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Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents

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ABSTRACT

Deep eutectic solvents (DESs) have been dramatically expanding in popularity as a new generation of environmentally friendly solvents with possible applications in various industrial fields, but their ecological footprint has not yet been thoroughly investigated. In the present study, three choline chloride-based DESs with glucose, glycerol and oxalic acid as hydrogen bond donors were evaluated for in vitro toxicity using fish and human cell line, phytotoxicity using wheat and biodegradability using wastewater microorganisms through closed bottle test. Obtained in vitro toxicity data on cell lines indicate that choline chloride: glucose and choline chloride:glycerol possess low cytotoxicity ($EC_{50} > 10$ mM for both cell lines) while choline chloride:oxalic acid possess moderate cytotoxicity (EC_{50} value 1.64 mM and 4.19 mM for fish and human cell line, respectively). Results on phytotoxicity imply that tested DESs are non-toxic with seed germination EC_{50} values higher than 5000 mg L^{-1}. All tested DESs were classified as'readily biodegradable' based on their high levels of mineralization (68–96%). These findings indicate that DESs have a *green* profile and a good prospect for a wider use in the field of *green* technologies.

1. Introduction

The design of environmentally friendly solvents in recent years finds its strategic place within the framework of *green* technologies (Anastas and Warner, 1998; Anastas and Eghbali, 2010; EEA, 2013). In the past 20 years, ionic liquids (ILs) have attracted attention as a new generation of *green* designer solvents with potential uses in various industrial fields (Petkovic et al., 2011). However, regardless of the ILs' declarative *green* properties (non-volatility, non-flammability, reusability), the limitations of conventional ILs, such as imidazolium- and pyridinium-based ones, are high cost (5–20 times higher than the cost of conventional organic solvents), toxicity similar to or even higher than organic solvents and generally poor biodegradability (Cvjetko Bubalo et al., 2014a). These facts have mobilized scientists to develop solvents that would retain excellent technological properties of ILs while being low-cost and exerting minimal environmental effects. In that manner, a type of solvents with similar physical properties and phase behavior to ILs, called deep eutectic solvents (DESs), have emerged (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Francisco et al., 2013). In the literature, DESs are sometimes referred to as the fourth-generation of ILs, even though they could not be considered ILs as they are not entirely composed of ionic species. The DESs are easily prepared by mixing two or three low-cost components (e.g., quaternary ammonium salts, amides, organic acids, polyalcohols), forming an eutectic mixture based on hydrogen bonding interactions with a melting point much lower than either of the individual components (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Dai et al., 2013a; Francisco et al., 2013). One of the most popular components used for the formation of these DESs is choline chloride (ChCl), a cheap, biodegradable and non-toxic salt, which is approved without a time limit under Council Directive 70/524/EEC8 for use as a nutritional additive in all species (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2011). Since their emergence, DESs as a *greener* version of ILs have attracted attention in synthesis, metal-catalyzed organic reactions, electrochemistry, nanomaterials, biochemistry, separation, and analysis. Accordingly, the number of DESs-related references has

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increased rapidly in recent years (almost 300 papers in this period) (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Francisco et al., 2013; Tang and Row, 2013; Vidal et al., 2014).

The industrial use can be expected based on numerous potential DESs applications in the *green* technologies, meaning that environmental impact and fate (e.g., biodegradation and ecotoxicity) of DESs has to be extensively and critically evaluated before their large-scale production (Tanneberger, 2010). So far, the assumption that DESs are benign is based on toxicity data for the components that make up DESs, which are biomaterial-derived and pharmaceutically acceptable. However, this theory does not take into account the possibility of synergetic effect of combining the compounds in the DESs (Hayyan et al., 2013a) which could have significant impact on biological properties of such mixtures. To date, three publications dealing with the toxicity of DESs have been published (Hayyan et al., 2013a, 2013b; Paiva et al., 2014). Hayyan et al. (2013a, 2013b) assessed toxicity of several ChCl- and phosphonium-based DESs toward brine shrimp and bacteria. For instance, it was shown that although ChCl based DESs were completely harmless for the tested bacteria, the phosphonium-based DESs exhibited slight antibacterial activity. However, the cytotoxicity of the DESs tested was much higher than that of their individual components, indicating a noticeable synergistic effect after forming DESs. Paiva et al. (2014) were the first ones who tested the effect of 11 different DESs on L929 fibroblast-like cell line and compared it to two ILs. However, the obtained results did not indicate a clear trend between the cytotoxic effect and the constitution of DES. Therefore, the use of terminology *green* for DESs should still be used with caution whereby DESs toxicity toward organisms at different trophic levels should be proactively assessed prior to their large-scale use. Also, since there are no available data on DESs biodegradability it is necessary to determine their biodegradation potential by wastewater organisms.

