PROFILING THE CYTOTOXICITY OF DEEP EUTECTIC SOLVENT SYSTEMS AND THEIR POTENTIAL APPLICATIONS AS ANTICANCER AGENTS

YVES PAUL VINCENT MBOUS

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ENGINEERING SCIENCE

FACULTY OF ENGINEERING

UNIVERSITY OF MALAYA

KUALA LUMPUR

2017

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Yves Paul Vincent Mbous

Registration/Matrix No.: KGA140045

Name of Degree: Masters of Engineering Science

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): Profiling the cytotoxicity of deep eutectic solvents and their potential applications as anticancer agents

Field of Study: Chemical Engineering

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;

(2) This work is original;

(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;

(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;

(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;

(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:

Designation

ABSTRACT

The natural origin of natural deep eutectic solvents (NADESs) primary constituents presumes negligible toxicity profiles comparing to DESs. As NADESs have become the subject of elaborate research in various sectors, it is imperative for them to be accurately established as safe mixtures. To meet this end, binary and ternary NADES systems prepared using choline chloride salt with several hydrogen bond donors-HBD (i.e., fructose, glucose, sucrose, ethylene glycol, glycerol, urea and malonic acid) were assessed *in vitro* using the human cervical adenocarcinoma (HelaS3), the human prostate (PC3), the human skin melanoma (A375), the human breast adenocarcinoma (MCF-7), the human gastric adenocarcinoma (AGS) and the human hepatic cell lines (WRL-68); and *in vivo* using ICR mice model. Concomitantly, binary DES systems were prepared using N,N-diethylethanol ammonium chloride salt and ethylene glycol, glycerol, urea, malonic acid and zinc chloride as HBDs for comparison. The results revealed that *in vitro* NADES systems including the water-based ternary ($127 \leq EC_{50} \geq 483$ mM) and binary ($15 \leq EC_{50} \geq 1260$ mM) were on average less toxic than binary DES systems ($34 \leq EC_{50} \geq 120$ mM). The negligible cytotoxicity profiles of NADESs compared to standard DESs stem from the natural origin of their constituents especially those of metabolic importance. It was also found that NADESs prepared using fructose, glucose or glycerol had lesser detrimental effect as opposed to solvents prepared using organic acids malonic acid or zinc chloride. A trend between the cellular requirements of cancer cells, the viscosity of ternary NADESs and their cytotoxicity was observed. High viscosities correlated with higher cytotoxicity. Further, the presence of water in ternary NADESs yielded less potent mixtures. Results also showed that the longer alkyl chain of the N,N-diethylethanol ammonium chloride salt -used for DESs preparation- significantly

increased their cytotoxicity in contrast with choline chloride based-NADESs even when the same HBDs (glycerol, ethylene glycol and urea) had been used to prepare both types of mixtures. It was discovered for the first time that binary NADESs are synthesized intracellularly to counterbalance the individual cytotoxicity of the chief materials; thus, making NADES the possible third liquid phase in mammalian cells and a potential tool for physiology-based work. Further, binary NADESs seemed to induce necrosis through increasing membrane porosity and redox stress. However, binary NADESs were, *in vivo,* more destructive than binary DESs and caused liver failure. The first time application of the Conductor-like Screening Model for Real Solvent (COSMO-RS) model for cytotoxic studies was demonstrated through the simulation of the interactions taking place between NADESs/DESs and cellular membranes. The results suggested that the accumulation and aggregation of these mixtures on the lipid bilayers defined their cytotoxicity. The selectivity index (i.e., EC_{50} ratio of cancer to healthy cell line) values of ternary and binary NADES systems ($0.24 \leq SI \geq 1.061$) were lower than those of binary DES systems ($0.269 \leq SI \geq 1.315$), implying that DESs were more active as anticancer material compared with NADESs, thereby reinforcing the conclusion that they are indeed more cytotoxic than the naturally synthesized NADESs.

ABSTRAK

Komposisi semulajadi asal pelarut eutektik dalam semulajadi (NADES) dianggap mempunyai kadar toksik yang terlalu rendah berbanding DES. Memandangkan NADES telah menjadi tumpuan kajian dalam pelbagai lapangan, ianya sangat penting bagi memastikan NADES ini diiktiraf sebagai pelarut yang selamat. Oleh itu, pelarut NADES sistem penduaan and pertigaan telah disediakan melalui pencampuran garam kolin klorida dengan pelbagai penderma ikatan hydrogen (HBD) (fruktosa, glukosa, sukrosa, etilen glikol, gliserol, urea, dan asid malonik) dan telah diuji kesannya secara *in vitro* manusia terhadap sel servik adenokarsinoma manusia (HelaS3), sel kanser prostat manusia (PC3), sel kulit melanoma manusia (A375), sel payu dara adenokarsinoma manusia (MCF-7), sel gastrik adenokarsinoma manusia (AGS) dan sel hepatik manusia (WRL-68) dan *in vivo* menggunakan model haiwan tikus. Disamping itu juga sistem penduaan pelarut DES menggunakan garam N,N-dietil etanolammonia klorida dengan etilena glikol, gliserol, urea, asid malonik and zink klorida sebagai HBD bertujuan untuk perbandingan. Hasil ujikaji menunjukkan sistem NADES secara *in vitro* yang terdiri daripadan sistem pertigaan berasaskan air ($127 \leq EC_{50} \geq 483$ mM) dan sistem penduaan ($15 \leq EC_{50} \geq 1260$ mM) terbukti kurang toksik berbanding sistem penduaan DES ($34 \leq EC_{50} \geq 120$ mM). Tahap ketoksikan NADES yang terlalu rendah berbanding konvensional DES berpunca daripada sumber asal komposisi mereka terutamanya kompoun-kompoun yang penting dalam sistem metabolik. NADES yang diterdiri daripada fruktosa, glukosa mahupun gliserol terbukti kurang toksik berbanding pelarut yang terdiri daripada asid malonik dan juga zink klorida. Trend antara keperluan asas bagi kanser sel, tahap kelikatan sistem pertigaan NADES dan juga tahap ketoksikan NADES dinilai.

Semakin tinggi tahap kelikatan maka semakin tinggi tahap ketoksikan. Kehadiran air dalam sistem pertigaan NADES dilihat mampu untuk menghasilan sebatian yang kurang poten. Hasil uji kaji juga menunjukkan semakin panjang rantai alkil bagi garam N,N-dietiletanolammonia klorida yang digunakan untuk penghasilan DES semakin meningkat tahap ketoksikannya, berbeza dengan NADES yang berasaskan klorin klorida walaupun menggunakan HBD yang sama (gliserol, ethilen glikol, dan urea) untuk penghasilan kedua-dua pelarut. Ianya telah terbukti buat pertama kalinya sistem penduaan NADES mampu menjadi penimbang balas terhadap ketoksikan kompoun individu; dan kemudiannya menjadikan sistem penduaan NADES berkemungkinan sebagai cecair ketiga dalam sel mamalia dan berpotensi untuk diaplikasikan dalam kerja-kerja berasaskan fisiologi. Kemudian, sistem penduaan NADES dilihat dapat meransang proses nekrosis untuk berlaku melalui peningkatan lohong membran dan tekanan redoks. Walaubagaimanapun, sistem penduaan NADES secara *in vivo*, menghasilkan lebih banyak kerosakan berbanding sistem penduaan DES dan menyebabkan kerosakan hati. Untuk pertama kalinya aplikasi model penyaringan konduktor untuk pelarut (COSMO-RS) digunakan dalam kajian ketoksikan melalui simulasi perhubungan yang berlaku antara NADES/DES dan membran sel. Keputusan hasil kajian daripada aplikasi ini mengandaikan pergumpulan cecair di lapisan lipid menyebabkan mereka menjadi toksik terhadap sel. Indeks sikap memilih (ratio untuk EC_{50} bagi sel kanser dan sel sihat) nilai untuk sistem pertigaan dan perduaan NADES ($0.24 \leq SI \geq 1.061$) adalah lebih rendah berbanding sistem penduaan DES ($0.269 \leq SI \geq 1.315$), membuktikan DES adalah lebih aktif sebagai anti-kanser berbanding NADES. Justeru itu, ianya dapat disimpulkan bahawa DES sememangnya lebih toksik berbanding pelarut semulajadi NADES.

ACKNOWLEDGEMENTS

The journey towards the completion of my Masters was met with several obstacles from the very start. Most of which were steadily overcome by the unceasing assistance from Prof. Dr. Mohd Ali Hashim, Dr. Maan Hayyan and Dr. Adeeb Hayyan. To them I wish to express my sincere gratitude.

My genuine recognition to my direct advisor Dr Maan Hayyan for the continuous support during my Master study and related research, for his patience, motivation and immense knowledge. His guidance helped me during the time spent doing research and writing up this thesis. I could not have imagined having a better advisor and mentor for my Masters study.

My sincere thanks also goes to Dr. Won Fen Wong and Dr. Chung Yeng Looi, who provided me with the opportunity to join their team and who gave access to the laboratory and research facilities for my work. Without they precious support, it would not have been possible to conduct this research.

I thank all members of the University of Malaya Centre for Ionic Liquids involved at both the academic and administrative levels for the support and advices they were not shy to provide.

Last but not least, I would like to thank my family; my mother, my father and my sisters for their unending encouragements.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

A	Acetamide
Ac	Acetate
Ad	Adonitol
APOx	Ascorbate peroxidase
Bet	Betaine hydrochloride
1,2-BD	1,2-Butanediol
1,3-BD	1,3-Butanebiol
1,4-BD	1,4-Butanediol
2,3-BD	2,3-Butanediol
BMIm / Bmim	1-Butyl-3-methylimidazolium
CA	Citric acid
CAT	Catalase
C1Him	1-Methylimidazolium
CCO	Channel catfish ovaries
ChCl	Choline chloride
ChAc	Choline acetate
DAC	N,N-diethylethanol ammonium chloride
DESs	Deep eutectic solvents
EG	Ethylene glycol
Gly / G	Glycerol
GPX	Guaiacol peroxidase
HBA	Hydrogen bond acceptor

HBD	Hydrogen bond donor
1,6-HD	1,6-Hexanediol
ILs	Ionic liquids
LDH	Lactate dehydrogenase
MA	Malonic acid
MAL	Malic acid
Mlt	Maltose
MCA	Methacrylic acid
MDA	Malondialdehyde
MTPPB	Methyltriphenylphosphonium bromide
NADESs	Natural deep eutectic solvents
Ph	Phenyl
PhAc	Phenyl acetate
Pro	Proline
Prop	Propionic acid
1,2-PD	1,2-Propanediol
RDF	Radial distribution fractions
ROS	Reactive oxygen species
TMAC	Tetramethyl ammonium chloride
SOD	Superoxide dismutase
U	Urea
VOCs	Volatile organic solvents
Xyl	Xylitol

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Overview

In chemistry, solventless reactions are the exception, whereas solvent-based reactions are the norm. The implementation of solvent-free systems being scarce; solvents and co-solvents are of the utmost importance in most industrial chemical processes. Therefore, solvents intrinsic and extrinsic characteristics must meet the anticipated economic, environmental and safety concerns. These conditions are at the heart of the fundamental focus of green chemistry, which aims to develop safe and sustainable chemical solvents to overcome the use of hazardous petroleum-based materials. Hence, the evolution of green solvents was a priority at the beginning of the 21st century, all the more so because of the inadequate characteristics of the existing primary industrial solvents: traditional organic solvents and water (Bart, 2011; Sowmiah et al., 2009). Due to their emissions of volatile organic compounds (VOCs), traditional solvents have long been associated with environmental and health hazards, such as toxic and carcinogenic effects on human; photochemical smog, ozone-depleting effects and air pollution. Further, their low biodegradability and low recycling potential coupled to their preparation costs are serious issues to account for. Certainly an adequate solvent for polar solutes, water is a relatively poor solvent for hydrophobic solutes and the energy required for its purification is considerably high (Bart, 2011). Therefore, the research focus shifted towards the discovery of greener, friendlier, cheaper and better solvents. The development of DESs was a downstream result of this effort. DESs are arbitrarily defined as mixtures whose average freezing points are lower than those of their individual components.

The key components of DESs are a hydrogen bond acceptor-HBA, which in most instances is a halide salt and a HBD such as polyols, sugars, organic acids, amides, amino acids, metal salts. HBD and HBA are chemically linked via hydrogen bonds (Smith et al., 2014). The hydrogen bonds result from a complexation between a HBA, typically choline chloride (ChCl) and a HBD. Besides their low melting points, DESs are well-known for other characteristics, namely: the low cost their starting materials, their inflammability, their low volatility, their thermal and chemical stability, tuneability, their dipolar nature, their low vapor pressure, their high solvability, their ease of storage and availability and their alleged negligible cytotoxicity (Tang & Row, 2013). These encouraging features prompted DESs applications in various fields, notably in biotransformations (Durand et al., 2012), downstream and upstream biodiesel processing (Hayyan et al., 2014; Hayyan et al., 2010), extraction of bioactive substances (Wei et al., 2015), drug transport and solubility (Sanchez-Leija et al., 2014) and nanotechnology (Wagle et al., 2014). As materials of "infinite" potential, one of the most pressing concern is the effective characterization of their toxicity profiles. As stated earlier, one of the pillars of green chemistry is the design of safe solvents. The most encountered design of DESs are based on cholinium, which is a material of significant biological activity. As expected, ChCl-based DES toxicity profiles were less potent than some first or second generation ILs; but not harmless enough to be deemed of negligible toxicity. A glaring pattern emerged from the results of DESs-based toxicity studies. The toxicity of these mixtures was firmly linked to the nature of its constituents. Biorenewable materials and ingredients of biological/natural provenance produced DESs of significantly low toxicity compared to typical organic acids and alcohols. Findings from Radošević et al. (2015; 2016), Zhao et al. (2015) and Juneidi et al. (2016) have amply described the deleterious effect of organic based DESs on the growth rate of bacteria as well

as on the viabilities of human cancer and fish cell lines. However, the same studies proved that the use of biomaterials-based DESs did not affect the selected organisms. These biomaterials-based DESs have been categorized as a subclass of DESs named natural deep eutectic solvents (NADESs). NADESs discovery reinforced the belief that harmless DESs could be obtained by using natural and biorenewable materials. As a subset of DESs, NADESs are prepared solely using starting materials of metabolic importance such as amino acids, sugars and amines. NADESs were identified via the analysis of the collected metabolome data from various plant materials. Choi et al. (2011) rationale stated that the purpose of the otherwise mysteriously high concentration of primary metabolites (choline, sugars, acids and amino acids) in plant cells could be the formation of NADESs, which would act as a third cellular media different from water and lipids phases. These intracellular eutectic mixtures would be strategic during plant developmental stages such as germination or cryopreservation and could be involved in other activities like the solubilization of intermediate polarity materials, the storage of metabolic products and/or drought resistance (Choi et al., 2011). Recent studies have provided a list of the composition of these mixtures as well as their molar ratios (Choi et al., 2011; Dai et al., 2013). One of the fundamental precepts of this class dictates that if cellular media produce NADESs, the propensity for cytotoxicity must be at minimal limits. Paiva et al. (2014a) briefly investigated the cytotoxic profile of several NADESs namely, [ChCl]-[Glucose], [ChCl]-[Citric acid], [ChCl]-[sucrose], [ChCl]-[Tartaric acid], [ChCl]-[Xylose], [Citric acid]-[Glucose], [Citric acid]-[Sucrose], [Glucose]-[Tartaric acid] at various molar ratios. Using fibroblast-like cells, the authors assessed cellular viability following NADESs' treatment. The results pointed to the role of the HBD (organic acids) as major enhancer of cytotoxicity, because the most toxic NADESs were [Glucose]-[Tartaric acid], [ChCl]-[Tartaric acid], [ChCl]-[Citric acid] and

[Citric acid]-[Glucose]. Although, the authors exalted in what they considered positive and encouraging results, the truth of the matter remain that no in-depth appreciation of these mixtures was performed, let alone an attempt at understanding their cytotoxic mechanism. As mentioned before, NADESs share several physical characteristics as DESs; and the examination of these properties can often lead to a better understanding of their cytotoxic mechanism. For instance, viscosity is one of the negative trait of DESs and NADESs alike. Viscosity impedes mass-transfer kinetics and ultimately influences the toxicity of these mixtures. Recently though, it has been shown that NADESs physical properties can be tailored by adding water as a tertiary component. The authors showed that the strong hydrogen interactions within NADESs -which account for their high viscosities- could be reduced upon addition of water (< 50% v/v). In fact, the resulting viscosities were found to be as low as those of water and other common organic solvents (Dai et al., 2015). Consequently, water-based NADESs may yet represent an alternative to DESs of high viscosities, poor conductivities and perhaps also low toxicities. In spite of the scarcity of NADESs physicochemical characterizations, the repeated calls for their industrial appropriation hinge on the enforcement of safety policies based on detailed toxicology assessments. The use of ChCl as one of DESs starting materials promised relatively non-toxic profiles. In contrast, it raised controversy with regards to the use of organic acids as DESs ingredients. The opportunity to remedy this issue have been provided with the advent of NADESs. Therefore, it is important that these novel mixtures undergo a thorough critical and an in-depth analysis of their toxicity profiles is needed; especially because limited data on this matter is available (Paiva et al., 2014a; Zhao et al., 2015).

1.2 Problem statement

NADESs are a novel class of chemical solvents with a wide range of applications. Their appealing characteristics are encouraging with regards to their large-scale use as industrial solvents. However, compliance with “green” standards remain necessary. Their wide tuneability implies an unbiased classification to acquire a complete or objective opinion of their cytotoxicity mechanism. Screening numerous NADESs will help distinguish those of therapeutic value. Further, as a class of neoteric solvents whose physical and chemical properties are being uncovered, it is relevant to determine the effect of these properties on their cytotoxic profiles to devise strategies to circumvent issues, which may arise as a result.

1.3 Research objectives

The aims and specific objectives of this study are listed below.

1. ***Binary natural deep eutectic solvent systems: In vitro and in vivo profiling***
    - Assessment of the cytotoxic parameters of binary NADESs *in vitro* in both cancer and normal cell lines
    - Evaluation of membrane porosity, redox stress and subsequent cellular necrosis
    - Examination of the physiological and biochemical responses following binary NADESs treatment *in vivo* (mice- animal model)
2. ***Comparative assessment of binary deep eutectic solvent systems against binary natural deep eutectic solvent systems***
    - Analyses of the cytotoxic values of binary DESs and NADESs
3. ***Investigation of the effect of water on ternary NADES solvent systems' toxicity and analysis of the interactions between lipid bilayer and the eutectics***
    - Interpret cytotoxic values of ternary NADESs prepared using water
4. ***Cytotoxic mechanism and anticancer potential of deep eutectic solvents***
    - Investigation of the critical interactions between DESs/ NADES and cellular membranes using the Conductor-like Screening Model for Real Solvents (COSMO-RS) model
    - Comparison of the selectivity index obtained following binary and ternary DES/NADES solvent systems treatment of cancer and normal cell lines

CHAPTER 2: LITTERATURE REVIEW

2.1 Green solvents

The concept of sustainability has largely influenced the undeniable impact of Green Chemistry towards the industry, the education, the environment and even the general public. Green chemistry emphasizes the concept of sustainable design by means of continuous novelty, planning and systematic conception. As such, this discipline strives to provide an effective path for the design of eco-friendly chemical substances and processes applicable at all levels including the molecular in order to reduce their hazard. Therefore, green chemistry aims are to implement hazard free processes at every stage of a chemical's life-cycle, through the design of safe and sustainable materials with little-to-no risk of toxicity, flammability or any other typical adverse conditions. Hence, its applicability and adoption in various fields. The framework of green chemistry gravitates around twelve guiding principles (Figure 2.1). Solvents account for one of the most active research area of green chemistry because they generally make up the bulk of the industrial waste accumulated during syntheses and processes (Anastas & Eghbali, 2010). Efforts to implement solvent-free processes have been mostly successful during the manufacture of bulk chemicals. However, in most specialized industries, such as the pharmaceutical industry, solvent-based reactions remain vital to the processing of reactants (Kerton, 2009). As such, two of the guiding principles of green chemistry focus on the modelling and use of novel, efficient and innocuous solvents. The need for alternative solvents cannot be emphasized enough in the face of volatile organic solvents shortcomings.

