



# Cluster aggregation of water-based deep eutectic solvents in water and evaluation of their cytotoxicity

Elison Lepore<sup>a</sup>, Gianluca Ciancaleoni<sup>b</sup>, Diego Romano Perinelli<sup>a</sup>, Giulia Bonacucina<sup>a</sup>,  
Serena Gabrielli<sup>c</sup>, Gaia de Simone<sup>d</sup>, Rosita Gabbianelli<sup>d</sup>, Laura Bordoni<sup>d</sup>, Matteo Tiecco<sup>a,\*</sup>

<sup>a</sup> School of Pharmacy, ChIP Research Center, University of Camerino, Via Madonna Delle Carceri, 62032 Camerino (MC), Italy

<sup>b</sup> Department of Chemistry and Industrial Chemistry, University of Pisa, Via Giuseppe Moruzzi 13, I-56124 Pisa, Italy

<sup>c</sup> School of Science and Technology, ChIP Research Center, University of Camerino, Via. S. Agostino 1, 62032 Camerino (MC), Italy

<sup>d</sup> Unit of Molecular Biology and Nutrigenomics, School of Pharmacy, University of Camerino, Via Madonna Delle Carceri, 62032 Camerino (MC), Italy

## ARTICLE INFO

### Keywords:

Deep eutectic solvents  
Physicochemical properties  
Structural properties  
Cytotoxicity

## 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.

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<sub>2</sub>O); betaine/water (TMG/H<sub>2</sub>O), choline chloride/water (ChCl/H<sub>2</sub>O). Ionic conductivity, viscosity, ultrasound spectroscopy and NMR techniques showed the presence of aggregates of *aquo*DESs in water dilutions. These aggregates are present in the solutions until values about 50 % w/w of added water, showing the presence of water-in-water structured systems. Moreover, 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. The water dilutions of water-based DESs have a great relevance for their applications, because their physical properties can be modulated as needed preserving their DESs' identities.

## 1. Introduction

The pollution prevention, the circular economy, the reduction of CO<sub>2</sub> 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

\* Corresponding author.

E-mail address: [matteo.tiecco@unicam.it](mailto:matteo.tiecco@unicam.it) (M. Tiecco).

<https://doi.org/10.1016/j.molliq.2024.126427>

Received 28 May 2024; Received in revised form 7 September 2024; Accepted 3 November 2024

Available online 6 November 2024

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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 downshift 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: 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 50–60 % 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<sub>2</sub>O 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<sub>2</sub>O); betaine/water (TMG/H<sub>2</sub>O); thymol/water (THY/H<sub>2</sub>O), a hydrophobic mixture thanks to the HBD role of thymol) and choline chloride/water (ChCl/H<sub>2</sub>O) [16,47–49]. This last ChCl/H<sub>2</sub>O 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<sub>2</sub>O, TMG/H<sub>2</sub>O and ChCl/H<sub>2</sub>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.

## 2. Experimental

### 2.1. 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<sub>2</sub>O<sub>5</sub> 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 were 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<sub>2</sub>O 1/4, TMG/H<sub>2</sub>O 1/5 and ChCl/H<sub>2</sub>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 (Merck), showing values spanning from 0.6 to 1.1 % w/w.

### 2.2. Ionic conductivity and viscosity measures

The ionic conductivity measures were performed using 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 through 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<sup>-1</sup>. The reported viscosity values were the mean of the viscosities determined in the shear rate range applied.

### 2.3. 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.

### 2.4. 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<sub>6</sub>D<sub>6</sub> or (CD<sub>3</sub>)<sub>2</sub>CO was used for external reference and technical operations (locking, shimming). <sup>1</sup>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 Eq. (1):

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

where  $I_0$  the intensity of the spin echo in the absence of gradient,  $D_t$  the self-diffusion coefficient,  $\Delta$  the delay between the midpoints of the

gradients (0.1 s),  $\delta$  the length of the gradient pulse (2 ms), and  $\gamma$  the magnetogyric ratio.

