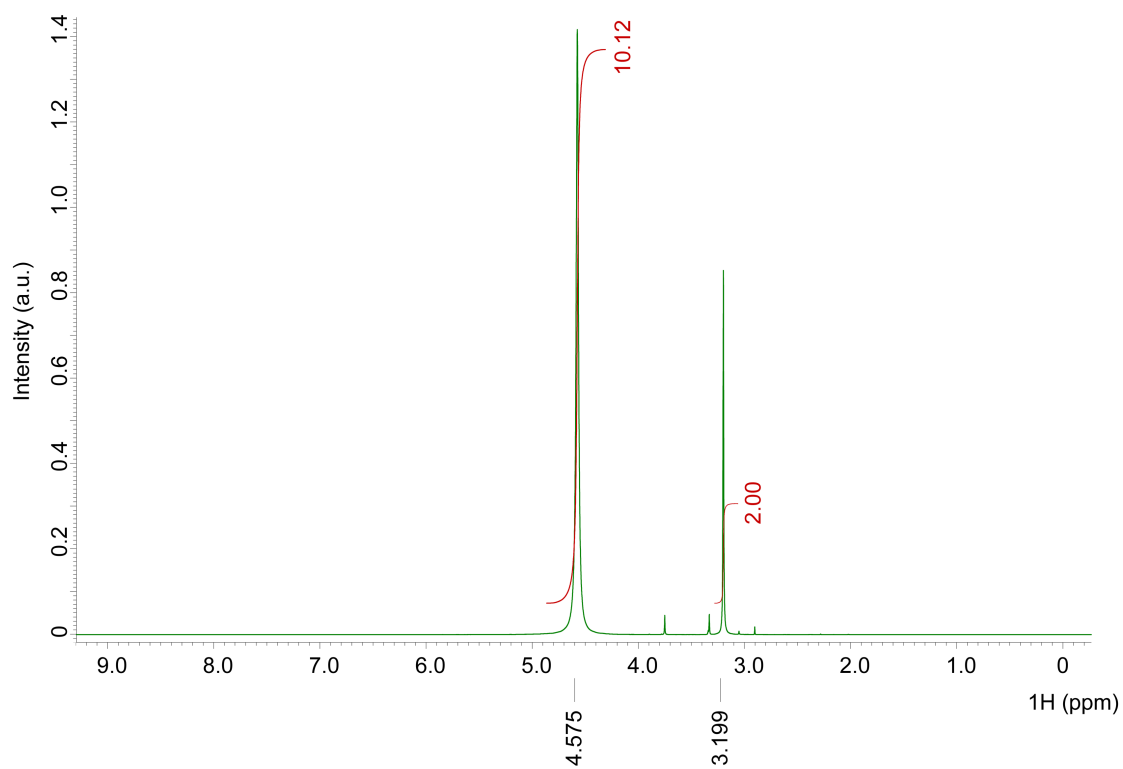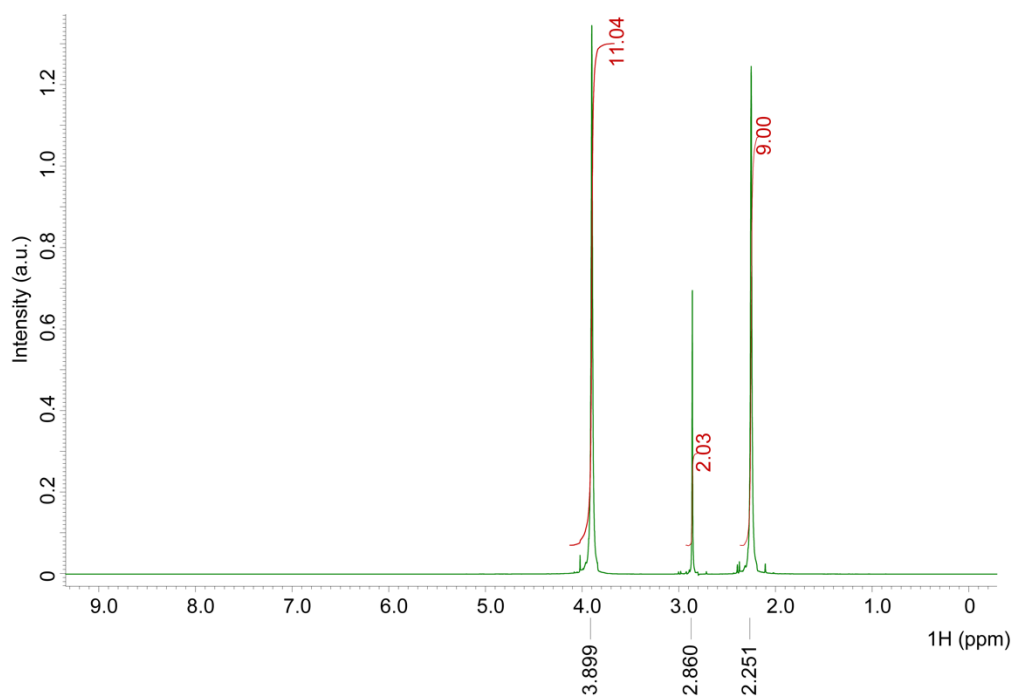
Figure S3. ^{1}H NMR spectrum of the DES GA/H_2O 1/4.