Figure 2.1: The twelve principles governing the design process in green chemistry (Anastas & Eghbali, 2010).

Organic solvents are a major environmental and health concern as shown in Figure 2.2. They are able to produce photochemical smog; have ozone-depleting effects; release emissions contributing to air pollution; and are not readily biodegradable. Health–wise, their adverse effect include eye irritation, headaches and allergic skin reactions, just to name a few. Studies have ascertained their highly toxic character coupled to their potential as carcinogens and genotoxins (Byrne et al., 2016; Clark & Tavener, 2007). Their toxic profiles prompted the enforcement of legislation and control measures to regulate their usage. For instance, the genotoxic and carcinogen properties of benzene, contributed to its volume adjustment in gasoline after 2000 from 5% to < 1%.

Dichloromethane was the preferred solvent for the extraction of caffeine from coffee. However, due to its suspected human carcinogenicity, supercritical carbon dioxide ($scCO_2$) is the current method of choice. In specialized industries, such as the pharmaceutical industry, the United States Food and Drug Administration (FDA) banned the use of several solvents including benzene, hexane or toluene, unless if unavoidable. Hexane, for instance is regularly used for the extraction of natural products and vegetable oils. However, the Environmental Protection Agency (EPA) Toxic Release Inventory, puts the hexane air emissions through this process, at more than 20 million kg per year (Kerton, 2009). The flaws of organic solvents particularly in the areas of environmental and health toxicity, recycling, inertness, handling hazard and energy-intensive purification accentuated the need for alternative solvents. The known alternatives are ionic liquids (ILs), supercritical carbon dioxide ($scCO_2$), fluorous solvents, water, bio-sourced sources. However, these alternatives were designed to address a particular issue. That is, to best attend for inorganic systems, water and $scCO_2$ are employed; for less volatility, ILs; to ease recycling, fluorous solvents and $scCO_2$; and ideally for all systems, no solvent. Hence to choose the best solvents for a wide range of applications is a tricky process as it generally involves EHS assessments and life-cycle analysis (LCA). In a wider context, one might not be able to fully conceive the possibilities that an alternative class of solvents offers may offer because of a lack of in-depth characterization. For instance, for most specialized solvents such as $scCO_2$ and ILs, life-cycle analysis (LCA) are not common. The function of a LCA is to evaluate environmental burdens of a product, process, or activity; quantify resource use and emissions; assess the environmental and human health impact; and evaluate and implement opportunities for improvements.

SOLVENT		Waste	Impact	Health	Safety
Alcohols	Ethylene glycol	4	9	8	10
	1-Butanol	5	7	8	8
	Diethylene glycol mono butyl ether	5	8	8	10
	Ethanol / IMS	3	7	9	6
	2-Propanol	3	10	7	7
	Methanol	3	8	4	8
	2-Methoxy ethanol	4	9	2	7
Esters	Butyl acetate	7	7	7	6
	Propyl acetate	7	6	7	6
	Isopropyl acetate	5	7	7	6
	Ethyl acetate	4	9	7	4
	Methyl acetate	2	6	5	5
Aromatics	Xylene	8	4	5	5
	Toluene	7	3	5	4
Ketones	Methylisobutyl ketone	7	4	6	7
	Acetone	2	7	6	5
	Methylethyl ketone	3	6	5	5
Acids	Propionic acid	5	8	5	9
	Acetic acid (glacial)	3	6	4	8
Alkanes	Cyclohexane	5	5	6	2
	Heptane	6	2	5	1
	Hexane	5	3	3	1
	Petroleum spirit / ether	4	2	5	1
Chlorinated	Dichloromethane	3	3	1	10
Ethers	1,2-Dimethoxyethane	3	5	4	2
	t-Butylmethyl ether	4	4	3	3
	Bis(2-methoxyethyl) ether	6	5	2	3
	Tetrahydrofuran	2	7	4	2
	Diisopropyl ether	5	2	6	1

Footnotes

1. **Waste** addresses: recycling, incineration, VOC and biotreatment issues.
2. **Impact** addresses fate and effect on the environment.
3. **Health** is based on acute and chronic effect on human health and exposure potential.
4. **Safety** considers explosivity, flammability and operational hazards.

The four key areas were assessed and given a scoring (The higher the better)

Legend

Major issues have been identified.
Appropriate control procedures need to be in place.

Issues have been identified.
The need for control procedures should be considered.

No major issues identified in this area.

Figure 2.2: Environmental and health factors of organic solvents (Curzons et al., 1999).

However, as pointed above, ILs-focused LCA are rare. Nevertheless, it is still possible to appreciate the overall strain of a particular solvent through the burden of its individual synthetic steps. Using a typical life-cycle flow chart, as shown in Figure 2.3, it is possible to investigate the step-wise EHS impact, the cost and the recycling strain of a particular solvent within a green chemistry framework by focusing on the manufacture, distribution, use and disposal stages. Although the focus of the work is on deep eutectic solvents, it is relevant to deal with ILs, as they considered the parent or analogue class to deep eutectic solvents.

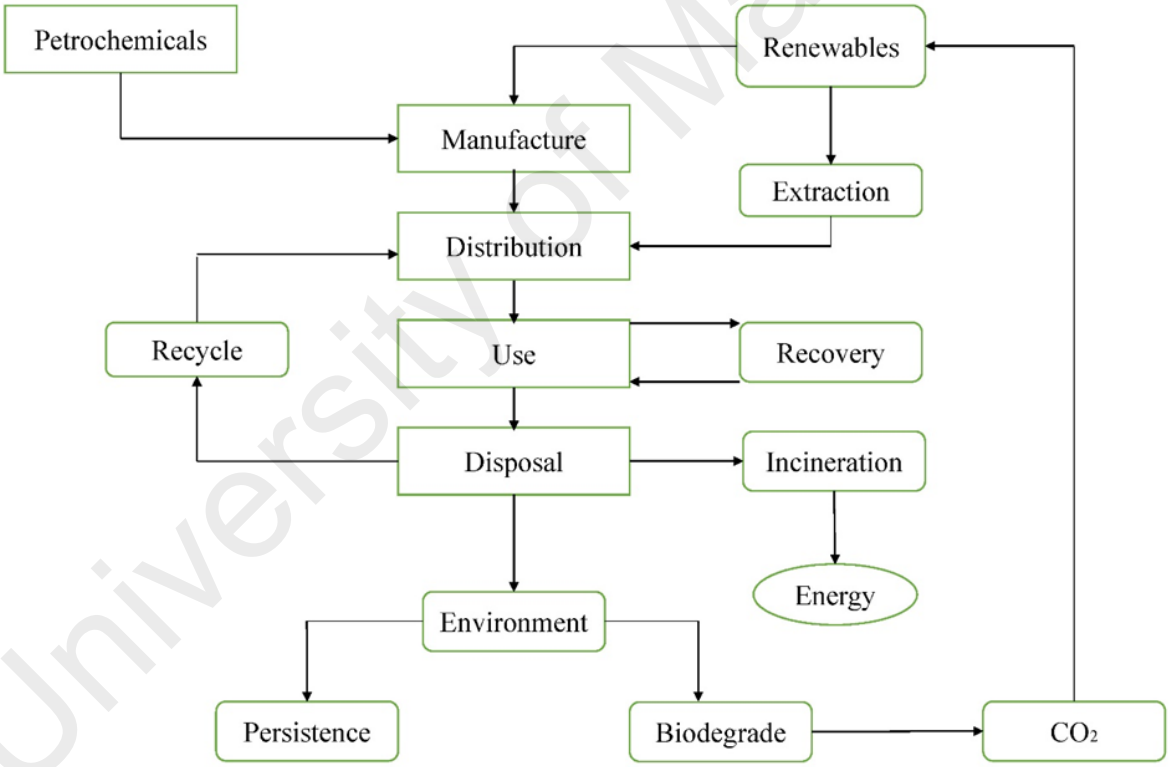


Figure 2.3: Typical steps to consider when analyzing or establishing the life-cycle of a solvent (Clark & Tavener, 2007).

2.2 Ionic liquids

ILs are arbitrarily defined as room temperature molten salts composed of cationic and anionic species, which form a liquid phase at temperatures lower than 100°C. ILs are generally divided into two groups with respect to their chemical structure and behavior: aprotic and protic ILs. Aprotic ILs which are typically referred to as "classic ILs" are prepared using bulky cations such as imidazolium or pyridinium in combination with a variety of anions such as Cl^- , Br^-, BF_4^- and PF_6^-. Protic ILs are a somewhat novel addition to the library and they are designed using simpler structured compounds such as substituted amines (monoethanolamine, diethanolamine or triethanolamine) as cations and organic acids (formic, propionic, butanoic, pentanoic acid) as anions (Oliveira et al., 2016; Peric et al., 2013). As a result of their simple synthesis, they are often associated with lower synthetic cost and less environmental burden.

ILs are generally prepared via metathesis, quaternization, "one pot" or proton transfer reactions using ingredients from both ionic and non-ionic sources as shown in Table 2.1 (Deetlefs & Seddon, 2010; Mirjafari et al., 2013). As a result, ILs are structured around various intermolecular and intramolecular attractions, such as ionic bonds, van der Waals forces, dipole-dipole forces and hydrogen bonds (Dong et al., 2006; Hunt et al., 2015). The basis of ILs "greenness" was centered on their low melting points, their dual polarity, their good thermal and chemical stability, their solvation ability, their low volatility and their non-flammability (Shamsuri & Abdullah, 2010). Their low volatility anticipated solvents of low toxicity and biodegrability profiles (Domínguez de María et al., 2012). Their versatility was especially celebrated because it allowed for the tuneability of their properties while retaining the core desired features, by shuffling the anionic and cationic species.

For instance, altering the length of the alkyl substituent of the cations significantly influenced the miscibility of ILs in organic solvents and water (Brennecke & Maginn, 2001). These characteristics justified their applications for extractions (Qi et al., 2015), biotransformations (Wu et al., 2014), nanoparticles assembly (Wagle et al., 2014), preservation of biomolecules (Dai et al., 2015), upstream and downstream biodiesel processing (Hayyan et al., 2013a; Hayyan et al., 2014; Hayyan et al., 2010; Muhammad et al., 2015), electrodeposition (Ru et al., 2015), organic synthesis, in biomass dissolution, as drug vehicles and as therapeutic agents among others (Sowmiah et al., 2009). These breakthroughs reinforced the belief that ILs could act as viable and green replacements for health-risk and toxic organic solvents. The core properties of ILs made the transition from organic solvents not only conceivable, but beneficial. Compared to ILs, organic solvents possess high volatility, high flammability (potentially hazardous chemicals), high vapor pressure and high melting points as listed in Table 2.2. These various characteristics considerably limit their applications in various industrial processes (Pham et al., 2009). While some of the properties listed in Table 2.2 are deemed attractive, ILs are not to be considered innocent, green, or non-coordinating solvents. The manufacturing, cost, disposal and environmental aspects of ILs life-cycles have thrown sufficient doubt on their claim of “greenness”. ILs are typically manufactured from petrochemical feedstocks, routinely acquired using cracking and distillation coupled with complex synthetic routes. Further, they require typically multistep synthetic stages and a lot of energy due to the esterification or acidification reactions coupled with washing, separation and purification steps (Hallett & Welton, 2011).

Table 2.1: Common ingredients used for ILs and DESs preparation.

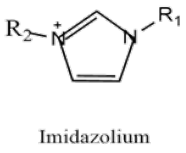
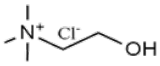
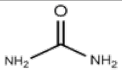
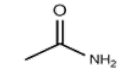
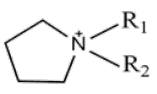
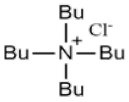
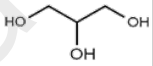
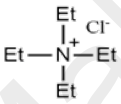
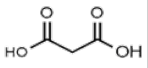
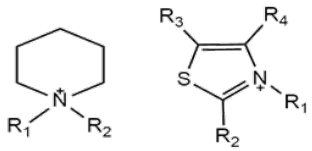
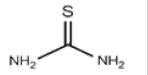
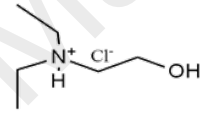
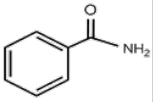
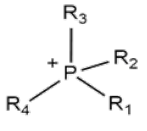
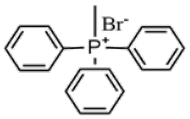
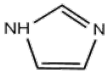
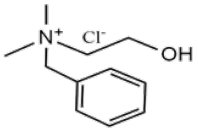
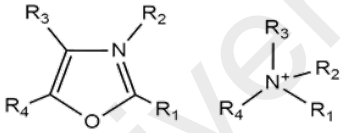
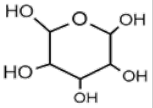
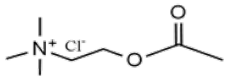
IL		DES	
Cations	**Anions**	**HBA**	**HBD**
 Imidazolium Pyridinium	Cl^-, Br^-	 Choline chloride	 Urea
	I^-, $Al_2Cl_7^-$	 Tetramethylammonium chloride	 Acetamide
 Pyrrolidinium Guanidinium	$CuCl_2^-$, $SnCl_2^-$	 Tetrabutylammonium chloride	 Glycerol
	NO_3^-, PO_4^{3-}		
	HSO_4^-, SO_4^{2-}	 Tetraethylammonium chloride	 Malonic acid
 Piperidinium Thiazolium	$Al_2Cl_7^-$, $Al_3Cl_{10}^-$		 Thiourea
	$Sb_2F_{11}^-$, $Fe_2Cl_7^-$	 N,N-diethyl-2-hydroxyethanamidium chloride	
	$Zn_2Cl_5^-$, $Zn_3Cl_7^-$		 Benzamide
 Phosphonium Morpholinium	$(CF_3SO_2)_2N^-$, $N(CN)_2^-$	 Methyltriphenylphosphonium bromide	
	$(CF_3SO_2)_3C^-$, BR_4^-		 Imidazole
	$RCB_{11}H_{11}^-$	 N-benzyl-2-hydroxy-N,N-dimethyl ethanamidium chloride	
 Oxazolium Quaternary ammonium	$CF_3SO_3^-$, $ROSO_3^-$		 Glucose
	$CF_3SO_2^-$, $C_6H_5SO_3^-$		
	PF_6^-, SbF_6^-, BF_4^-	 2-Acetate-N,N,N-trimethyl ethanamidium chloride	

Table 2.2: Comparison of ILs, organic solvents and DESs main properties.

Property	ILs	Organic solvents	DESs	Ref
Vapor pressure	Negligible	Obeys the Clausius-Clapeyron equation	Negligible	(Sowmiah et al., 2009)
Flammability	None	High	None	(Gorke et al., 2010)
Volatility	None	High	None	(Pena-Perreira & Namiesnik, 2014; Wagle et al., 2014)
Toxicity	Moderate to low	High	Low to none	(Frade & Afonso, 2010; Hayyan et al., 2013b,c; Petkovic et al., 2010)
Biodegradability	Moderate to High	0.2-100	High	(Coleman & Gathergood, 2010; Latała et al., 2009; Radošević et al., 2015)
Viscosity/cP	22-40,000	0.2-100	1.4-85,000	(Anna & Wypych, 2014; Sowmiah et al., 2009; Zhang et al., 2012)
Density/g.cm-3	0.8-1.33	0.6-1.7	1.041-1.980	(Shahbaz et al., 2012; Sowmiah et al., 2009)
Recyclability	High	Low and expensive	High	(Singh et al., 2010)
Surface tension/nM.m^{-1}	22.8-64.8	11.91-64	22.39-77.3	(Instruments, 2006; Sedev, 2011; Smith et al., 2014)
Dielectric constant	8.0-61.0	1.89-37.5	-	(Abbott et al., 2011; Hallett & Welton, 2011; Pandey et al., 2014; Scurto et al., 2002; Tang et al., 2012)
Cost	Expensive	Cheap	Cheap	(Kerton, 2009)
Preparation	Requires time and skills	Time consuming	Easy	(Kerton, 2009)

In the eyes of the industry, the current high prices of ILs do not facilitate the large-scale replacement of generic organic solvents. Kunz and Häckl (2016) articulated that in light of their hefty prices, interest in ILs –other than their applications as bulk solvents- should be rather focused on their use in small quantities and as high performance chemicals. Mai et al. (2014) listed several issues specifically pertaining to the high energy consumption, the high

cost of the procedure and the equipment, the need for technical skills, the use of organic solvents, the low recovery yields, partial decomposition and the pretreatment prerequisites of the various mixtures during recycling and recovery procedures which seriously undermine the sustainability aspect of ILs with regards to the prevention of waste (one of the twelve guiding principles of "green chemistry").

2.2.1 Pre-release and post-release toxicity

The toxicity of ILs is two-fold. Generally, the bulk of ILs-based studies focus on the post-release consequences of ILs in the environment and neglect to address the environmental burden produced during their synthetic steps. Organic solvents are often used during ILs synthesis; and these release air emissions contributing to air pollution and smog formation. Table 2.3 lists the emissions recorded during the synthesis of three different ILs: 1-butyl-3-methylimidazolium bromide ([BMIm]-[Br]), 1-butyl-3-methylimidazolium chloride ([BMIm]-[Cl]), 1-butylpyridinium chloride ([BPy]-[Cl]). It shows the percentage of emissions of various compounds with the potential of being environmentally noxious. Hence, pre-release impact should not be omitted from ILs-based toxicity studies, as it accounts for a considerable percentage of ILs toxicity. In spite of the non-volatility of ILs suggesting no atmospheric release of toxic substances and no toxicity, several studies have described ILs post-release acute toxicity profiles in terms of phytotoxicity, ecotoxicity, cytotoxicity and biodegrability (Egorova et al., 2015; Latała et al., 2009; Liu et al., 2016; Oliveira et al., 2016; Ranke et al., 2004; Ventura et al., 2014). It is now known that ILs can be considerably toxic especially those prepared using imidazolium, pyridinium and phosphonium cations.

Table 2.3: Emissions during the synthesis of various ILs (Mehrkesh & Karunanithi, 2015).