The shape of the gradients was rectangular, and their strength  $G$  was varied during the experiments. The self-diffusion coefficient,  $D_b$ , 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)):

$$D_t = \frac{k_B T}{c \pi \eta r_H} \quad (2)$$

where  $k_B$  is the Boltzmann constant,  $c$  is a numerical factor,  $\eta$  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)):

$$c = \frac{6}{1 + 0.695 \left( \frac{r_{\text{soln}}}{r_H} \right)^{2.234}} \quad (3)$$

### 2.5. Cell culture and cytotoxicity assay

The cytotoxicity of Deep Eutectic Solvents (glycolic acid/water (GA/H<sub>2</sub>O), betaine/water (TMG/H<sub>2</sub>O), and choline chloride/water (ChCl/H<sub>2</sub>O)) 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<sub>2</sub>. 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-dite-trazolium 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  $1 \times 10^4$  cells/well in 100  $\mu$ l 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 24 h. Both DESs non-water components have been sterilized with a 0.22  $\mu$ m syringe filter. For TMG/H<sub>2</sub>O, 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 *aquo*DESs dilutions for GA/H<sub>2</sub>O and ChCl/H<sub>2</sub>O, 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  $\mu$ l 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  $\pm$  standard deviation (SD). Results from each test (GA/H<sub>2</sub>O, TMG/H<sub>2</sub>O, ChCl/H<sub>2</sub>O and the individual compounds) were analysed by Kruskal-Wallis test followed by Bonferroni's correction for multiple testing. A value of  $p < 0.05$  was considered statistically significant.

## 3. Results and discussion

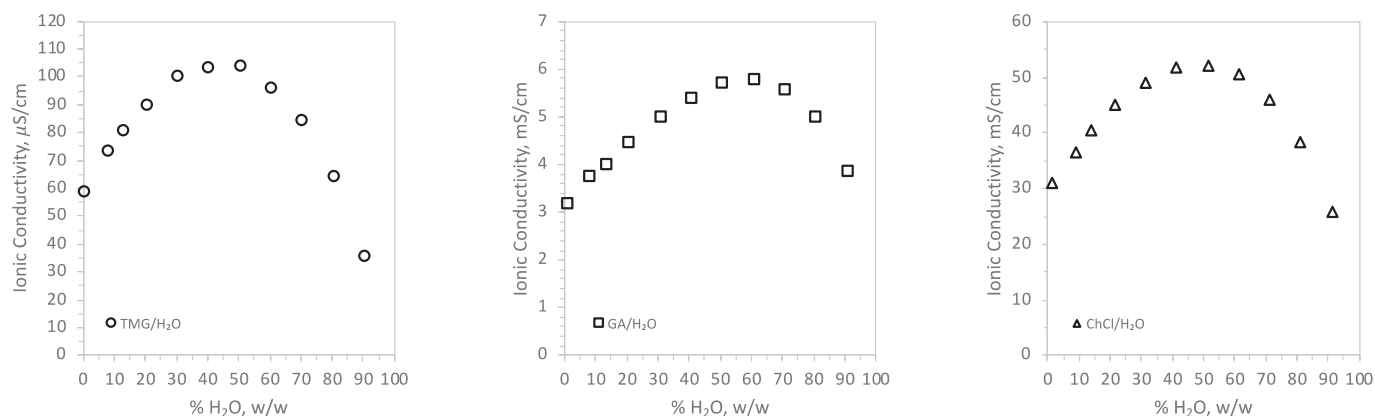
### 3.1. Ionic conductivity measures

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. This is usually reported in the literature at about 50 % w/w of added water [46]. In Fig. 1 the conductivity measures of the water dilutions of the three analyzed *aquo*DESs (TMG/H<sub>2</sub>O, GA/H<sub>2</sub>O, ChCl/H<sub>2</sub>O) are reported, in the dilution range from 5 % to 90 % w/w at the temperature of 25 °C.