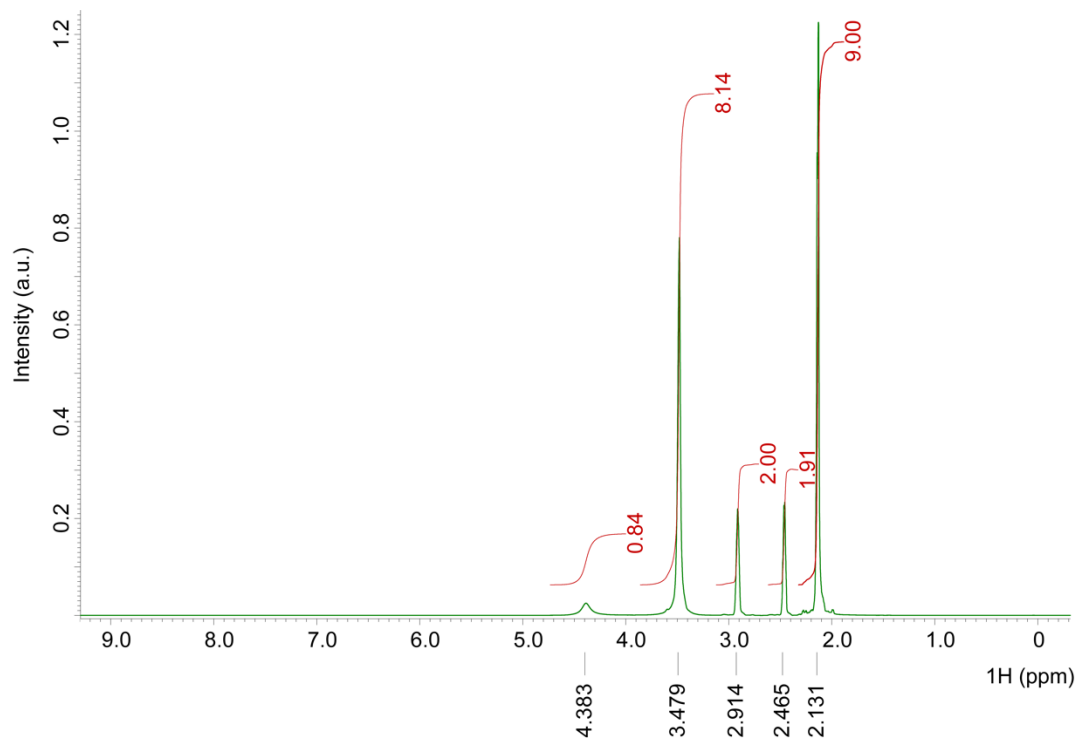

Figure S4. ^{1}H NMR spectrum of the DES TMG/H_2O 1/5.

Figure S5. ^{1}H NMR spectrum of the DES ChCl/H_2O 1/4.