IL	Impact (CTUe)	Reactants	Emissions	Impact (CTUe)	%
$[BMIm]^+[Br]^-$	48.3	1-methylimidazole 1-Bromobutane	Metals Bromobenzene Benzene 1,2-dibromobenzene	6.1 38.6 2.0 0.8	13 80 4 2
$[BMIm]^+[Cl]^-$	16.4	1-methylimidazole 1-chlorobutane	Metals Chlorobenzene Benzene 1,2-dichlorobenzene	7.7 6.4 1.2 0.5	47 39 7 3
$[BPy]^+[Cl]^-$	53.2	Pyridine 1-chlorobutane	Chloramine Chlorobenzene Benzene 1,2-dichlorobenzene	40.5 6.7 1.2 0.5	76 13 2 1

Frade and Afonso (2010) have attributed these moderate to high toxicity and low biodegrability profiles to the structural formation of both ions, the effect of the cation alkyl chain length, the side reactions engendered by ILs' anions and the lipophilicity of ILs' anions which allows for their penetration in biological membranes (Latała et al., 2009). Hence, the belief that the versatility and tuneability of ILs ionic species could resolve this issue. In fact, several studies have shown that the alteration of ILs structures or the shuffling of their conjugates yield less toxic fluids. For instance, imidazolium-based ILs, generally considered toxic, can be converted into relatively safe ILs by introducing an oxygen atom into the lateral alkyl chain of the imidazolium cation (Samorì et al., 2010). Moreover, Ventura et al. (2012) showed that the toxicity of phosphonium and guanidinium-based ILs can be altered by introducing ether or esters moieties in the cation alkyl chain. Concomitantly, ILs of low toxicity can be designed using biomaterials or biorenewable metabolites.

ILs prepared using quaternary ammonium salts recorded lower toxicity and high biodegrability than common ILs (Coleman & Gathergood, 2010). The use of biomaterials or bio-compatible substances represents a safer and cheaper route for the design of viable ILs. Given that these components have a natural origin, their acquisition is rather simple and their price affordable (Gorke et al., 2010). Physical properties such as, viscosity and polarity also impact ILs toxicity profiles. ILs possess high viscosities, which are known to affect mass-transfer kinetics, causing poor operability and poor diffusion, poor catalytic properties, difficult recycling, mixing and pumping operations and most importantly increase the overall toxicity of the solvent (Olivier-Bourbigou et al., 2002; Zhao & Baker, 2012). Moreover, ILs wide polarities entail their dissolution in aquatic organisms and wastewaters. This translates into clean-up cost and energy, alongside potential toxicity to ecosystems (Hallett & Welton, 2011). Hence, the design of better ILs must include biorenewable materials with enough versatility to reduce the impeding physical properties. Another solvent class namely, deep eutectic solvents (DESs) seems to satisfy these conditions altogether. The next section carefully examines these solvents with regards to their safety, sustainability and environmental acceptability. Due to their relatively recent appearance, the available literature on DESs' properties is fairly limited. In spite of this issue, the available literature will serve as a canvas for a logic and comprehensive description of these solvents.

2.3 Deep eutectic solvents

Although Abbott et al. (2004a) pioneered the emergence of DESs in the fields of chemistry and electrochemistry, the utility of eutectics had long been evident to the pharmaceutical industry as either facilitators of enzymatic reactions, or as separation and purification phases for pharmaceutical active salts (Davey et al., 1995; Gill & Vulfson, 1994). Currently, DESs are gaining increasing attention, mostly in industrial processes, as highlighted by the number of patents that have been awarded in the last five years (Figure 2.4). DESs differ from ILs in two specific aspects, i.e., their chemical formation process and the source of their starting materials. DESs result from a complexation (shown in Figure 2.5) between a halide salt or a HBA and a HBD. Most DESs are obtained from non-ionic species, such as salts and molecular components listed in Table 2.1 (Zhang et al., 2012). In contrast, ILs are synthesized from ionic components that combine through ionic bonds. The interactions between HBA and HBD involve mostly hydrogen bonding, occasional electrostatic forces and van der Waals interactions (Zhang et al., 2012). As trademark of eutectics, DESs possess melting points lower than those of their individual components as shown in Figure 2.6. Different explanations have emerged to explain the freezing point depression of DES, but the most plausible one assigns the cause to electrostatic interactions, hydrogen bonding and the asymmetry of the ionic species in DESs. Abbott et al. (2004) argued that the freezing points of DESs repose on several factors such as, the lattice energies of their asymmetric ions, the manner in which the HBA-HBD interacts, cationic charge delocalization and the entropy change resulting from the formation of a liquid phase.

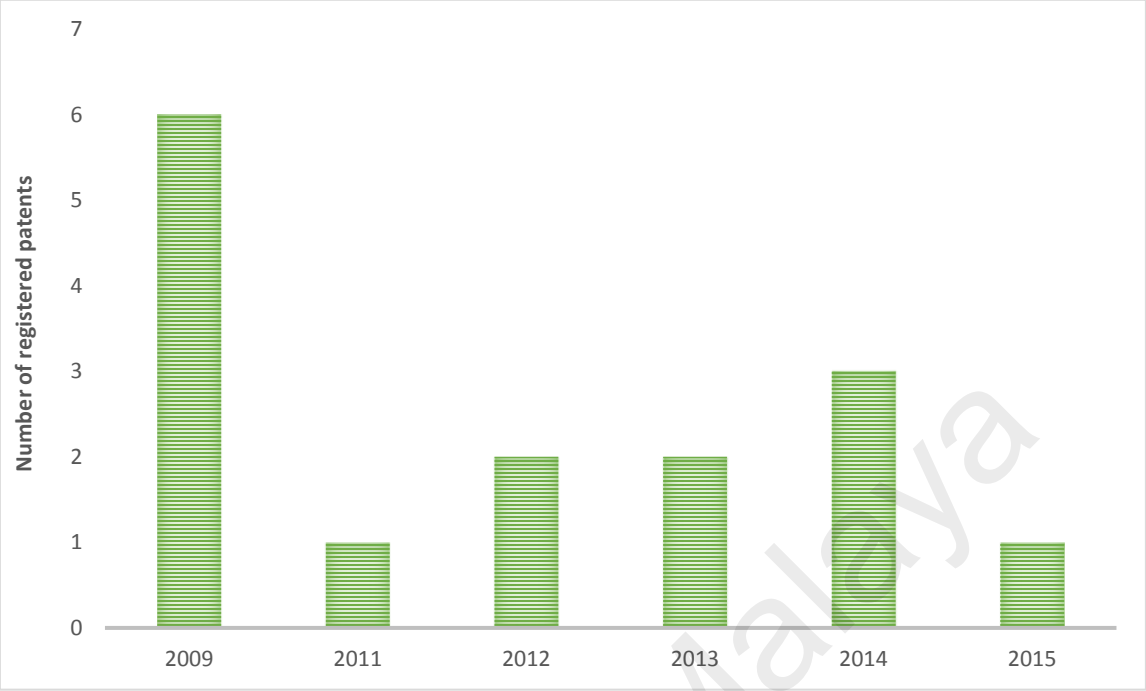


Figure 2.4: Number of patents on DESs registered the past 5 years (WIPO Patent Scope).

That is, hydrogen bonding between HBD and HBA as well as the sequestration of the halide ion by the HBD result in supramolecular complexes, which alter the free energy of the solid compared to that of the liquid phase, thereby creating a freezing point depression (Florindo et al., 2014; Jibril et al., 2014). The strength of the hydrogen bonding network is associated with a deeper decrease in the freezing point and a more stable mixture. Moreover, hydrogen bonds and van der Waals interactions interfere with the crystallization of the initial constituents, thereby stabilizing the liquid phase of the mixture (Francisco et al., 2013). Although the interactions between HBA and HBD include electrostatic attractions and van der Waals interactions, hydrogen bonding nevertheless remain the most important interactions. The most important intramolecular bonds in DESs are the hydrogen bonds between the HBD and the halide anion, as shown during the formation of [ChCl]-[Urea] DES in Figure 2.5.

Structural analysis of four different ChCl-based DESs by Perkins et al. (2014) indicated that the largest fraction of hydrogen bond interactions generally occurs between the HBD and the anion as shown in Figure 2.7. Depending on the occurrence of functional groups in the starting ingredients, some DESs can exhibit higher HBD-HBD hydrogen bond interactions. Nevertheless, they typically are followed in intensity by HBD-anion hydrogen bond interactions. In Figure 2.7, with the exception of the [ChCl]-[Urea] DES, the highest hydrogen bond fractions in the remaining three DESs are shared between the HBDs and their respective anions. In the [ChCl]-[Urea] DES, the oxygen atom in urea acted relatively as a strong HBA, which competed for hydrogen bonds, thereby resulting in weaker HBD-anion interactions. Among the examined DESs, namely [ChCl]-[Urea], choline chloride-ethylene glycol ([ChCl]-[Ethylene glycol]) and choline chloride-glycerol ([ChCl]-[Glycerol]), the authors recorded similar anion-cation, anion-anion, cation-cation, HBD-HBD and HBD-anion radial distribution function (RDF) distances. The most significant RDF variations across all DESs occurred between the anion-HBD, thereby implying that hydrogen bonds form the basis of DESs' neoteric properties (Perkins et al., 2014). The vast hydrogen bond network is responsible for the intrinsic characteristics of DESs such as, their low melting points, low volatility, non-flammability, low vapor pressure, dipolar nature, chemical and thermal stability, high solubility, tuneability, high viscosity, high density, ease of storage due to their chemical inertness with water, alleged low toxicity and high biodegradability (Florindo et al., 2014; Zhang et al., 2012). The low lattice energies of HBDs' and HBAs' large asymmetric ions result in low melting temperatures, whereas hydrogen bonding is responsible for the generation of DESs' room temperature liquid phases (Smith et al., 2014). These aspects ultimately derive from the chemical natures of DESs' chief ingredients.

Table 2.1 shows the common components of DESs. Given that numerous HBDs and HBAs can be used to prepare DESs, it is relevant to discuss the different types of DESs known to date (Table 2.4).

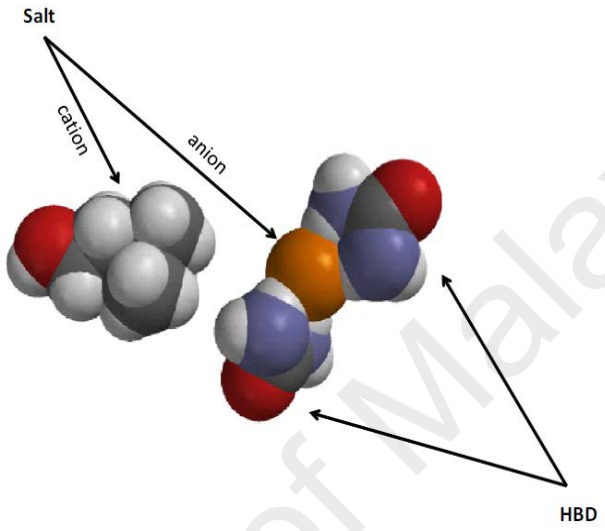


Figure 2.5: Formation of DES through complexation of a quaternary ammonium salt with a hydrogen bond donor (metal halide, alcohols, carboxylic acids or carbamides) (Abbott et al., 2004a).

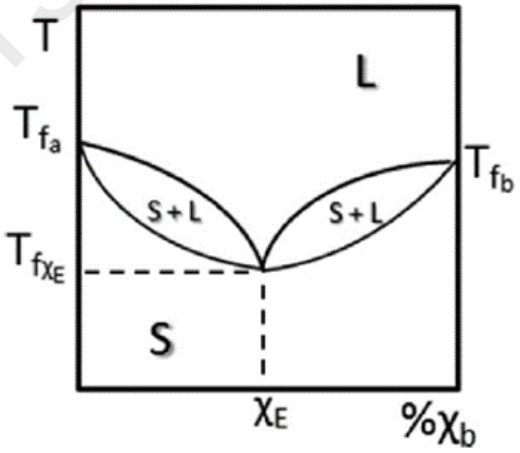


Figure 2.6: Phase diagram depicting the freezing point depression characteristic of DESs (Paiva et al., 2014a).

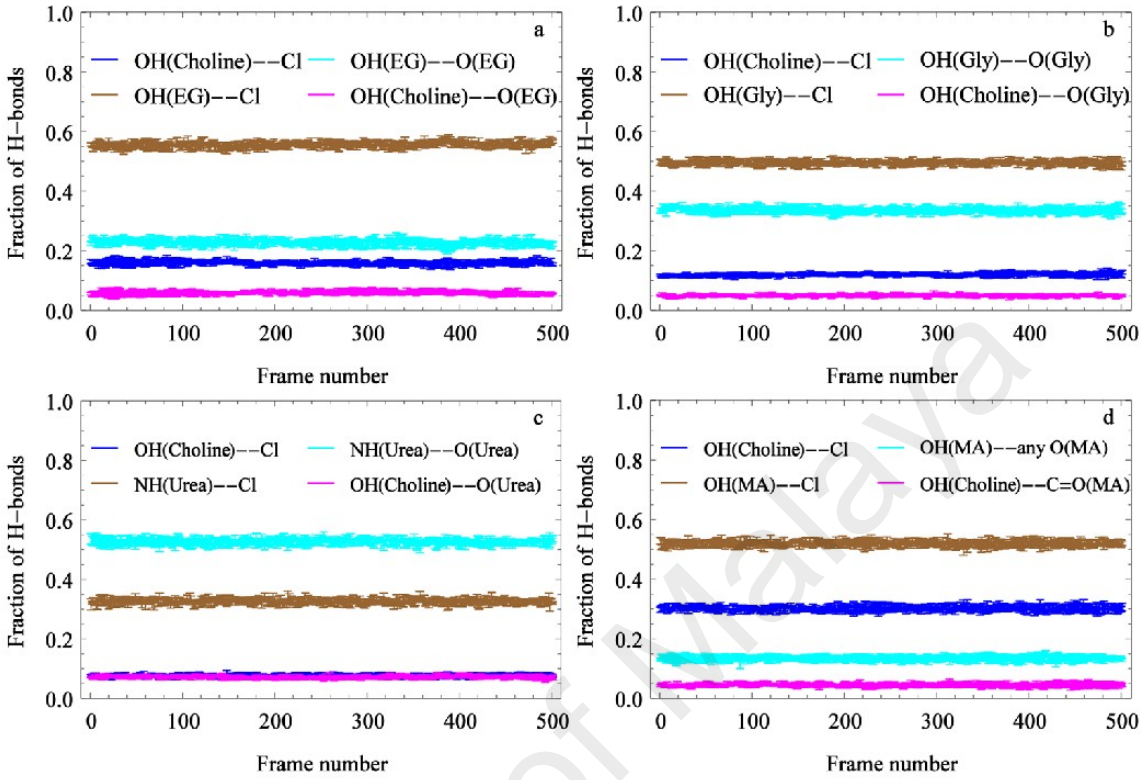


Figure 2.7: Hydrogen bonds fractions in a) [ChCl]-[EG], b) [ChCl]-[Gly], c) [ChCl]-[U], d) [ChCl]-[MA] ChCl: Choline chloride; EG: Ethylene glycol; Gly: Glycerol; U: Urea; MA: Malonic acid (Perkins et al., 2014).

Smith et al. (2014) described DESs by the following formula: Cat^+X^-zY; in which Cat^+ represents the cations of various sulfonium, phosphonium or ammonium salts, X^- is the halide anion of the salt, Y is a Lewis or Brönsted acid and z is the number of molecules of Y. Among the four types of DES listed in Table 2.4, the third type is the most encountered in literature because of their simple preparation, high biodegradability, low cost of their starting materials, the versatility of chief ingredients and nonreactivity with water. Examples include the well-known [ChCl]-[Amide/Carboxylic acids] engineered by Abbott et al. (2003) and Abbott et al. (2004a).

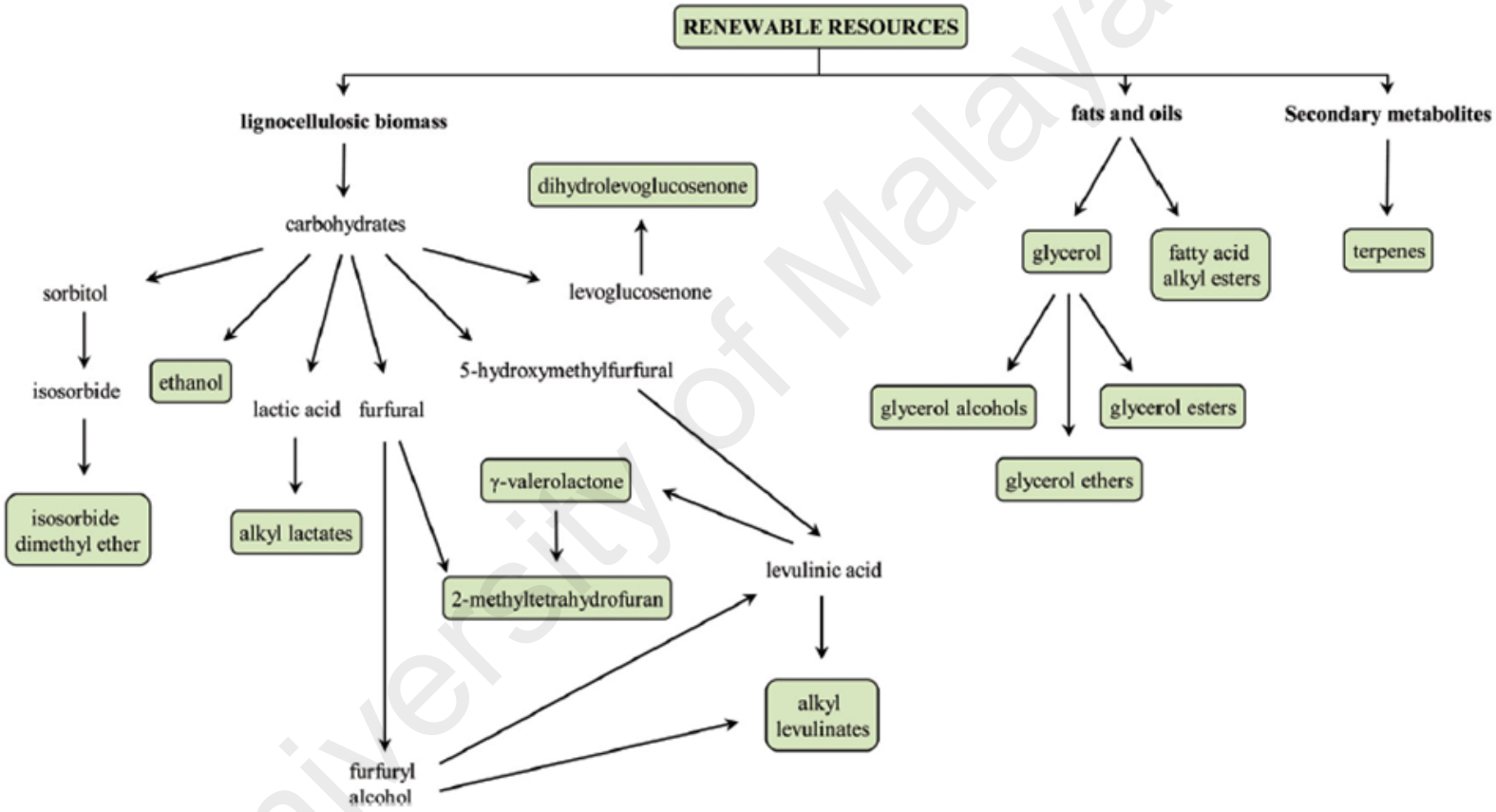
Table 2.4: Main types of deep eutectic solvents (Smith et al., 2014).