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 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/H<sub>2</sub>O DES (GA maximum solubility in water = 7.894 mol/L); 45 % w/w of added water for TMG/H<sub>2</sub>O DES (TMG maximum solubility in water = 5.216 mol/L); about 40 % w/w of added water for ChCl/H<sub>2</sub>O DES (ChCl maximum solubility in water = 4.655 mol/L) [58]. 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 [44,59]. In the case of *aquo*DESs the curves are straight lines in this region. Moreover, the DES liquid formed by the two non-water components of these systems (glycolic acid/betaine 2/1 M 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 Figs. S1 and S2). This behavior of the *aquo*DESs dilutions was more deeply analyzed in this work with NMR techniques (see further 3.4 section).

The overall ionic conductivity values observed were strictly related



**Fig. 1.** Ionic Conductivities at  $25.0 \pm 0.1$  °C of water dilutions from 5 % to 90 % w/w of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H<sub>2</sub>O; center, squares = glycolic acid/water GA/H<sub>2</sub>O; right, triangles = Choline chloride/water ChCl/H<sub>2</sub>O.

to the nature of the components of the DESs: the presence of ionic species in ChCl/H<sub>2</sub>O DES determined its highest values in the set (in the order of magnitude of tens of mS/cm); the GA/H<sub>2</sub>O DES showed intermediate values, which derived from acid/base equilibria 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<sub>2</sub>O DES showed the lowest values in the set in the order of magnitude of tens of μS/cm.

### 3.2. Viscosity measures

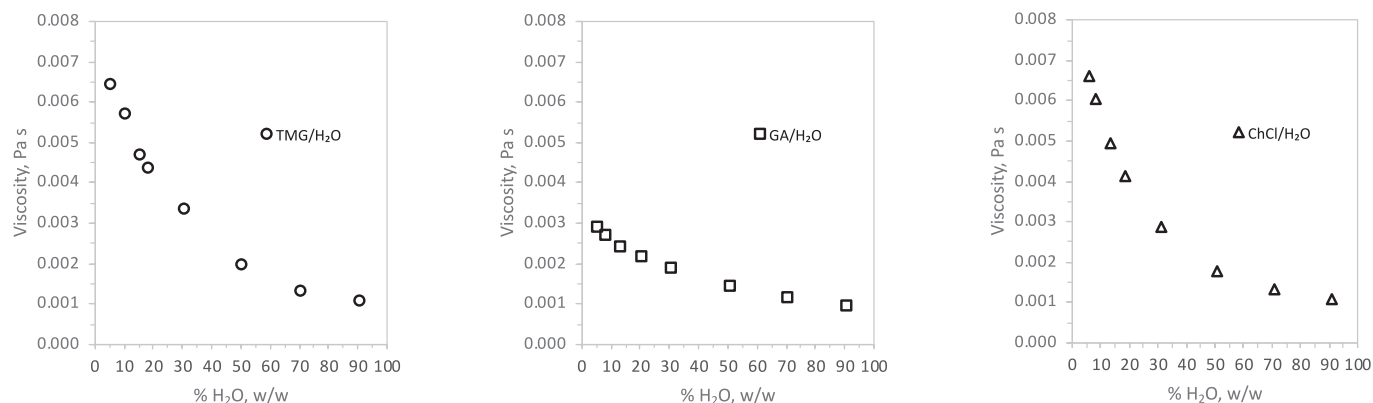
The viscosity is a relevant physical property for the characterization of DESs. The added water reduces significantly the viscosity of the liquids, facilitating their applications [41,45,60]. Indeed, the decrease of the measured viscosity of DESs as a function of water dilutions has been reported to be affected by the aggregation state of the molecules and their interaction with water [41]. Fig. 2 shows the viscosity of the water dilutions of the three analyzed *aquo*DESs (TMG/H<sub>2</sub>O, GA/H<sub>2</sub>O, ChCl/H<sub>2</sub>O) in the dilution range from 5 % to 90 % w/w at the temperature of 25 °C.

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 [45,60], 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<sub>2</sub>O and ChCl/H<sub>2</sub>O with respect to GA/H<sub>2</sub>O. 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<sub>2</sub> extractions.

### 3.3. 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 [61,62]. 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)



**Fig. 2.** Viscosity (mPa s) at  $25.0 \pm 0.1$  °C of water dilutions from 5 % to 90 % w/w of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H<sub>2</sub>O; center, squares = glycolic acid/water GA/H<sub>2</sub>O; right, triangles = choline chloride/water ChCl/H<sub>2</sub>O.