Based on the aforementioned, the aim of this work was to evaluate three commonly used ChCl-based DESs containing sugar (glucose), alcohol (glycerol), and organic acid (oxalic acid) as hydrogen donor for in vitro toxicity using fish and human cell line, phytotoxicity on wheat, and their biodegradability using wastewater microorganisms in the closed bottle test. The data obtained would serve to fill the existing gaps in the knowledge about environmental fate of DESs and could be used to predict their effects on human health and environment.

2. Experimental

2.1. Biological and chemical materials

All chemicals for DESs syntheses (choline chloride, glucose, oxalic acid and glycerol) were purchased from Sigma Aldrich (purity of $\geq 99\%$) and used without further purification. The CCO fish cell line (ATCC no. CRL-2772) and MCF-7 human tumor cell line (ATCC no. HTB-22) were used in this work. WST-1 assay was purchased from Roche, Germany. Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco, UK. Wheat seeds (*Triticum aestivum*) were obtained from the Mladen Commerce d.o.o. market, Croatia. All other chemicals were from commercial sources (Sigma Aldrich) and were of the highest purity available.

2.2. Preparation of DESs

The DESs samples were synthesized as reported previously (Dai et al., 2013b; Hayyan et al., 2013c). Briefly, the mixture of choline chloride (ChCl) and hydrogen bond donor was stirred in a flask at 80 °C for 2–6 h until a homogeneous transparent colorless liquid was formed. The hydrogen donors used were glucose, oxalic acid and glycerol which were mixed with choline chloride in molar ratios 2:1, 1:1, and 1:2, respectively. DESs samples were vacuum dried prior to toxicity and biodegradability assessment. Average molecular weights of synthesized DESs were calculated as the sum of the molecular weight of each compound forming the mixture multiplied by its mass fraction. For DESs cholin chloride:glucose (ChCl:Glc), cholin chloride:oxalic acid (ChCl:OA) and cholin chloride:glycerol (ChCl:Gly) average molecular weights were as follows: 156.80 g mol^{-1}, 133.30 g mol^{-1} and 112.67 g mol^{-1}.

2.3. Cytotoxicity assay

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM,) supplemented with 10% (v/v) fetal bovine serum (FBS) in the incubator with 5% CO_2 and humidified atmosphere at 30 °C for CCO cells and 37 °C for MCF-7 cells.

The effect of synthesized DESs on cell proliferation was examined by the WST-1 assay, which is a modification of the classical MTT test (Mosmann, 1983), according to the manufacturer's instructions. Briefly, CCO and MCF-7 cells were seeded in 96-well plates at a density of 5×104 cells per well in 100 μL of media. After overnight incubation, cells were treated with tested compounds (DESs, DESs′ components or organic solvents) and incubated for 72 h, wherein the nominal tested concentrations were from 1 mg L^{-1} to 2000 mg L^{-1}. Following exposure, 10 μL of tetrazolium salt WST-1 was added to each well and cells were incubated for another 4 h, after which absorbance at 450 nm was measured on the microplate reader (Tecan, Switzerland). Cell viability was expressed as percentage of treated cells versus control cells, and corresponding EC_{50} values, defined as the concentration of tested compounds that resulted in 50% growth inhibition, were calculated from the dose–response curves using equations of best-fitted trend-lines. The pH values of DES solutions, as well as the solutions of forming compounds, in DMEM were measured by digital pH meter (Mettler Toledo, Switzerland).

Light microscopy was used to observe morphological changes during the exposure. Therefore, CCO cell were seeded (1×105 cells mL^{-1}) in 6-well plate and exposed to EC_{50} concentration, if determined, or to the highest tested concentration of DESs (2000 mg L^{-1}). Images of CCO cells were taken using an inverted microscope (Carl Zeiss, Germany) and Dino-Eye digital camera (AnMo Electronics Co., Taiwan).

2.4. Phytotoxicity assay

Prior to germination, wheat seeds (*T. aestivum*) were sterilized in 1% NaOCl (v/v) for 30 min and then thoroughly washed 3 times with distilled water. Seeds were incubated for 24 h in the darkness at 24 °C. After that, 30 seeds were placed in a Petri plate ($d=15$ cm) on a piece of filter paper covered with a thin layer of cotton wool and moistened with 30 mL of DESs′ solutions. The treatment concentrations of DESs′ were set to 100, 500, 1000, 5000, 10,000 and 20,000 mg L^{-1}. The pH values of prepared DESs aqueous solutions were also measured by digital pH meter. The control was maintained with 30 mL of distilled water. Wheat seedlings were grown under controlled conditions at 24 ± 1 °C with a shift cycle of 14 h/day and 10 h/night. The solutions were daily renewed to keep DES concentration stable. All treatments were replicated 3 times. After 7 days of exposure, seedlings were harvested and the effect of DESs on germination and early growth of wheat was determined. Wheat seeds were considered germinated when both the plumule and radicle extended to more than 3 mm from their junction. Results were expressed as germination