Type	Formula	Term
I	$Cat^+X^-zMCl_x$	M: Zn, Sn, Fe, Al, Ga, In
II	$Cat^+X^-zMCl_x{\bullet}yH_2O$	M: Cr, Co, Cu, Ni, Fe
III	Cat^+X^-zRZ	Z: $CONH_2$, COOH, OH
IV	$MCl_x + RZ = MCl_{x-1}^+{\bullet}RZ + MCl_{x+1}$	M: Al, Zn; Z: $CONH_2$, OH

The first type of DESs did not receive as much attention as the third type because of the inherent hygroscopic character of the metal salts (e.g., $AlCl_3$) used to prepare them. The second type entailed better applications due to their insensitivity to moisture and the wider array of hydrated metal salts available for their synthesis (Abbott et al., 2004b). The fourth type has rarely been characterized. Abbott et al. (2007) initiated research on this category by characterizing the properties of $ZnCl_2$ as a complexing agent, together with amides and carboxylic acids as HBDs. Recently, various scientists have sought to promote and enforce a so-called novel class of DESs. These DESs are obtained using combinations of HBDs from biorenewable starting materials. Thus, the mixtures in this category are referred to as natural deep eutectic solvents (NADESs). Their discovery resulted from an attempt to explain and understand the solubility of intracellular compounds that have intermediate polarity and are otherwise insoluble in both water and lipid phases. At the moment it is still difficult to categorize these mixtures as DESs in the proper sense of the term according to the general formula of DESs because, very few physicochemical characterizations of NADESs exist (Craveiro et al., 2016).

2.4 Natural deep eutectic solvents

NADESs discovery was a consequence of an attempt to explain and understand the solubility of intracellular compounds of intermediate polarity, otherwise indissoluble in both water and lipid phases. Choi et al. (2011) categorized NADESs as a possible third liquid phase in plant cells, necessary for biological processes such as cryoprotection, drought resistance, germination and dehydration. Some NADESs can fit in the 3rd category of DESs as they are prepared using ChCl. Most of their starting materials are derived from renewable resources shown in Scheme 2.1. Therefore, their toxicity was expected to be negligible. Just like their parents, NADESs exhibit attractive properties such as versatility, affordability and non-coordinating. As a subcategory of DESs systems, NADESs physicochemical characteristics are a consequence of the extensive hydrogen bond network supporting their supramolecular complexes. As NADESs can be prepared using exclusively HBDs or using HBAs in combination with HBDs hydrogen bonding remain the predominant intermolecular force in these mixtures. Consequently, they exhibit similar appealing and undesirable characteristics as their DESs. For instance, NADESs viscosities and densities are on average higher than typical organic solvents, but comparable to ILs and DESs (Table 2.2). However, it is possible to reduce the high viscosities and densities of NADESs via the addition of water. Following their discovery, few attempts have been made at characterizing the full potential of NADESs. Most applications of NADESs revolve around their use as extraction solvents for plants' natural bioactive compounds, drug/compounds solubility enhancers and biomass treatment (Kumar et al., 2016; Li & Lee, 2016; Liu et al., 2016; Radošević et al., 2016). Table 2.5 lists the potential combinations, which can be used to prepare NADESs.



Scheme 2.1: Inventory of NADESs starting materials (Pena-Pereira et al., 2015).

Table 2.5: Various NADESs combinations (Dai et al., 2013).

Components			**Molar ratio**
Component 1	**Component 2**	**Component 3**	
Choline chloride	Malonic acid		1:1
Choline chloride	Maleic acid		1:1, 2:1
Choline chloride	Xylitol		5:2
Choline chloride	Glycol		1:1, 1:2
Choline chloride	Glycerol		1:1, 3:2
Choline chloride	D-(+)-Glucose		1:1, 2:1
Betaine	Sucrose		4:1, 1:1
Betaine	D-Sorbitol		3:1
Betaine	Sucrose	Proline	1:1:1
Betaine	Malic acid	D-(+)-Glucose	1:1:1
Betaine	Oxalic acid	D-(+)-Glucose	1:1:1
Malic acid	D-(+)-Glucose		1:1, 1:2
Malic acid	D-Xylose		1:1
Malic acid	D-Mannose		1:1
Malic acid	Maltose		2:1
Malic acid	Raffinose		3:1
Citric acid	Sorbose		1:1
Citric acid	Ribitol		1:1
Citric acid	Adonitol		1:1
Citric acid	D-(+)-Trehalose		2:1
Citric acid	Malic acid		1:1
Citric acid	Sucrose		1:1
D/L-Proline	Sucrose		2:1, 3:1
D/L-Proline	D-Sorbitol		1:1
D/L-Proline	Lactic acid		1:1
D/L-Proline	Malonic acid		1:1
D/L-Proline	DL-Malic acid		1:1
Phytic acid sodium	Betaine		1:6
Phytic acid sodium	Glycerol		1:6
Phytic acid sodium	Choline chloride		1:3
Phytic acid sodium	L-Proline		1:6

The lack of extensive physical characterization of NADESs is an important factor to be addressed if their potential is to be exploited. Moreover, it is important to understand these properties in order to study their cytotoxic profiles in-depth. In spite of the repeated allegations about their low cytotoxicity, only a few cytotoxic studies are currently available. Hence, a lot more remains to be done in that regard. During the discussion on ILs, the issues currently slowing down their large-scale use have been discussed. It is important to examine DESs in the same light to truly ascertain their superiority to ILs. DESs only can be recognized as green solvents if they match the expected, multi-layered safety and sustainability requirements. In order to define DESs as ideal solvents, they must be assessed and compared with their predecessors in this case, ILs and organic solvents.

2.4.1 DESs preparation

Four methods are typically used for DESs preparation: heat-and-stir synthesis, freeze-drying and evaporation and the grinding method. The heat-and-stir procedure is by far the most common because it requires minimal energy input as the preparation of the solvent involves heating the components together at 80 ºC – 100 ºC with constant stirring. One of the hallmarks of DESs-based research was the non-requirement for purification steps during their preparation, in contrast to ILs (Dai et al., 2013). This assumption was recently challenged by the discovery of impurities in DESs following the heat-and-stir synthesis. In some cases, the hygroscopic character of the starting materials generates water impurities. Although, these impurities can be removed via freeze drying of aqueous solutions of individual DESs counterparts (Gutiérrez et al., 2009); other types of impurities are still a concern, especially when organic acids are used as HBDs.

Florindo et al. (2014) showed the formation of esters impurities in a [ChCl]-[Glutaric acid] DES, following the heat-and-stir based preparation. The authors uncovered that using a carboxylic acid (in this case glutaric acid) as HBD, in combination with ChCl salts, promotes the formation of HCl as a co-product. HCl in turns favors the formation of ester-impurities. These impurities increase the viscosities of DESs, which are already critical with regards to their use and applications. For this reason, Florindo et al. (2014) recommended the grinding method -mostly used in the pharmaceutical industry- in order to synthesize pure, esters-free DESs. The grinding approach entails the mixture of raw materials in a mortar with a pestle at room temperature until a homogeneous liquid is formed (Florindo et al., 2014). This method does not require heat or energy, although the workload could be a cause of concern. The spectra in Figure 2.8 show the formation of esters moieties during the heat-and-stir preparation of DESs, whereas the NMR spectra in Figure 2.9 highlights that the grinding method provides highly pure DESs mixtures. Another method, namely, the mechanochemical synthesis although mostly used during industrial applications can also be applied to obtain pure DESs in bulk. Crawford et al. (2016) recently showed the benefits of the mechanochemical synthesis by twin screw extrusion for the rapid and continuous synthesis of pure DESs. The process provides space and time yields of higher magnitude than currently employed batch process based on the endothermic heat and stir method. It also facilitated the collection and transfer of viscous DESs. Overall, DESs synthesis can generate impurities depending on both the method and the chief materials used. Nevertheless, the workload is vastly reduced compared to ILs where purification steps are amply required. Further, DESs preparation does not involve organic solvents, thereby limiting air pollution and health hazards. Recycling of DESs will not discussed extensively here due to the limited amount of information available.

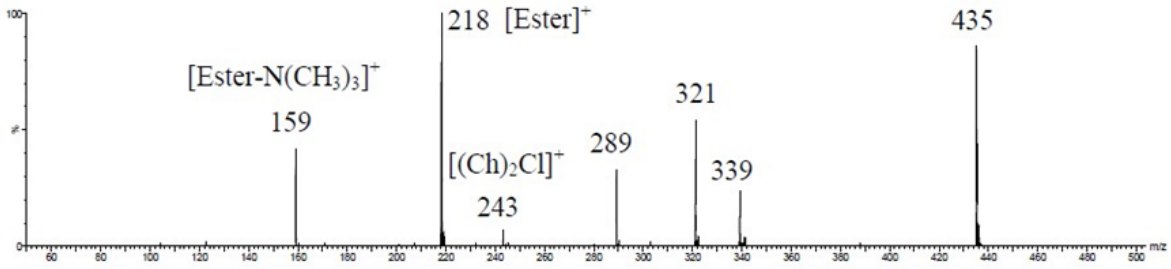


Figure 2.8: Fragmentation spectra of cholinium chloride and glutaric acid DES (Florindo et al., 2014).

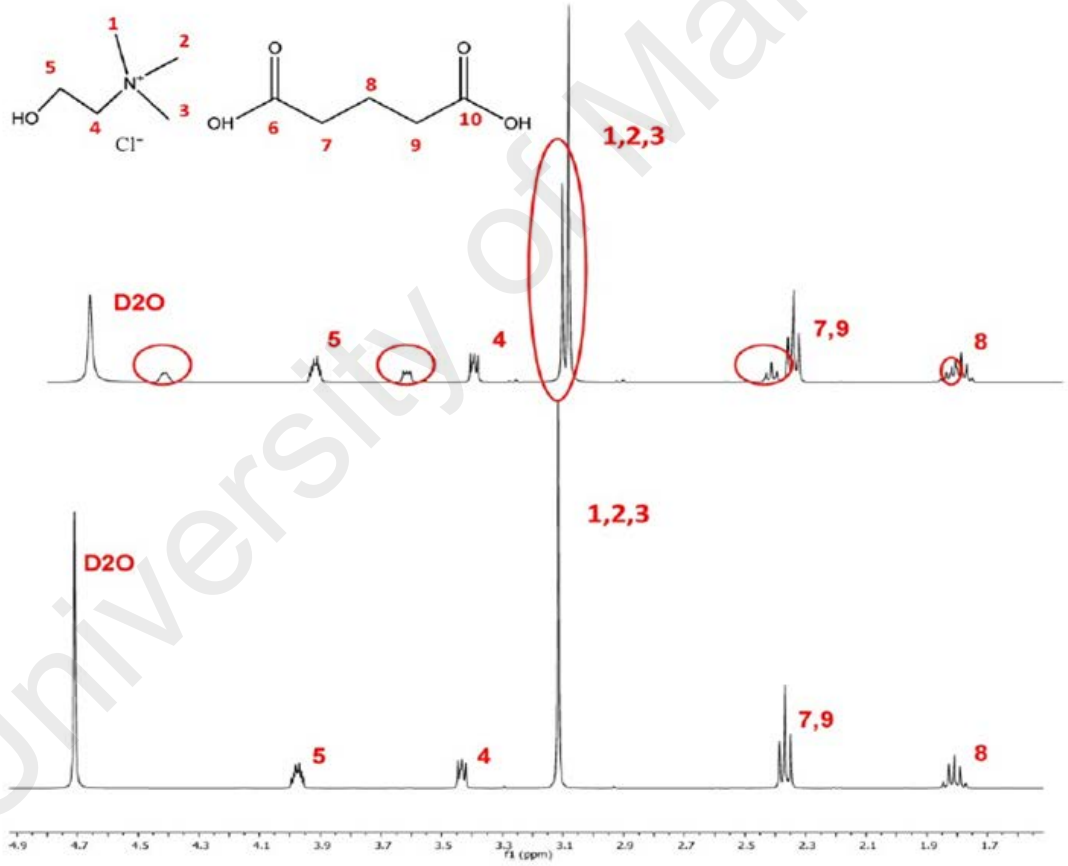


Figure 2.9: NMR spectra of [ChCl]-[Glutaric acid] prepared by heating at 100°C (upper quadrant) and by grinding (lower quadrant) (Florindo et al., 2014).

Nevertheless, DESs have been recycled countless times in extraction, separation, biotransformations and organic synthesis procedures; some of which involve the use of organic solvents (Jeong et al., 2015; Lobo et al., 2012; Singh et al., 2014).

2.4.2 Impact on health and environment

As previously mentioned when discussing ILs, the impact on the health and environment should include pre and post release assessments. Unfortunately, due to the recent advent of DESs, no life-cycle assessments have yet been performed. Hence, this section will solely examine the post-environmental release impact of these solvents. The "green" attributes of DESs are based on their non-volatility and the alleged benign nature of their chief constituents. Most DESs are ChCl-based and this most likely is due to the cholinium characteristics. Cholinium (Ch^+), an essential ingredient of vitamin B4, is used extensively as a food additive, indicating that the appropriate authorities have classified it as a safe compound (Shahriari et al., 2013). In addition, cholinium derivatives have important metabolic functions linked to the storage of carbohydrates, enzyme activity and the synthesis of vitamins (Zeisel & da Costa, 2009). Consequently, cholinium derivatives are frequently used as raw materials for the synthesis of both DESs and ILs (Zhao et al., 2011). However, based on the information that is available about ILs, the toxicity of choline-based mixtures hardly could be deemed to be negligible. Figure 2.10 summarizes up-to-date, post-release studies of the effects of DESs on various organisms.

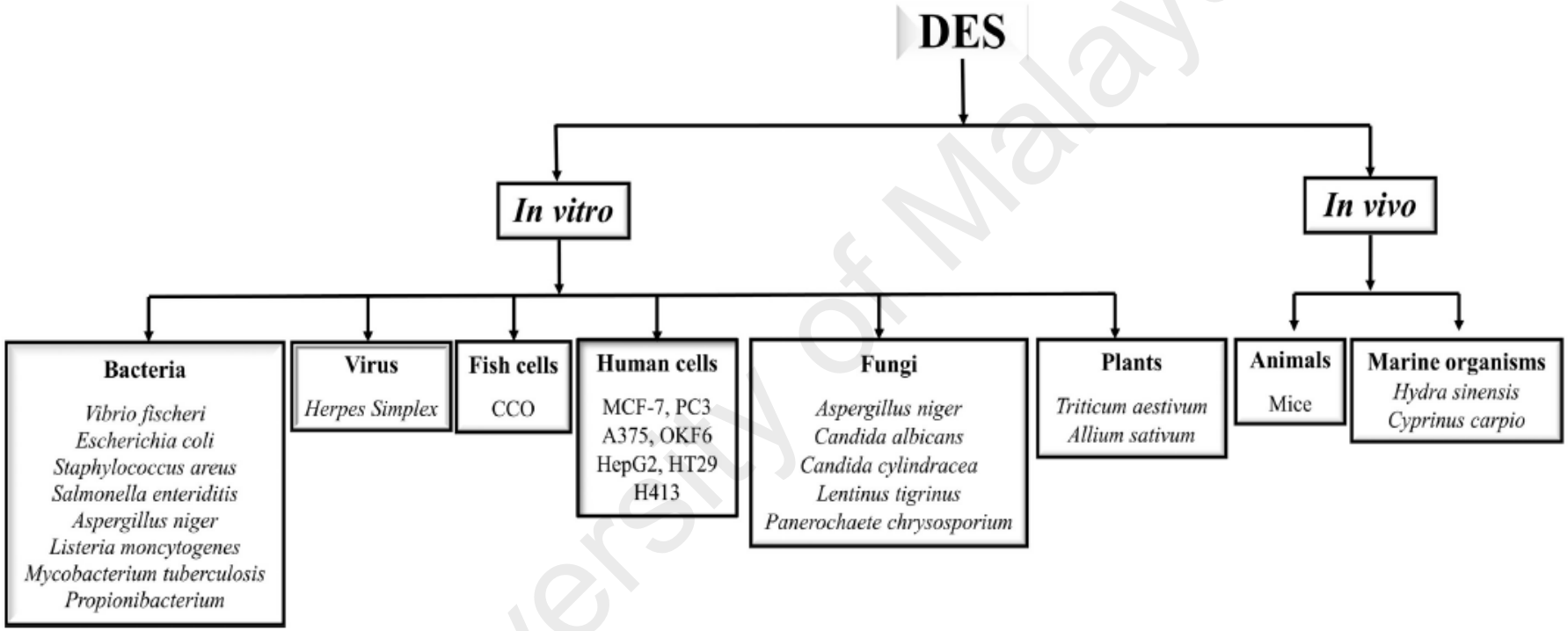


Figure 2.10: Flowchart of organisms used in previous DESs and NADESs toxicity assessments.

2.4.2.1 Cytotoxicity and phytotoxicity of DESs

The first set of DESs' cytotoxic studies assessed the effects of cholinium and phosphonium-based DESs on various bacteria (Table 2.6) (Hayyan et al., 2013b, c). During Hayyan et al. (2013c) study, ChCl-based DESs exhibited no toxicity against bacteria, but they showed lethal cytotoxicity in brine shrimp (Table 2.7). In contrast, Hayyan's group revealed that phosphonium-based DESs had lethal toxicity towards both bacteria and brine shrimp. In both cases, the toxicity of the DESs was significantly higher than the toxicities of the starting materials (Hayyan et al., 2013b, c). This observation has been stated in several assessments of the toxicity of DESs. It has been reported that this occurs because the intrinsic characteristics of the starting ingredients are altered during the formation of DESs, resulting in different properties (Smith et al., 2014; Zhang et al., 2012). The ensuing toxicity profiles that result from these synergistic interactions depend on multiple variables, including the salt's cation, the counteranion species, the molar ratio and the chemical nature of the HBD. Some researchers have suggested that the charge delocalization that occurs during the formation of DESs is responsible for their having higher toxicity than their individual constituents (Hayyan et al., 2013b). Delocalized cations, such as cholinium, enhance the toxicity of certain mixtures through the interaction of their side chains and head groups with cellular membrane groups (Modica-Napolitano & Aprille, 2001; Wen et al., 2015). The accumulation of positively-charged cations creates electrostatic attraction on the surfaces of cells' membranes and this eventually damages the negatively-charged bilayer. For instance, Kurtoglu and Lampidis (2009) emphasized that the sensitivity of cancer cells and cardiac muscle cells to anthracyclines and delocalized lipophilic cations was due to their positive charge, which, in turn, defined their selective accumulation and toxicity in these two types of cells.

Although it has yet to be conclusively shown that this occurs with DESs, studies using cholinium-based ILs have reached the same conclusion (Gal et al., 2012; Ventura et al., 2014). The findings indicated that the retention of small cations on the bilayer at high IL concentrations disrupts the membrane, thereby increasing the permeability of the membrane, which allows the free flow of IL species into the cytoplasm.

Table 2.6: Influence of DESs on the inhibition of bacteria (Hayyan et al. 2013b, c).

Bacterium	**Bacterial inhibition (cm)**							
	[ChCl]-[Gly]	**[ChCl]-[EG]**	**[ChCl]-[U]**	**[ChCl]-[TEG]**	**ChCl**	**HBD**	**[MTPPB]-[Gly]**	**[MTPPB]-[EG]**
E. coli	NI	NI	NI	NI	NI	NI	NI	0.70
S. aureus	NI	NI	NI	NI	NI	NI	NI	0.15
P. aeuriginosa	NI	NI	NI	NI	NI	NI	0.7	0.45
B. subtilis	NI	NI	NI	NI	NI	NI	NI	0.75

*****NI: No Inhibition;

MTPPB: Methyltriphenylphosphonium bromide; **Gly:** Glycerol; **EG:** Ethylene glycol; **U:** Urea. **TEG:** Triethylene glycol

Table 2.7: Survival time of brine shrimp in DESs (Hayyan et al. 2013b, c).