[63]. The variations of ultrasound attenuation for the water dilutions of the aqueous DESs are reported in Fig. 3.

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<sub>2</sub>O and ChCl/H<sub>2</sub>O 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. Despite ultrasound spectroscopy has never been employed so far for the characterization of the cluster aggregation of DESs in water, the slope changes of the ultrasound profiles have been already recognized as reflecting the formation of heterogeneities resulting from the aggregation in water of different species. Perinelli et al. have reported the slope variation of the attenuation profiles for the surfactants sodium dodecyl sulfate, sodium deoxycholate, and N-lauroyl sarcosinate in the nearby concentrations at which the change of slopes of the conductivity data also occurs [64]. From these breakpoints, the critical micelle concentrations (CMCs) were calculated, which were in agreement with those obtained from other techniques. Moreover, Kargerová and Pekař showed that a sudden increase in attenuation can reflect the formation of microaggregates of hyaluronan–DTAB complexes in aqueous media [65]. GA/H<sub>2</sub>O 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 self-aggregation or more complex structures in water as also reported in the literature [66,67]. TMG/H<sub>2</sub>O and ChCl/H<sub>2</sub>O 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.

### 3.4. 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*<sub>6</sub> or acetone-*d*<sub>6</sub>) as an external reference. The proton spectra of the three systems are in the Supporting Information (Figs. S3, S4 and S5). It is interesting to note that in the case of DESs GA/H<sub>2</sub>O and TMG/H<sub>2</sub>O 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<sub>2</sub>O 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 [68]. 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.

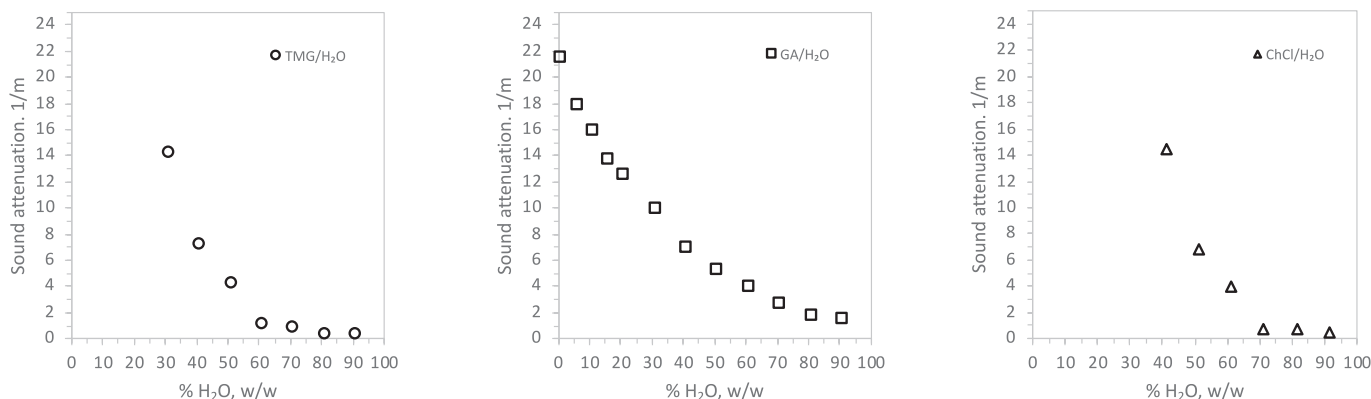
To obtain structural information, diffusional NMR techniques have been used on pure and diluted DESs. The diffusion coefficients  $D_t$ , the average hydrodynamic radii  $r_H$  and the volumes  $V_H$  of the aggregates as a function of the percentage of added water are reported in Table 1.

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<sub>2</sub>O, 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 than their maximum water solubility.

**Table 1**  
Diffusion coefficients ( $10^{10} D_t$ , m<sup>2</sup>/s), average hydrodynamic radii ( $r_H$ , Å) and volumes ( $V_H$ ) as a function of the percentage of added water (%H<sub>2</sub>O, w/w).