Number of Nauplii[a]	**Time (min)**						
	[ChCl]-[Gly]	**[ChCl]-[EG]**	**[ChCl]-[U]**	**[ChCl]-[TEG]**	**[MTPPB]-[Gly]**	**[MTPPB]-[EG]**	**[MTPPB]-[TEG]**
2	0.15±0.05	1.59±0.41	0.45±0.05	0.20±0.004	0.33±0.15	0.45±0.07	0.30±0.04
4	0.44±0.05	3.12±0.62	1.35±0.08	0.35±0.06	0.47±0.13	1.10±0.06	0.55±0.06
6	1.01±0.02	5.55±0.42	1.56±0.11	0.56±0.03	1.01±0.32	1.30±0.07	1.32±0.03
8	3.20±0.12	8.46±0.49	2.30±0.15	1.50±0.11	1.42±0.19	2.40±0.04	1.50±0.11
10	5.32±0.03	10.13±0.88	4.05±0.27	2.58±0.26	2.32±0.03	3.27±0.11	3.58±0.26

[a]Nauplii: The free-swimming first stage of the larva of Brine shrimp (*Artemia salina*)

MTPPB: Methyltriphenylphosphonium bromide; **Gly:** Glycerol; **EG:** Ethylene glycol; **U:** Urea. **TEG:** Triethylene glycol

Typically, this is followed by redox stress because delocalized cations target the mitochondria and trigger oxidative stress via an increased synthesis of reactive oxygen species (ROS) (Modica-Napolitano and Aprille, 2001). The toxicity of DESs also varies according to the salt's counteranion species. During a cytotoxic assessment, Wen et al. (2015) prepared two different sets of DESs using choline acetate (ChAc) and ChCl as salts. These two different DESs were administered to Hydra sinensis to evaluate their survival times. In Figure 2.11, although ChAc and ChCl-based DES had a deleterious effect on the survival times of Hydra sinensis, the ChCl-based DES had a greater detrimental effect than the ChAc-based DES (Figure 2.11 and Figure 2.12). In theory, when DESs dissociate in cellular media, it was assumed that cholinium cations aggregate and disrupt the cellular membranes (Gal et al., 2012).

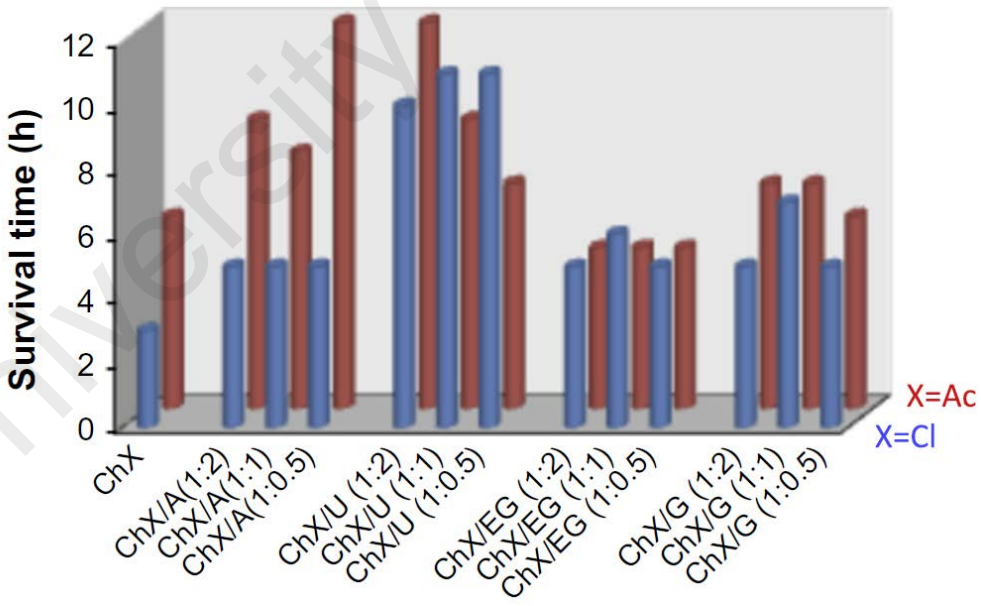


Figure 2.11: Survival time of hydras in different combinations of DESs (Wen et al., 2015). A: acetamide; Ac: acetate; U: urea; EG: ethylene glycol; G: glycerol.

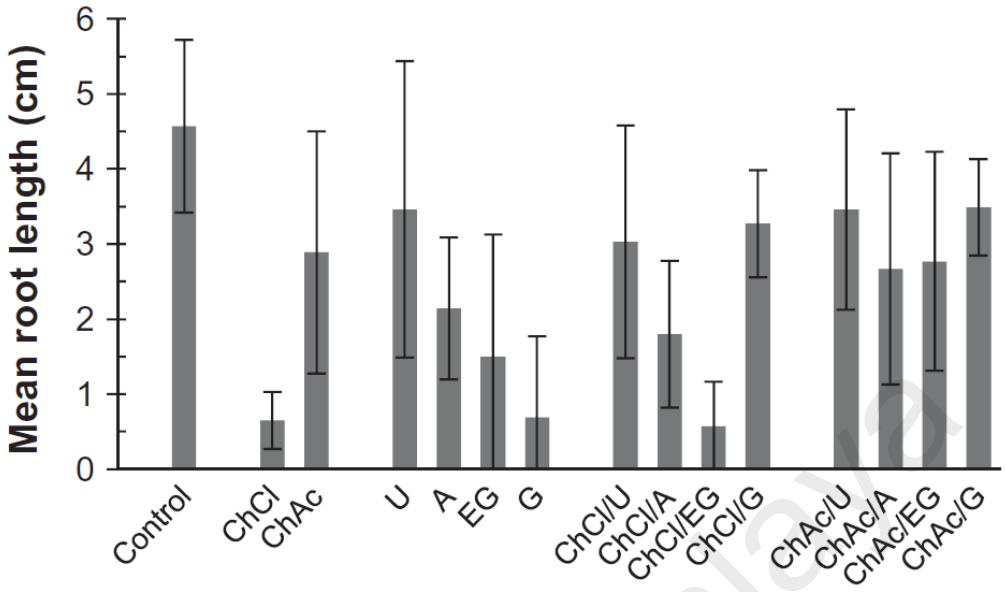


Figure 2.12: Assessment of root length growth upon treatment with DESs and its individual components separately (Wen et al., 2015).

This aggregation is dictated supposedly by the ionic interactions between the charged groups that are present on the surface of the membranes and the DESs salts' cations and respective counteranions (Wen et al., 2015). According to the Hofmeister series, ions are categorized as either kosmotropic or chaotropic depending on the Jones-Dole viscosity B coefficients (Broering & Bommarius, 2005). Kosmotropicity and chaotropicity relate to the degree of hydration, which is high for kosmotropic ions and low for chaotropic ions Based on these coefficients, acetate is a kosmotropic anion, chloride is a chaotropic anion and cholinium is a chaotropic cation (Jenkins & Marcus, 1995). The law of matching water affinities proposed by Collins (2004) suggests the favorable and stable pairing of chaotropic anions to chaotropic cations and the pairing of kosmotropic anions to kosmotropic cations. Since the surfaces of cell membranes are mostly composed of chaotropic cations (choline headgroups), chloride anions interact and form close ion pairs with cell surface moieties, whereas acetate anions are less favored.

The stronger affinity of Cl^- to the bilayer's cationic residues results in its disruption of cellular membranes to a greater extent than acetate. The disruption of cellular membranes is followed by an increase in membrane porosity. During an investigation of the levels of lactate dehydrogenase (LDH) following the treatment of human breast cancer cells (MCF-7) with DESs, Hayyan et al. (2015) confirmed the resulting altered permeability of the cellular membranes. LDH is an enzyme that is present in the cytosol of cells. When the cell membrane is disrupted, LDH is released into the cell media. The high levels of LDH observed when cells were treated with increasing concentrations of DESs are further proof that the cells' membranes have ruptured (Figure 2.13). Once the DESs' species enter the cytoplasm, they promote the synthesis of ROS, which ultimately lead to cellular necrosis via oxidative stress. Redox stress is marked by a decrease in antioxidants and a surge in free radicals. As Figure 2.14 shows, the immediate consequence is the increased apoptotic behavior of the exposed cells. However, it must be noted that other studies have attributed the cytotoxicity of DESs to cell dehydration (Cardellini et al., 2015, 2014). Whether that is a subsequent consequence of membrane porosity or a post-effect of redox stress has yet to be determined. Likewise, in plant systems, DESs treatment disturbs the natural homeostasis. In Figure 2.15, the analysis of the antioxidant defenses of wheat seeds following DESs treatment indicated an accumulation of ROS, such as malondialdehyde (MDA), alongside a gradual decrease in the concentrations of antioxidant enzymes and chlorophyll (Radošević et al., 2015). The overproduction of radical species in plants often leads to the oxidative degradation of lipids in cellular membranes, a phenomenon known as lipid peroxidation. Figure 2.16 shows the enzymatic activities of four antioxidants, i.e., superoxide dismutase (SOD), guaiacol peroxidase (GPx), catalase (CAT) and ascorbate peroxidase (APOx).

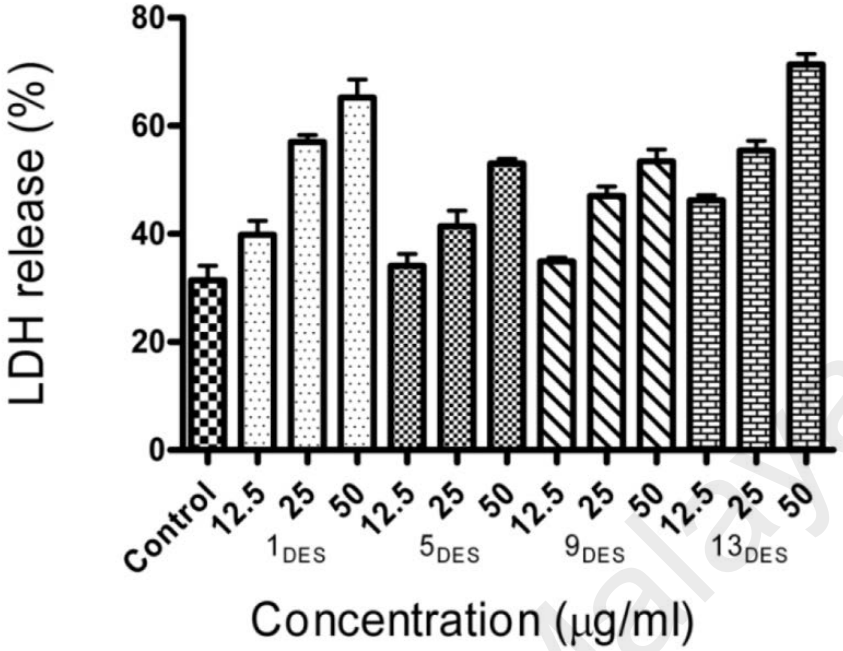


Figure 2.13: Concentration of LDH released upon DES treatment. 1_{DES}: [ChCl]-[Glycerol]; 2_{DES}: [ChCl]-[Ethylene glycol]; 9_{DES}: [ChCl]-[Urea]; 13_{DES}: [ChCl]-[Triethylene glycol] (Hayyan et al., 2015).

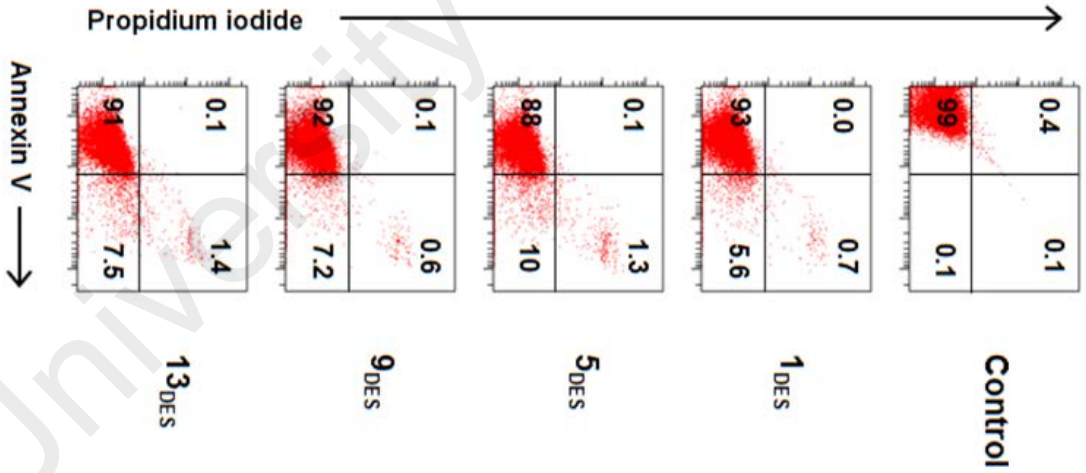


Figure 2.14: Induction of apoptosis in MCF-7 cells treated with DESs (Hayyan et al., 2015). Using annexin V and propidium, the percentage of apoptotic cells compared to control was assessed. Depending on dye concentration, the stage of apoptosis was determined.

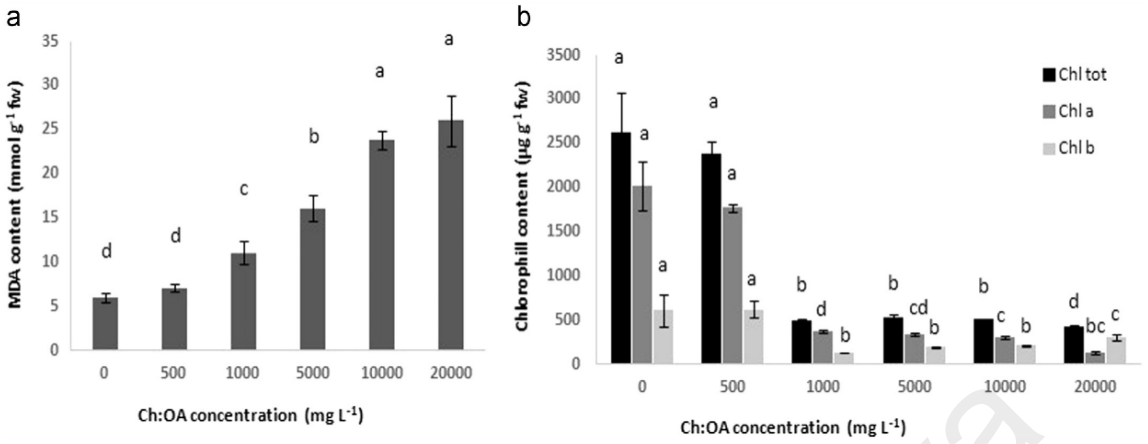


Figure 2.15: Effect of [ChCl]-[OA] on a) MDA and b) total chlorophyll levels (Radošević et al., 2015).

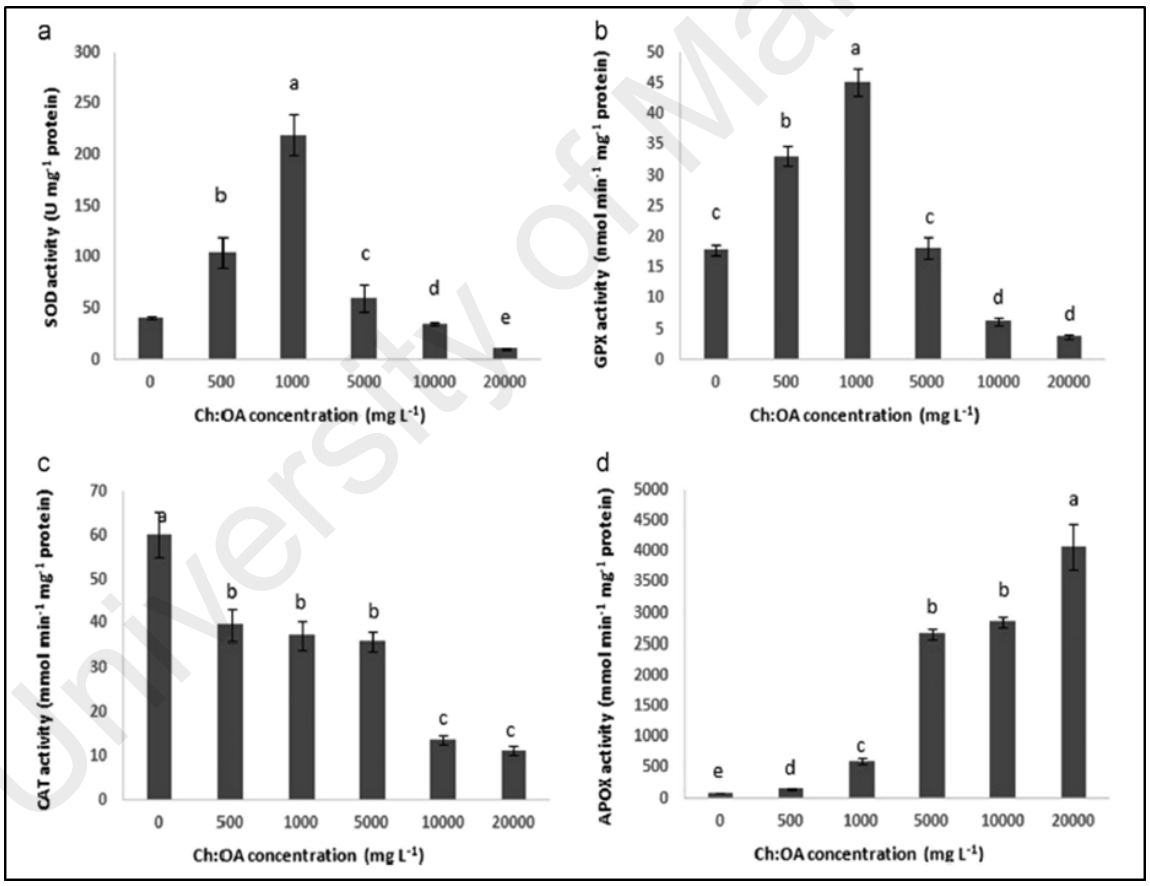


Figure 2.16: Effect of [ChCl]-[OA] DES on antioxidant enzymes. a) Superoxide dismutase (SOD), b) guaiacol peroxidase, c) catalase and d) ascorbate peroxidase (Radošević et al., 2015).

The activities of SOD and GPx increased to accommodate the toxic effects of the increasing concentrations of [ChCl]-[Oxalic acid] DES. However, after a threshold concentration of 1000 mg L^{-1}, the cellular machinery could no longer cope with the high levels of ROS. The catalase concentration decreased gradually from the onset of DESs treatment, whereas the concentration of APOX increased continuously. One of the end-products of lipid peroxidation is MDA. Therefore, the over-synthesis of MDA is indicative of ongoing oxidative stress. Chlorophyll, a pigment that is critical for photosynthesis, is indirectly responsible for plant growth and development. As such, its concentration can be used as an indicator of biochemical stress. Figure 2.15 shows the fluctuation in the concentrations of both MDA and chlorophyll after treatment with a choline chloride-oxalic acid ([ChCl]-[Oxalic acid]) DES. The concentration of MDA increased gradually, whereas the concentration of chlorophyll decreased continuously. Radošević et al. (2015) correlated the increasing levels of MDA with damage to the cellular apparatus due to oxidative stress. Furthermore, the authors examined the concentrations of antioxidants following treatment with [ChCl]-[Oxalic acid] DES.