%H <sub>2</sub> O	$D_t$	$r_H$	$V_H$
TMG/H <sub>2</sub> O			
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 <sub>2</sub> O			
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 <sub>2</sub> O			
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



**Fig. 3.** Ultrasound attenuation (1/m) at  $25.0 \pm 0.1$  °C of water dilutions from 5 % to 90 % w/w of the *aquo*DESs: left, circles = trimethylglycine/water TMG/H<sub>2</sub>O; center, squares = glycolic acid/water GA/H<sub>2</sub>O; right, triangles = choline chloride/water ChCl/H<sub>2</sub>O.

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

- for TMG/H<sub>2</sub>O,  $V_H$  goes from 698.7 to 230.9 Å<sup>3</sup> (%H<sub>2</sub>O = 71.2 % w/w) which can be compared with the volume of a single TMG molecule, 145 Å<sup>3</sup>;
- for GA/H<sub>2</sub>O,  $V_H$  goes from 519.4 to 262.9 Å<sup>3</sup> (%H<sub>2</sub>O = 64.4 % w/w), which can be compared with the volume of a single GA molecule, 86 Å<sup>3</sup>;
- for ChCl/H<sub>2</sub>O,  $V_H$  goes from 860.3 to 184.7 Å<sup>3</sup> (%H<sub>2</sub>O = 79.6 % w/w), which can be compared with the volume of a single ChCl molecule, 185 Å<sup>3</sup>.

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

As it can be seen in Fig. 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].

### 3.5. Cytotoxicity of aquoDESs 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 [69]. Therefore, the study of the toxicity of water dilutions of DESs has a prominent importance for their use.

In this work, the suitability of aquoDESs 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 cytotoxicity of the aquoDESs as well as of the DESs' forming molecules is shown in Fig. 5. Results show the viability of the Caco-2 cells (in percentage) after exposure for 24 h to different dilutions of DESs (lines) or DESs' forming molecules (dashed lines) The reduction in cell viability to below 100 % indicates cytotoxicity of the solution under these experimental conditions. The cytotoxicity observed for aquoDESs

dilutions (lines) did not exhibit significant differences compared to the cytotoxicity of the non-water components of the liquids (dashed lines) (Fig. 5).

In particular, ChCl/H<sub>2</sub>O and GA/H<sub>2</sub>O DESs did not show any toxic effect when tested at concentrations lower than 0.125 % v/v. TMG/H<sub>2</sub>O did not show any toxicity at concentrations lower than 1 % v/v. 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 [70,71], 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<sub>2</sub>O 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 [72]. This suggests the effect of the toxicity to be related to the choline chloride molecule considering the very low toxicity of glycerol molecule [73]. 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 [74].

## 4. Conclusions

The DESs formed by water as a component of binary mixtures, i.e. aquoDESs 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 is 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<sub>2</sub>O and TMG/H<sub>2</sub>O aquoDESs, 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<sub>2</sub>O and TMG/H<sub>2</sub>O aquoDESs, so these data seem

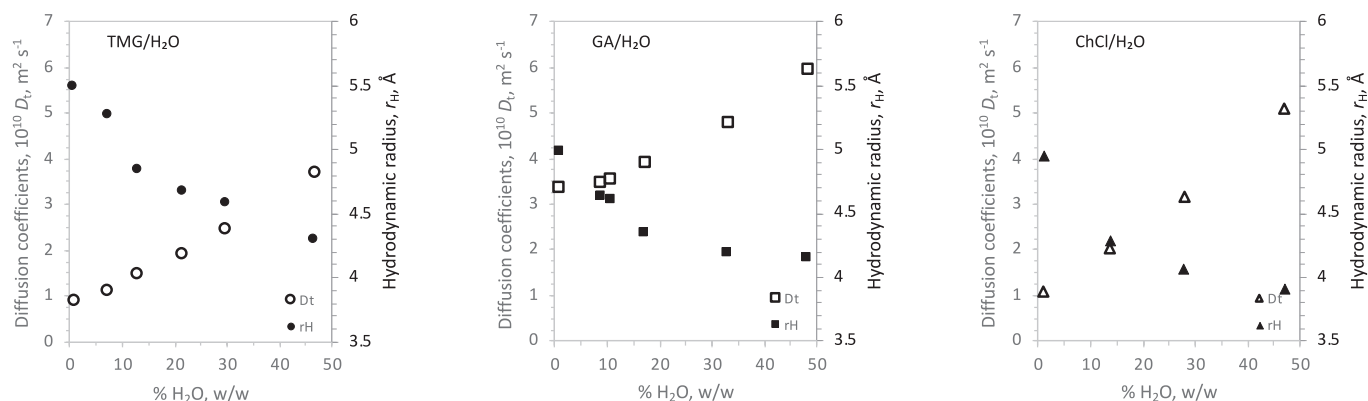
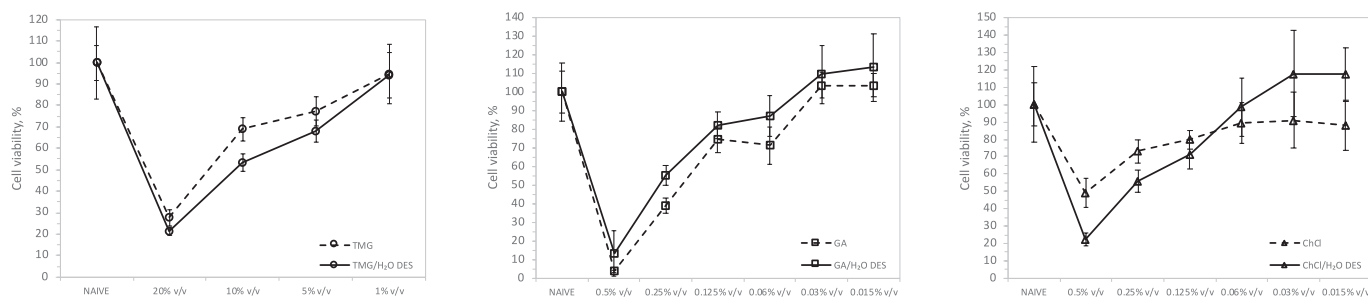


Fig. 4. NMR Diffusion Coefficients  $D_t$  (empty symbols) and average hydrodynamic radii  $r_H$  (filled symbols) at  $25.0 \pm 0.1$  °C of water dilutions from 0 % to 50 % w/w of the aquoDESs: left, circles = trimethylglycine/water TMG/H<sub>2</sub>O; center, squares = glycolic acid/water GA/H<sub>2</sub>O; right, triangles = choline chloride/water ChCl/H<sub>2</sub>O.



**Fig. 5.** Results from MTT assay showing cytotoxicity on Caco2 cells of *aquo*DESs water dilutions (lines) and DESs' forming molecules (dashed lines): left, circles = TMG/H<sub>2</sub>O DES and TMG; center, squares = GA/H<sub>2</sub>O DES and GA; right, triangles = ChCl/H<sub>2</sub>O DES and ChCl.

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 having a low toxicity, related to the betaine itself, which is an edible molecule that is found in many different food matrices.

The capability of modulation of the physical properties of these systems by preserving the DES' structural features permit their use in many different topics. The reduction on the viscosity, for example, could be useful for their application as co-solvent in supercritical CO<sub>2</sub> extractions; the possibility to regulate the conductivity values permit to use them in process where it is necessary to have them at specific values, such as for example in PEF (pulsed electric field) extraction processes and so on.

#### CRediT authorship contribution statement

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

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

This work has been funded by NGEU – PNRR, DM 117/2023 (Elison Lepore's scholarship) and DM 352/2022, M4 C2 I3.3 "Innovative PhD (Industrial)", CUP J11J22001830006 (Gaia de Simone's scholarship).

This research was in part funded by the Unicam institutional fund (FPA300015) to Rosita Gabbianelli.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2024.126427>.

[org/10.1016/j.molliq.2024.126427](https://doi.org/10.1016/j.molliq.2024.126427).

#### Data availability

Data will be made available on request.

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