The simple interactions between chaotropic/kosmotropic ions and chaotropic/kosmotropic surface groups described by Collins (2004) are not sufficient to entirely understand the mechanism of ChCl toxicity. With regards to bacteria, the individual components (HBD and HBA) generally are less toxic than the DES itself (Figure 2.17). However, marine organisms and plants follow an opposite trend. For instance, Figure 2.11 shows that the individual components of the DESs had greater deleterious effects on the survival times of Hydra sinensis than either of the two solvents. The salts exclusively caused a drastic contraction of the tentacles and a gradual disintegration of the hydras' bodies. Conversely, the HBDs did not seriously affect the hydras' lifetimes (Wen et al., 2015).

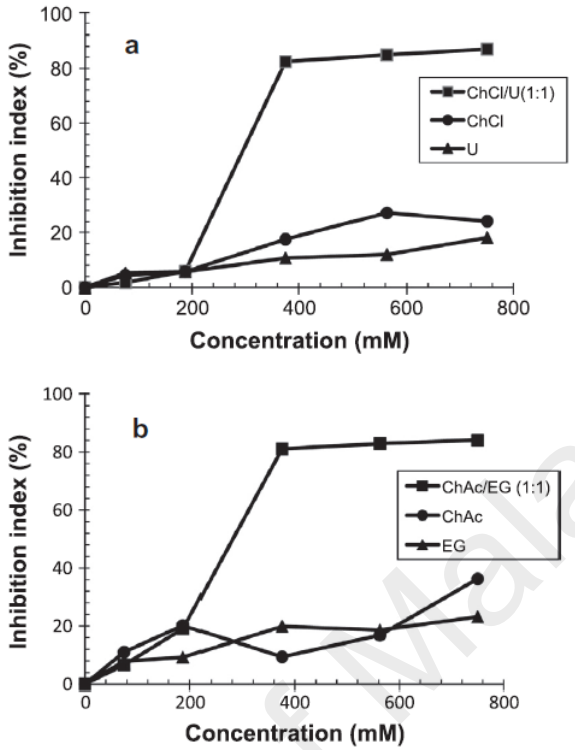


Figure 2.17: Inhibition index of *E. coli* cultures against [ChCl] and [ChAc]-based DESs (Wen et al., 2015). ChAc: choline acetate; u: urea; EG: ethylene glycol.

An identical observation was made by Huang et al. (2014) during an investigation of the survival times of Hydra sinensis after treatment with DESs. The authors reported that hydras subjected to ChCl treatment had faster disintegration rates than those treated with urea or [ChCl]-[Urea] DES. This suggested that the interactions between the salt and the surface groups of the cells (with different distributions of charge density depending on the organism) were more complex than originally thought and were not limited simply by the relationship derived from the water affinities principle described by Collins (2004). Typically, mixtures of DESs at minute concentrations are harmless, but fluctuations in their concentrations or in their molar ratios alter their toxicity significantly (Wen et al., 2015).

Figure 2.11 shows the influence of the molar ratio on the survival times of the hydras. At a salt/HBD ratio of 1:2, ChCl-based DESs (prepared using ethylene glycol, urea and glycerol as HBDs) reduced the survival times of the hydras. Longer survival times occurred as soon as the ratio was readjusted from 1:2 to 1:1. Since the improvement in the survival times occurred only after a change in the concentration of HBD, one can argue either that the HBD is not as safe as previously thought or that the molar ratio yields an unstable DES with remnants of the salt's toxic effect. Now, let us consider the impact of HBDs on the toxicity profiles of DESs. The negative impact of HBDs is best highlighted by considering DES systems prepared using organic acids (de Morais et al., 2015; Juneidi et al., 2016; Zhao et al., 2015). Florindo et al. (2014) noted that eutectic mixtures were highly viscous when they were prepared using citric acid or tartaric acid. Depending on the synthesis method, organic acids are known to generate waste during the preparation of DESs, i.e., the gentle heat-and-stir method has been associated with the formation of impurities, which increase the acidity of the system, ultimately making the proliferation of microorganisms and cells unsustainable. In addition, these impurities indirectly exacerbate the toxicities of the DESs by increasing their viscosities (Florindo et al., 2014). Radošević et al. (2015) provided additional evidence of the deleterious impact of organic acids by assessing the cytotoxicity and phytotoxicity of ChCl-based DESs on channel catfish ovaries (CCO), the human breast (MCF-7) cell lines and wheat seeds. Table 2.8 shows that combinations of [ChCl] with glucose or glycerol yield DESs that have low toxicity profiles. However, the complexation of ChCl with oxalic acid produced a highly toxic mixture. The acidity of the resulting DESs affected the cellular machinery and terminated the proliferation of the cells. Also, the use of [ChCl]-[Oxalic acid] promoted the intracellular formation of calcium oxalate crystals (Radošević et al., 2015). The detrimental effect of these crystals is obvious.

Table 2.8: EC_{50} values of the different DESs obtained during cytotoxicity and phytoxicity tests (Radošević et al., 2015).

Cell line	EC_{50} values of DES (mM)								
	[ChCl]-[Glu]	**[ChCl]-[Gly]**	**[ChCl]-[OA]**	**ChCl**	**OA**	**Glu**	**Gly**	**Ethanol**	**Toluene**
CCO fish	> 10	> 10	1.64	> 10	5.02	> 10	> 10	> 10	> 10
MCF-7 human	> 10	> 10	4.19	> 10	13.23	> 10	> 10	> 10	> 10
Process									
Germination	> 100	> 100	39.94	–	–	–	–	–	–
Shoot growth	9.85	32.81	3.67	–	–	–	–	–	–
Root growth	5.41	28.83	3.06	–	–	–	–	–	–

Glu: Glucose; **Gly:** Glycerol; **OA:** oxalic acid

These observations make the importance of the chemical structures of the salt and HBDs to the toxicity profiles of the DESs abundantly clear. Thus, the screening process for the HBD must be conducted very carefully with the aim of selecting relatively safe candidates, such as renewable biomaterials. Based on these results, several authors advocated the use of NADESs. The arguments raised in favor of these mixtures were focused on the natural origin of their constituents and the stable liquid phases obtained at room temperature (compared to some DESs that solidified). However, the available literature does not provide sufficient evidence for the previous generalization concerning the phases of NADESs, because the area is relatively new and very few physical characterization studies have been conducted (Craveiro et al., 2016; Dai et al., 2013, 2015). NADESs are comparable to DESs, especially in terms of the effect of organic acids as starting materials. Upon examination of bacterial growth following treatment with NADESs, Zhao et al. (2015) observed the highly deleterious attributes of organic acids based-NADESs, whereas other types of NADESs (sugars, amines and alcohols) were non-inhibitory (Table 2.9).

Table 2.9: Inhibition of bacterial growth (in cm) following treatment with NADESs (Zhao et al., 2015).

DES	*E. coli*	*S. enteritidis*	*S. aureus*	*L. monocytogenes*
[ChCl]-[U] (1:2)	NI	NI	NI	NI
[ChCl]-[At] (1:2)	NI	NI	NI	NI
[ChCl]-[EG] (1:2)	NI	NI	NI	NI
[ChCl]-[Gly] (1:2)	NI	NI	NI	NI
[ChCl]-[BD] (1:4)	NI	NI	NI	NI
[ChCl]-[TEG] (1:4)	NI	NI	NI	NI
[ChCl]-[Xyl] (1:1)	NI	NI	NI	NI
[ChCl]-[So] (1:1)	NI	NI	NI	NI
[ChCl]-[PTSA] (1:1)	1.71 ± 0.09	1.20 ± 0.01	1.12 ± 0.02	0.70 ± 0.01
[ChCl]-[OA] (1:1)	2.48 ± 0.03	1.93 ± 0.07	1.97 ± 0.07	1.50 ± 0.01
[ChCl]-[LA] (1:2)	1.65 ± 0.05	1.60 ± 0.10	1.00 ± 0.01	0.97 ± 0.08
[ChCl]-[MA] (1:1)	1.53 ± 0.03	1.17 ± 0.03	1.32 ± 0.03	0.93 ± 0.07
[ChCl]-[MAL] (1:1)	1.92 ± 0.08	1.22 ± 0.03	1.50 ± 0.05	1.10 ± 0.10
[ChCl]-[CA] (1:1)	1.93 ± 0.13	1.77 ± 0.03	1.58 ± 0.08	1.30 ± 0.05
[ChCl]-[TA] (2:1)	1.76 ± 0.14	1.50 ± 0.01	1.50 ± 0.19	1.10 ± 0.05
[ChCl]-[Xyl]-[W] (1:1:1)	NI	NI	NI	NI
[ChCl]-[Suc]-[W] (5:2:5)	NI	NI	NI	NI
[ChCl]-[Fru]-[W] (5:2:5)	NI	NI	NI	NI
[ChCl]-[Glu]-[W] (5:2:5)	NI	NI	NI	NI
[ChCl]-[Mlt]-[W] (5:2:5)	NI	NI	NI	NI

Also, Paiva et al. (2014a) assessment certainly highlighted excellent viability for specific NADESs. The data, presented in Figure 2.18, show that the cytotoxicity of NADESs depends on their chemical compositions and the interactions between their individual components. For instance, the most toxic NADESs were [ChCl]-[tartaric acid] and [ChCl]-[citric acid] and the least toxic were [tartaric acid]-[sucrose] and [ChCl]-[glucose]. It can be deduced that the toxic mechanism of ChCl depends on the HBDs used; in this case organic acids. However, organic acids might yield less toxic eutectics if they are mixed with ingredients other than ChCl.

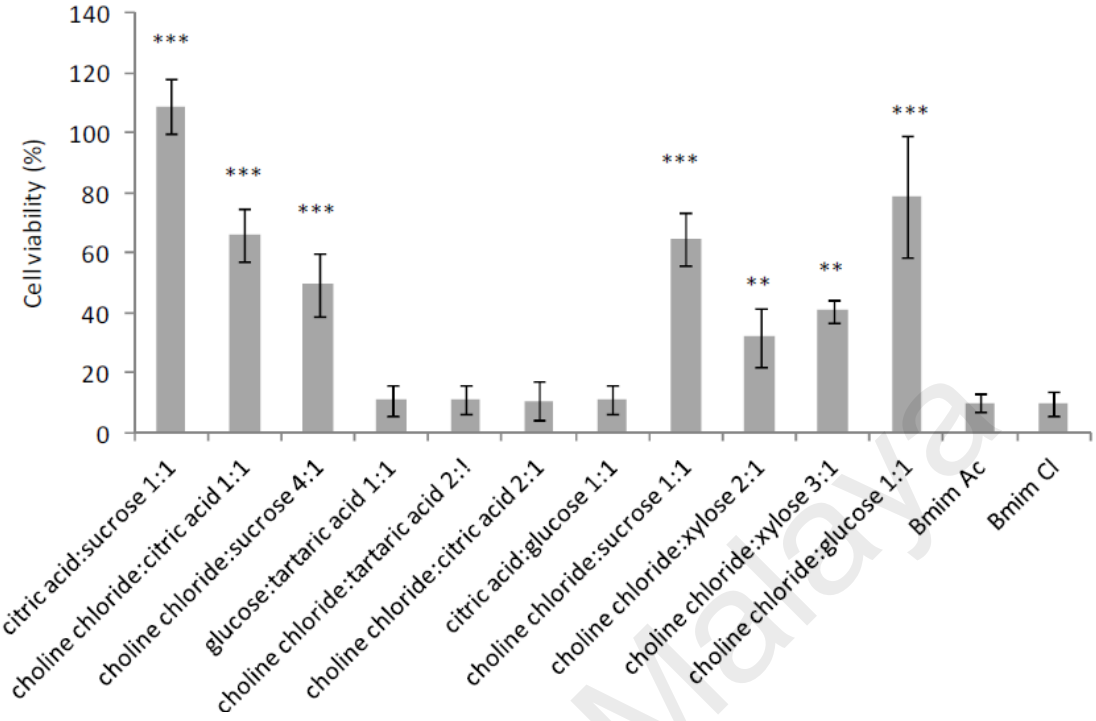


Figure 2.18: Viabilities of L929 fibroblast like cells in NADESs and ILs (Paiva et al., 2014a). Bmim Ac: 1-butyl-3-methylimidazolium acetate; Bmim cl: 1-butyl-3-methylimidazolium chloride.

2.4.2.2 *In Vivo* toxicity of DESs

The toxicity of DESs for mature mammals, namely mice, was assessed in a study led by Hayyan et al. (2015). The authors acted on the premise that an assessment of the toxicity of DESs for mice would be a very good indication of the toxicity for humans, because the two organisms share a significant degree of genetic homology. Table 2.10 shows that DESs at high concentrations were deadly to mice. The study also showed that the individual components of DESs were less toxic than the solvent itself. In general, the trend of the order for the toxicity between DESs and their components is similar between bacteria and mammals, but it is opposite in plants (with the only exception being wheat seeds in Radošević et al. (2015) study) and marine organisms. Changes in the molar ratio of the DESs, specifically with regards to the HBD, correlated with the fluctuating toxicity.

For instance, the [ChCl]-[Urea] DES with a molar ratio of 1:3 resulted in immediate death of the mice, whereas, at a molar ratio of 1:2, an LD_{50} value of 5.64 g/Kg was obtained.

Table 2.10: LD_{50} values of DESs tested with mice (Hayyan et al., 2015). The molar ratio of the DESs was 1:3 unless stated otherwise.

Solvent	LD_{50} (g/kg)
[ChCl]-[Gly]	6.39±0.53
[ChCl]-[EG]	5.33±0.49
[ChCl]-[U]	Toxic
[ChCl]-[U] 1:2	5.64±0.36
[ChCl]-[TEG]	5.31± 0.62
Pure Gly	20.6±2.16
Pure EG	9.71±1.95
Pure TEG	16.98±2.04
Pure U	>20
Pure ChCl	>20

Gly: Glycerol; **EG:** Ethylene glycol; **U:** Urea; **TEG:** Triethylene glycol

The over-confidence concerning the characteristics ChCl must be backed by in-depth studies to analyze the different interactions of cholinium with lipid bilayers, counteranions and HBDs. The toxicity of DESs has considerable ramifications in their numerous biological applications; therefore, a rigorous assessment of this property is required. However, the tuneability of DESs might be their greatest asset. This characteristic makes it possible to modify the structure and properties of HBAs and HBDs, which seem to be of significant importance in the toxicity profiles of DESs. The vast array of different HBAs and HBDs provides encouragement that there might be viable solutions for the toxicity concerns.

2.5 Biological perspectives of DESs

As alternatives to ILs, DESs can be applied to a wide range of biological industrial processes, such as extractions and separations, or biocatalysis. They can accomplish these tasks because they possess both solvent and non-solvent properties. These applications are examined in the subsequent sections.

2.5.1 Biotechnological applications

2.5.1.1 Extraction/purification/separation of natural products

Typically, the aim of extracting and recovering natural products from living organisms is to collect secondary metabolites. Despite being second in importance to primary metabolites (i.e., sugars, amino acids and nucleic acids), secondary metabolites in living organisms (especially in plants) play critical functions related to natural defense and stress-responses. Consequently, their absence or deficit indirectly leads to death via long term impairment of vital metabolic activities. Natural bioactive compounds (NBCs) are found in different organisms i.e. plants, microorganisms, algae and fungi. Hence the methods used to extract them are specific to each and every type of organism (Agostini-Costa et al., 2012). For instance, the extraction of NBCs from plant materials requires mechanical approaches, whereas biotransformations and genetic engineering are used to extract NBCs from microorganisms. NBCs are classified in five categories namely, polyketides, isoprenoids, alkaloids, phenylpropanoids and flavonoids (Oksman-Caldentey & Inzé, 2004). The interest in NBCs stems from their relevance in several fields, such as cosmetics, pharmaceuticals, nutraceuticals, agrochemicals and the food industry, in which they are used to manufacture biologically-active substances (Song et al., 2014). NBCs also represent major intermediates or synthetic affiliates of commercially-available drugs; it is estimated that at least 60% of approved anti-cancer drugs are derived from NBCs (Joana Gil-Chávez et al., 2013).

NBCs also are used as components of various food additives, surfactants, nutritional supplements, flavor enhancers, preservatives, flagrances and pesticides (Wang and Weller, 2006). Given their innumerable applications, various techniques exist to extract NBCs from plant material. These techniques are roughly divided into two categories, i.e., traditional and modern extraction methods. The traditional methods include solvent extraction, Soxhlet extraction, maceration and percolation. The modern techniques cover pressurized liquid extraction, subcritical fluid extraction, supercritical extraction, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and enzyme-assisted extraction (EAE), among others (Bart, 2011). The recovery of NBCs from solid phytomaterials using both traditional and modern techniques often is overshadowed by various issues. Traditional techniques mostly have long extraction periods, require large amounts of solvents, have inadequate solvent disposal and recycling processes, use toxic solvents and thermally degrade NBCs. Modern techniques represent a marked improvement over traditional techniques in terms of better extraction time and efficiency, higher purity of the extract and lower cost. However, they still are adversely affected by various physical and chemical issues, including the thermal instability of solvents, the toxicity of solvents, the solubility and polarity of solvents, the types and concentrations of solvents, the recovery of compounds from the solvents, moisture content, modification of NBCs during extraction and the degradation of the NBCs among others (Wang and Weller, 2006). In general, the major concerns about extraction procedures are the physicochemical properties of the solvents that are used. Table 2.11 lists the required qualities for a solvent and simply stated, an adequate solvent must have properties that are compatible with those of the solute. The various solvents currently in use have been approved by several authorities (FDA and the European Union) and these authorities monitor their use (Bart, 2011; FDA, 2012).

To date, water and VOCs remain the most commonly-used solvents in the food, pharmaceutical, cosmetic and agricultural industries. Using water as a solvent satisfies most of the required conditions listed in Table 2.11. Water is an effective solvent for hydrophilic and polar NBCs. However, it is far less effective as a solvent for hydrophobic and non-polar substances (Bart, 2011). These ambiguous qualities of water are further exacerbated by the presence of water-soluble impurities, which affect extraction procedures. Despite the successful applications of VOCs as solvents, they are, at least to some extent, the cause of solvent-based issues among the extraction techniques cited above. Therefore, the requirement for green and cheap compounds is at the heart of the issues in the biological industry. The scientific community has slowly become accustomed to the applications of various ILs and their DES analogs. The success of ILs has accentuated the interest of the scientific community in DESs, which is understandable because both exhibit the same core characteristics. In general, DESs meet most of the qualifications listed in Table 2.11, except those related to corrosivity, viscosity and toxicity, which are still under scrutiny. Numerous publications have focused on the role of DESs as suitable candidates for the extraction of NBCs. The recent successes in using DESs in this field are substantially significant when one compares the amounts of extracts and the recovery yields obtained using them rather than conventional solvents. For instance, Zhang et al. (2014) showed that the extraction of astaxanthin from shrimp waste with DESs rather than ethanol provided a 47.3% higher maximal output.

Table 2.11: Primary features of desired solvents (Bart, 2011).

Feature	Comments
Solubility	Solubility is crucial for extraction purpose. It is usually determined by the polarity of both the solvent and solute in question. Hydrophilic solutes will dissolve easily in hydrophilic solvents and vice-versa.
Selectivity	A solvent with high selectivity for a specific group of molecules decreases the amount of steps needed for extraction.
Recyclability	The more reusable a solvent, the better it is. Of course, the efficiency must be directly correlated with the reusability.
Thermal and chemical stability	Thermal and chemical stability entails the use of the solvent at high temperatures and substantially reduces the risk for side reactions encountered with the use of the solvent.
Toxicity	The toxicity profile of the solvent must be low, preferably negligible. This is extremely important for both the pharmaceutical and food industry.
Flammability	A solvent with no flammability is preferred, but extra safety measures can be taken if the solvent is flammable.
Corrosivity	Corrosivity is not desired for any solvent used in chemical or biochemical process. A highly corrosive solvent can damage both the reactant and the target product. Moreover, solvents with high corrosivity necessitate pre-treatment and post-treatment for adequate recovery of the target compound. The possibility of recyclability is also decreased.
Ecological impact	The ecological impact must be reduced; therefore, the solvent should be easily biodegradable and the volatility should be low or inexistent.
Surface tension	Low surface tension is critical in terms of porosity and wetting ability. These dictate the ability of a solvent to penetrate biological matrices and attach to the compound of interest. This comes in handy during extraction procedures.
Viscosity	High viscosity directly affects mass transfer kinetics and as such, is detrimental for reaction rates, dispersion, pumping, recycling and mixing operations.
Melting point	For ease of handling, low melting point is preferred.
Dielectric constant	High dielectric constant is important for solvents since it is a measure of the polarity of the latter. Using this value, the correct match can be made between the target compound and the solvent.
Availability and cost	The availability and low cost of a solvent are important aspects to take into consideration especially regarding the economic strategy of the industry.

Table 2.12 shows the progress achieved using DESs as solvents or co-solvents in extraction procedures. Park et al. (2014), in their study of the extraction of chlorogenic and caffeic acids from *Herba Artemisiae Scopariae* using a DES as a co-solvent, reported increases of 177 and 138%, respectively, over the amounts obtained by Han et al. (2009), who used a mixture of methanol and water as the solvent (60:40, v/v). The ability to design DESs with different solubilities is a great asset in that, hypothetically, they could be viewed as a universal solvent given their expected wide range of polarities. These studies pinpointed the crucial factors that have significant influences on DESs-based extraction procedures (Table 2.13). The applications of DESs include separations, analyses and sometimes, the recovery of both NBCs and metal ions from environmental sources. Gu et al. (2014) separated polar compounds, such as phenols, amines and acids, from hexane by independently using [ChCl]-[Gly], [ChCl]-[EG], [ChCl]-[U] and [MTPPB]-[EG] DESs as the acceptor phases in ultrasound-assisted LLE. Since polar DESs were selected, they were able to trap the phenols easily from the non-polar solvent. The enrichment factors obtained were similar or superior to those obtained with ILs. In a different study, Zeng et al. (2014) achieved the purification of proteins (bovine serum albumin) in various samples using DESs in a two-phase aqueous system. The migration of the proteins to the DES phase was attributed to hydrophobic interactions, the salting-out effect and the hydrogen bond network. Choi et al. (2011) proposed that the intracellular requirement of NADESs for the dissolution of NBCs in plants suggest that they can act as viable substitutes for both VOCs and ILs, especially in extraction procedures in which a prerequisite is that the NBCs must be soluble in the extraction media.

Table 2.12: Recent applications of DESs in extraction procedures.

Extract	Source	Extract function	DES	Role of DES	Method	Comments on extraction procedure	Ref.
Astaxanthin	Shrimp by-products	Antioxidant	[ChCl]-[EG] [ChCl]-[Gly] [ChCl]-[1,2-BD] [ChCl]-[1,3-BD] [ChCl]-[1,4-BD] [ChCl]-[2,3-BD] [ChCl]-[1,6-HD]	Solvent	UAE	Optimum performance achieved in 30 min, at a solid/liquid ratio of 1/15 g ml^{-1}, under ultrasonic power of 85W and using 1.0 mL of solvent. Best extraction efficiencies were obtained using [ChCl]-[1,2-BD] (1:5) mol mol^{-1}. The amount of astaxanthin derived from shrimp shells was 146 µg g^{-1}; the maximum amount extracted from shrimp heads was 218 µg g^{-1}. The recoveries of astaxanthin following DESs-based extractions were in the range of 76 - 102%.	(Zhang et al., 2014)
Phenolic acids (chlorogenic acid and caffeic acid)	*Herba Artemisiae Scopariae*	Antibacterial Anti-inflammatory Antioxidant Nutraceutical Food additive	[ChCl]-[EG] [ChCl]-[1,2-BD] [ChCl]-[MCA] $[ZnCl_2]$-[EG] $[ZnCl_2]$-[1,2-BD] $[ZnCl_2]$-[1,6-HD] [TMAC]-[Ph] [TMAC]-[OA] [TMAC]-[U] [MTPPB]-[EG] [MTPPB]-[1,2-BD]	Co-solvent	UAE	Optimum performance was achieved in 30 min, at a solid/liquid ratio of 1/10 g ml^{-1}, under ultrasonic power of 89 W and using 1.0 mL of solvent. The optimal solvent was an aqueous solution of 50% v/v [TMAC]-[U] DES (1:4) mol mol^{-1} with methanol/water (60:40, v/v). The amount of chlorogenic and caffeic acids extracted were respectively 9.35 mg g^{-1} and 0.31mg g^{-1}. The recoveries of the phenolic acids were in the range of 97.3 - 100.4%.	(Park et al., 2014)

EG: Ethylene glycol; **1,2-BD:** 1,2-Butanediol; **1,3-BD:** 1,3-Butanediol; **1,4-BD:** 1-4-Butanediol; **2,3-BD:** 2,3-Butanediol; **1,6-HD:** 1,6-Hexanediol, **MA:** Malonic acid; **MCA:** Methacrylic acid; **TMAC:** Tetramethyl ammonium chloride; **MTPPB:** Methyl triphenyl phosphonium bromide; **Ph:** Phenol; **OA:** Oxalic acid; **U:** Urea; **Gly:** Glycerol; **Ac:** Acetate; **Prop:** Propionic acid; **Lac:** Lactic acid; **PhAc:** Phenylacetate; **Xyl:** Xylitol; **Glu:** Glucose; **Pro:** Proline; **CA:** Citric acid: **Ad:** Adonitol; **Bet:** Betaine hydrochloride; **MAL:** Malic acid; **Suc:** Sucrose; **1,2-PD:** 1,2-Propanediol; **Mlt:** Maltose

Table 2.13: Summary of the effects of several factors on DESs-based extraction procedures.

Factor	Comment	Ref.
Solid/Liquid ratio	Due to the advances of modern techniques, solvents are needed only in small amounts for the extraction of target compounds. Nevertheless, minute amounts are not recommended, as they complicate extraction procedures and only yield very small quantities of extract.	(Park et al., 2014)
Molar ratio	The ratio of the DESs influences viscosity and as such must be carefully assessed.	(Nam et al., 2014)
Viscosity	The extensive network of hydrogen bond allows for strong interactions between HBA and HBD. The resulting high viscosities impede the mobility of these mixtures leading to a slow rate of mass transfer.	(Zhang et al., 2014)
Temperature	The high temperatures used during extraction procedures decrease the viscosities of DESs, thereby enhancing extraction efficiencies.	(Bi et al., 2013)
Nature of the HBD	DESs cohesion reposes on a strong hydrogen bond network, which in turn is dependent on the HBD. Usually the target compound is also able to form hydrogen bonds and will thereby interact competitively with the HBA. The resulting interactions directly influence the extraction process.	(Zhang et al., 2014)
Polarity	As the expression states: “like dissolve like”. Therefore, an adequate selection of DESs (based on the polarity of the extracts) must take place prior the start of the procedure.	
Water content	The increase in water content of DESs leads to enhanced solubility of NBCs and a decrease in viscosity of the solvent. The same effect is encountered when DESs are used as co-solvents or additives to an organic solvent. Care must be taken however as addition of high amounts of water to DESs results in the breakdown of their hydrogen bond network and thereby, leads to the inefficacy of the DESs.	(Dai et al., 2013; Park et al., 2014)
The selection of technique	The technique also greatly impacts the physical properties of the solvent and inherently affects extraction procedures. For instance, using mechanical agitation instead of ultrasound waves for the extraction of phenolic metabolites from safflower resulted in higher efficiency, as the agitation contributed to a higher transport rate, thereby increasing the mobilities of the DESs species.	(Dai et al., 2014)

The use of NADESs in extractions procedures supported these assertions. Radošević et al. (2016) extracted phenolic compounds from grape skins using [ChCl]-[Glu], [ChCl]-Fru], [ChCl]-[Xyle], [ChCl]-[Gly] and [ChCl]-[MA] NADESs and yielded amounts comparable to the fractions obtained using an aqueous solution of methanol (methanol/water 70:30, v/v). The determination and quantification of metal ions in environmental sources have been performed using DESs. This is important because it facilitates the detection of compounds in environmental and food sources that can have negative effects on people's health. For instance, Helalat-Nezhad et al. (2015) extracted polycyclic aromatic hydrocarbons (PAHs) from marine fish spiked with acetonitrile-containing PAHs and macroalgae samples. PAHs are considered to be mutagenic and carcinogenic materials, so their concentrations in food sources are carefully monitored. The authors used high-performance liquid chromatography fluorescence detection (HPLC-FL) to measure the extraction recoveries of PAHs using [ChCl]-[OA] DES as a solvent. Minute amounts of cyclohexane were used to recover the PAHs extracts from the DES. The evaluation of the extraction yields indicated that the efficiency of the HPLC-FL was greater when [ChCl]-[OA] DES was used than when the Soxhlet method was used with organic solvents (methanol, hexane and dichloromethane). Ghanemi et al. (2014) used DESs in an MAE to determine the levels of Cu, Ni, Zn and Fe in fish and macroalgae and the results were similar to the results obtained using conventional methods. This has inherent significance for the use of DESs in biological waste treatment and pollution control in both marine and terrestrial environments.

2.5.2 Biotransformations in deep eutectic solvents

2.5.2.1 Biocatalysis based-DESs

Biotransformations represent an alternative route for the synthesis of NBCs. Typically, they are divided into two groups depending on the synthesis method that is used. Enzyme-based biocatalysis use harvested enzymes for the synthesis of metabolites, whereas microorganism-based biotransformations use the enzymatic structures of microorganisms to produce specific metabolites. For optimal performance, biocatalysis requires adequate solvents and/or co-solvents. The possibility of using non-aqueous solvents for biocatalysis emerged after several concerns were voiced against biocatalysis in aqueous media. That is, the use of water as a solvent was characterized by the limited solubilities of various substrates, unwanted side reactions, the instability of enzymes, the risk of microbial contamination and the post-treatment of products. In contrast, most DES-based biocatalytic reactions are characterized by a significant increase in stability of both the reactants and the products, coupled with the enhanced solubility of enzymes and chemical inertness (Štěpánková, 2014; Juneidi et al., 2017). In addition, DESs increased enzymatic efficiencies by enhancing the enantioselectivity and regioselectivity of the enzymes. Table 2.14 presents selected biocatalytic reactions that use DESs as solvents. In general, the results obtained indicate the superiority of DESs over traditional solvents, in terms of the yields of the reaction, the purity of the products, the availability of reactants, the reaction time, the enantioselectivity and regioselectivity of the catalyst, the recycling of the catalyst and solvent and the stability and activity of the biocatalyst. These superior traits are attributed to the intrinsic characteristics of DESs, such as their wide and strong hydrogen bond network.

For instance, the stability of an enzyme in DESs originates from the interactions between the DES components (HBD, anion and cation of the salt) and the enzyme's surface moieties, which usually involve hydrogen bonding. As a result, of this rigid supramolecular complex, the enzyme's surface is docked or immobilized to the DESs structure, hence promoting protein stability (Monhemi et al., 2014). Due to a strong hydrogen bond donor system, DESs components (especially the HBD) can compete with the catalyst for the substrate (depending on its polarity), thereby reducing its reactivity. Hence, it is often necessary to add a co-solvent in order to make the substrate more available for reaction. Durand et al. (2013) argued that when water is added as a co-solvent, hydrogen bond interactions between water molecules and the DESs increase thus, freeing the substrate and rendering the DESs' structure more rigid. While most enzymes require aqueous environments to be catalytically active, excess water often leads to competitive hydrolysis and unwanted side reactions that occur between enzyme groups and polar molecules. These side reactions can result in the degradation of the substrate and product or/and the denaturation of the catalyst (Durand et al., 2013). It has been hypothesized that DESs shield enzymes from unwanted interactions, thereby conserving the original conformation of the catalyst, which is essential for its activity and stability. In fact, some DESs can modify specific structural features of enzymes, thereby enhancing their efficiencies. Wu et al. (2014) noted slight structural variations of the horseradish peroxidase enzyme (HRP) when it was used in different DESs, i.e., 12 ChCl-based DESs and 12 ChAc-based DESs. In the DESs, the enzyme exhibited increased α-helix content, lower β-sheet content and lower random coil content. These variations are encountered predominantly in the highly-active form of HRP.

Table 2.14: Selected enzymatic performances in the presence of DESs.

Substrate	Product	Product use	DES	Enzyme	Final Solvent composition	Immobilization of enzyme	Enzyme performance in DES	Ref.
Vinyl laureate and aliphatic alcohols (Butanol, Octanol, Octadecanol)	Ethylene glycol esters, Vinyl alcohols	Cosmetics, pharmaceuticals, fragrances, cleaning products	[ChCl]-[U] (1:2)	iCALB	DES	Yes	The activity range of iCALB in DES (6.7 - 8.7 µmol min^{-1} g^{-1}) was lower than its activity range in toluene (5.5 – 12.8 µmol min^{-1} g^{-1}).	(Durand et al., 2012)
			[ChCl]-[Gly] (1:2)	iCALB	DES	Yes	The activity range of iCALB in DES (7.1 - 7.5 µmol min^{-1} g^{-1}) was lower than the activity range in toluene (5.5 – 12.8 µmol min^{-1} g^{-1}).	
Aromatic aldehydes, active methylene group and urea/thiourea	DHPM moieties	Pharmaceutical agents	[ChCl]-[U] (1:2)	*Rhizopus oryzae* lipase	DES	No	The use of DES reduced the reaction time (4h) and increased the yield of the product to 95%.	(Borse et al., 2012)
Aromatic aldehyde and nitromethane,	Nitroaldols, β-Hydroxy nitriles, β-Hydroxy carboxylic acids	Pharmaceutical industry	[ChCl]-[U] (1:2)	[ChCl]-[U] (1:2)	10%DES in Hexane; 10%DES in Toluene; 10 – 40% DES in methanol	No	Best performance and the highest yields were obtained using 20% DES in methanol in very short reaction times. The yields of β-hydroxy derivatives using DES as catalyst improved markedly compared to reported methods.	(Singh, 2012)
Aldehydes	Gem-bisamides	Pharmaceutical industry	[ChCl]-[U] (1:2)	[ChCl]-[U] (1:2)	[ChCl]-[U] (1:2)	No	The bisamides derivatives were obtained in an adequate time frame (15–120 min) with moderate to good yields (52–90%).	(Azizi and Alipour, 2015)

Gly: Glycerol: **EG:** Ethylene glycol; **U:** Urea

Circular dichroism studies have indicated that the secondary structure contents for native/active HRP were 38% α-helices, 14% β-sheets and 48% random coils, whereas those of denatured HRP were 26, 18 and 56%, respectively. These variations were independent of the HBDs as indicated by the fact that an increase in the concentration of the HBDs did not improve HRP activity. However, a similar increase of the salt ratio (especially ChCl) promoted higher HRP activity (Wu et al., 2014). Although these findings are encouraging, a few concerns must be addressed before we can capitalize on the potential of these solvents/catalysts (Table 2.15). For instance, the hydrogen bond network formation in DESs depends on the structure and properties of the HBDs. Individual components of DESs such as urea or ChCl are known to denature enzymes, but hydrogen bond formation between HBAs and HBDs and between DESs and the residues on the catalyst's surface, promoted and enhanced the stability and activity of the latter. This is based on the fact that HBDs with the highest propensity for the formation of hydrogen bonds (based on the number of hydroxy groups in their chains) will stabilize and improve enzyme activity better than HBDs with lower propensity for the formation of hydrogen bonds. However, the propensity of an HBD to form excess hydrogen bonds may lead to the formation of unwanted side products, especially in the presence of substrate of alternate polarities (Maugeri & Domínguez de María, 2014; Monhemi et al., 2014). For instance, Durand et al. (2012) showed that [ChCl]-[MA] and [ChCl]-[OA] DESs competed for the substrates, resulting in the formation of byproducts and the subsequent degradation of the DESs. Therefore, prior analysis is required to identify suitable HBDs for biocatalysis. Although enzymes rarely perform well in excess amounts water, the addition of small amounts of water to DESs increases both their activity and stability.

The addition of water decreases the high viscosities associated with DESs and allows better performance of the solvent. However, it is important for the water content of DESs to be kept at minimum levels. Dai et al. (2015) advised that the ideal water concentrations in DESs should not be greater than 50% (v/v). The reason being that under high water concentrations, some HBDs form extra hydrogen bonds with H_2O molecules, which often lead to the disintegration of the DESs. Nevertheless, the limited solubility of enzymes in a solvent that consists of water and DESs can be improved by adding solid-binding carriers or by grinding the enzyme, thereby facilitating its dispersion in the DESs (Durand et al., 2012). Zhao et al. (2011b) reported that, while the selectivity of subtilisin decreases dramatically when the water/DES solvent has high water content, the immobilization of the protein via covalent cross-linking with a solid carrier increased both its stability and activity.

2.5.2.2 Whole cell biotransformations in DESs

Even though the advances in biocatalysis are remarkable, they have not kept pace with the demand for enzymatic products. This is mostly due to the extensive and laborious processes required to synthesize and isolate enzymes (Dako et al., 2012). However, one recourse has been the use of genetically-engineered microorganisms (e.g., bacteria and fungi) to stimulate the mass production of enzymatic products for biological use. Engineered cells provide a natural environment for enzymes, thereby reducing the risk of denaturation or conformational change. In cells, cofactors are continuously regenerated and there is no requirement for unconventional media such as ILs or DESs. Therefore whole cell biocatalysis are preferred to isolated enzyme systems. Whole cell biocatalysis are typically performed in aqueous solvents, but the benefits that ILs and DESs offered to enzyme-based biocatalysis prompted their use in whole cell biocatalytic processes.

Table 2.15: Summary of the effects of several factors on DESs-based biocatalysis.

Factor	Comment	Ref.
Viscosity	The high viscosity of DESs is an important concern. It is known that the viscosity affects mass transfer kinetics and lowers mobilities of DESs species. The dissolved particles diffuse much more slowly and collide less frequently thereby decreasing reaction rates	(Abbott et al., 2007)
Hydrogen bond formation	HBDs with the highest propensity for the formation of hydrogen bonds (number of hydroxy groups in their chain) will better stabilize and improve enzyme activity than those of lower propensity. However, the propensity of a HBD to form excess hydrogen bonds may lead to the formation of unwanted side products, especially in the presence of various substrates during biocatalysis.	(Durand et al., 2012; Maugeri & Domínguez de María, 2014; Monhemi et al., 2014)
Water content	Although enzymes rarely perform well in water, the addition of small amounts of water to DESs increases both their activity and stability. Water addition decreases the high viscosities associated with DESs and allows for a better solvent performance.	(Durand et al., 2013, 2012; Zhao et al., 2011b)
DES concentration	At high concentrations, DESs decrease the activity of certain enzymes, while increasing their stability. This has an obvious impact on the substrate bioconversion. The concentration of DESs is linked to their physical properties. Therefore, by increasing the concentration of the DESs, one runs the risk of increasing negative aspects such as viscosity.	(Wu et al., 2014)
Molar ratio	The molar ratio may positively or negatively influence aspects such as viscosity and enzyme activity through the formation of auxiliary HBDs, which may at one point, influence DES's structural stability.	(Wu et al., 2014; Zhang et al., 2014)
pH	The pH affects enzyme activity and may lead to superior performance. It ultimately depends on the composition of the DES and the solvent (if DES is used as an adjuvant).	(Durand et al., 2013)
Temperature	The temperature not only influences viscosity as discussed previously, but also the conversion and yield ratio of the biocatalysis products.	(Stepankova et al., 2014)
Byproduct formation	Some hydrophilic solvents enhance byproducts formation For instance, [ChCl]-[Gly] DES induced the formation of monoglycerides byproduct during the esterification of oleic acid and decanol into decyl oleate using Lipase B from *Candida antartica*.	(Kleiner and Schörken, 2014)

In spite of the qualities (versatility, enzyme stability, non-reactive with water) which made ILs and DESs highly successful in enzyme-based biocatalysis, the situation was reversed in whole cell biotransformations. The reason for this being that the incorporation of microorganisms in pure ILs is a challenging process. Whole microorganism biotransformations in ILs typically result in biphasic or monophasic systems in which a water-miscible or immiscible IL is combined with an aqueous solution of the microorganism (Xu et al., 2016). Likewise, whole microorganism biotransformations in DESs are hampered because they require the inclusion of aqueous solutions. Hydration destroys the supramolecular structure of DESs and eventually leads to their disintegration (Dominguez de Maria & Maugeri, 2011). Gutierrez et al. (2010) devised a unique approach to circumvent this issue. Their setup relied on the incorporation of freeze-dried bacteria (*E. coli*) solutions in pure DESs. The results showed that the cells' integrity and viability were preserved in pure [ChCl]-[Gly] DES. This was verified through the expression of a reporter gene. The genetically-engineered cell included a reporter gene (GFP protein) downstream of an inducible system, which was continuously expressed in the cell based on a specific stimulus. This continuous, intracellular expression indicated that the DES caused limited damage to the cellular membranes over a relatively long period of time. Another solution was proposed in which DESs were used as co-solvents in biphasic systems consisting of buffer solutions or water. Xu et al. (2014) demonstrated the improved performance of immobilized *Acetobacter* sp. CCTCC M209061 cells during the asymmetric oxidation of methoxyphenyl ethanol (MOPE) to enantiopure (S)-MOPE. This was conducted in a solvent that was prepared using a [ChCl]-[Gly] DES together with a triethylamine hydrochloride buffer.

Slight modifications of the pH, the concentration of the DES, temperature and the concentration of the substrate resulted in improved reaction time and enhanced conversion of MOPE. However, a bold increase in these factors resulted in a sharp decrease in the conversion ratio and the reaction rate. Overall, the use of DES greatly enhanced both the resolution efficiency of racemic MOPE and the stability of the biocatalyst, which most likely was due to the excellent solubility of MOPE in the [ChCl]-[Gly] DES and its benign biocompatibility with *Acetobacter* sp. cells.

2.5.3 Preservation of biological materials in DESs

Nucleic acids encode the genetic message required for the metabolic processes that occur in all living organisms. DNA exists as duplexes of different conformations, i.e., A-DNA, B-DNA and Z-DNA, which form via Watson-Crick base pairing (Tateishi-Karimata & Sugimoto, 2014). The discovery of DNA catalytic activity enhanced its use as catalyst for the synthesis of pharmaceutical and agrochemical intermediates. Most reactions that use DNA-based hybrid catalysts are performed in aqueous media. However, researchers soon noticed that the use of water resulted in longer reaction times and lower catalytic efficiency as a result of the low solubility of organic substrates in water (Zhao, 2015). Thus, the stage was set for conducting experiments to determine the value of non-aqueous media (notably for the DNA duplexes mentioned above). This research began with the assessment of the stability and preservation of DNA molecules in neoteric ILs and DESs. Mondal et al. (2013) showed that [ChCl]-[EG] and [ChCl]-[Gly] DESs dissolved salmon testes' DNA at the concentrations of 5.5 and 2.5% w/w, respectively.

The DESs adequately preserved the integrity of the nucleic acid at various temperatures (4, 25, 60 and 75 ºC) and pH ranges (4.2, 7.2 and 8.0), whereas DNA stored in Tris-buffer did not withstand long dissolution periods and deteriorated rapidly. Using infrared spectroscopy data, the authors concluded that the interactions between cholinium cations and the DNA's phosphate backbone were responsible for the efficient dissolution of DNA in DESs. Tateishi-Karimata and Sugimoto (2014) added more precision by using molecular dynamics simulations to understand the complex interactions between cholinium ions and the structures of DNA molecules. The data indicated that the cholinium cations interacted with the phosphate groups as well as with bases and ribose sugars, preferentially in the minor groove (by means of hydrogen bonding between the hydroxyl groups of cholinium and the DNA). In general, cholinium cations were located preferentially in the minor groove, where they stabilized DNA by forming hydrogen bonds. Recently, other structural forms of DNA, such as triplexes and quadruplexes, have attracted the interest of researchers due to their biological significance. For instance, G-quadruplexes have been proposed as promising therapeutic targets in oncology and as inhibitors of HIV-1 integrase due to their repeated occurrence in biologically-significant regions (human telomeres, oncogene promoter regions) (Figure 2.19) (Palumbo et al., 2009; Pedersen et al., 2011). For this reason, they have been hailed as, for example, promising catalysts, biosensors and DNA-nanomaterials. However, G-quadruplexes (just like their duplex relatives) require a strictly anhydrous environment for adequate performance and preservation. Therefore, any type of operation involving these structures requires an appropriate, non-aqueous medium. The stability and preservation of G-quadruplexes rely on the formation of different intra-molecular conformations (hybrid, parallel, antiparallel).

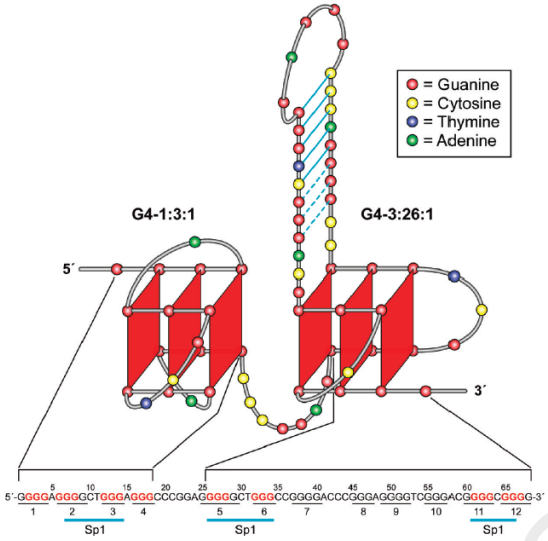


Figure 2.19: The proposed model of the tandem G-quadruplex structure formed in the hTERT core promoter. Reprinted with permission from (Palumbo et al., 2009).

They assume these conformations in dilute solutions of Na^+ and K^+ (Zhao et al., 2013). However, the parallel orientation is the most favored because it is associated with increasing biological significance, given the narrow cellular environment. Zhao & Qu (2013) examined the stability of G-quadruplexes using DESs as solvents or co-solvents. As shown in Figure 2.20, the occurrence of G-quadruplexes (in various genomic regions of different organisms) in parallel orientation is greater in DESs than in traditional solvents (water and bases). These observations prompted the authors to conclude that DESs enhanced the stability of G-quadruplexes. In addition, the thermal stability of these nucleic acid structures was significantly higher in DESs (judging by the melting temperatures of the quadruplexes in DES solvent) than in traditional solvents. Table 2.16 shows the results of the dissolution of G-quadruplexes in DESs. The stability of quadruplexes in DESs is thought to originate from the distinctive properties of the solvents, especially their thermal and chemical stabilities.

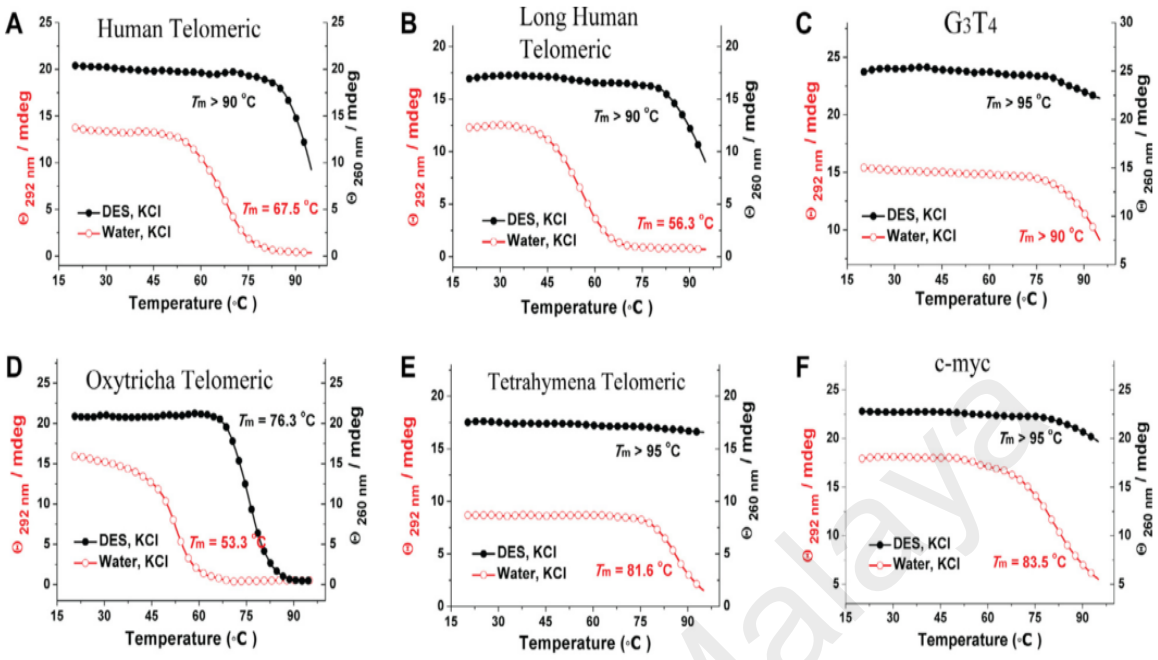


Figure 2.20: Melting curves of various quadruplexes DNA in DESs containing 100 mM KCl (black) and in water containing 100 mM KCl (10 mM Tris, pH = 7.2) (red) (Zhao & Qu, 2013).

Table 2.16: Orientation of quadruplexes structures in water and water-free solutions. (Zhao & Qu, 2013).

DNA region	Structure				
	Na^+/Water	K^+/Water	DES	Na^+/DES	K^+/DES
Human telomeric (Tel_{22})	Antiparallel	Hybrid	–	–	Parallel
Long Human telomeric	Antiparallel	Hybrid	–	–	Parallel
Oxytricha telomeric	Antiparallel	Antiparallel	–	–	Parallel
G_3T_4	Antiparallel	Antiparallel	–	–	Parallel
Tetrahymena telomeric	Hybrid	Hybrid	Parallel	Parallel	Parallel
c-myc	Parallel	Parallel	Parallel	Parallel	Parallel
c-kit	Parallel	Parallel	–	–	Parallel
KRAS	Parallel	Parallel	–	–	Parallel
TBA	Antiparallel	Antiparallel	–	–	Antiparallel
A_4G_6	Parallel	Parallel	Parallel	Parallel	Parallel

2.5.4 Synthesis of nanomaterials

In the past two decades, there has been considerable progress in the fields of nanoscience and nano-medicine. This progress is evident in the extensive information that has been generated concerning the shape, composition, design and properties of nanomaterials. The properties of nanomaterials are the subject of increasing research efforts, especially in biological research areas, where the targets are usually microscopic (Jain et al., 2007). Table 2.17 lists the different applications of nanomaterials in several biological fields. Previous studies have shown the potential applications of ILs during the synthesis and assembly of nanomaterials (Duan et al., 2014; Kang et al., 2016; Li et al., 2008). These applications have been extrapolated to DESs with considerable success.

Table 2.17: Various biological applications of nanomaterials.

Field	Application	Nanoparticle	Ref.
Therapeutic	Photothermal	Gold nanoshells	(Huang and El-Sayed, 2010)
	Chemotherapy	Polymer nanoparticles, anticancer drugs	(Cho et al., 2008)
	Gene therapy	Dendrimers, inorganic nanoparticles, cationic lipids, carbon-based nanoparticles	(Pérez-Martínez et al., 2012)
	Magnetic hyperthermia	Iron oxide based nanoparticles	(Thomas et al., 2009)
Imaging	Fluorescence	Carbon nanotubes, dye-doped silica	(Wolfbeis, 2015)
	MRI	Iron oxide	(Bardhan et al., 2011)
	Ultrasound	Polymeric nanoparticles	(Bardhan et al., 2011)
Food industry	Food packaging	Silica nanoparticles	(Duncan, 2011)
	Encapsulation of flavor	Polymer nanoparticles	(Duncan, 2011)
	Food textural	Polymer nanoparticles	(Duncan, 2011)
Agriculture	Fertilizer	Synthetic apatite nanoparticles	(Liu and Lal, 2014)
	Pathogen detection	Supraparamagnetic nanoparticles	(Sanvicens et al., 2009)
	Genetic engineering	Calcium phosphate nanoparticles	(Ardekani et al., 2014)

For instance, Raghuwanshi et al. (2014) highlighted that the formation and self-assembly of gold nanoparticles in DESs (inside and on the surface) occur via sputter deposition (Figure 2.21). Self-assembly refers to the process in which discrete components of nanomaterials assume an ordered and hierarchical arrangement. In a [ChCl]-[U] DES, deposition onto the surface of the solvent led to the formation of constant shapes and sizes of gold nanoparticles that were dispersed homogeneously in the solvent matrix. The authors hypothesized that the presence of the hydrogen bond network coupled with other mechanisms (single atom attachment, Ostwald ripening, coalescence) enhanced the coordination of gold particles into self-assembled nano-structures.

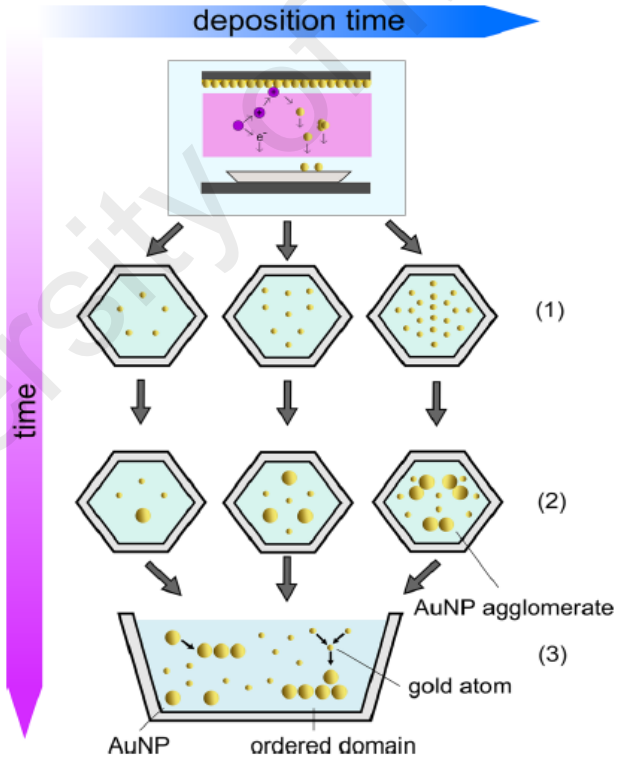


Figure 2.21: Aggregation model of the gold nanoparticles in DESs at different sputtering times; 1) initial sputtering, 2) final sputtering, 3) 5 hr after sputtering (Raghuwanshi et al., 2014).

These processes ultimately depend on surface tension, viscosity and the diffusion coefficient of the DESs. Also, water in the DES was important because of its ability to disrupt the strong hydrogen network of DESs, but the subsequent addition of a choline chloride-based stabilizer reversed its negative effect. At low volumes, water does not disrupt the short- or long-range ordering of nanoparticles, but, at high volumes, it induces a disordered agglomeration and partial sedimentation of the nanoparticles. Other studies have focused on the use of DNA as a component of nanoparticles. Mondal et al. (2014) used [ChCl]-[EG] DES to facilitate the assembly of a DNA-based material composed of Fe_3O_4 nanoparticles, protonated layered dititanate sheets (H_2•TiO_2•.H_2O) and salmon testes' DNA. The scanning electron microscope (SEM) images in Figure 2.22a show the morphology of the H_2•TiO_2•H_2O sheets and they also show that the tin sheets were attached preferentially on the periphery of the DNA, specifically on the phosphate groups.

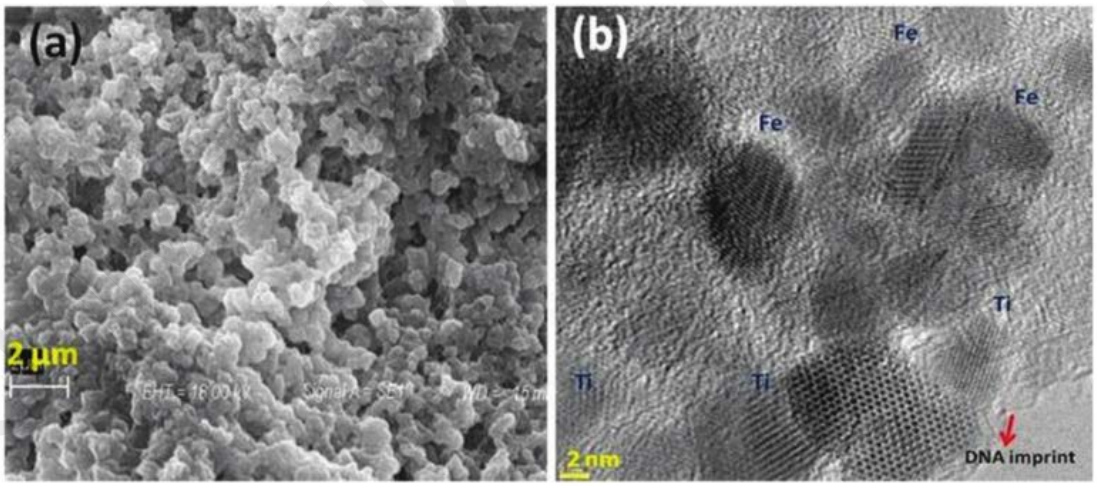


Figure 2.22: a) SEM and b) HR-TEM imagery of the hybrid DNA nanomaterials (Mondal et al., 2014).

The transmission electron microscope (TEM) images in Figure 2.22b indicated the presence of Ti, Fe, phosphorus, carbon and oxygen in the composite and they also confirmed that Fe particles attached preferentially to the DNA base pair in the center of the DNA molecule. Figure 2.23 shows the atomic force images of the dual-functionalized DNA hybrid (Mondal et al., 2014).

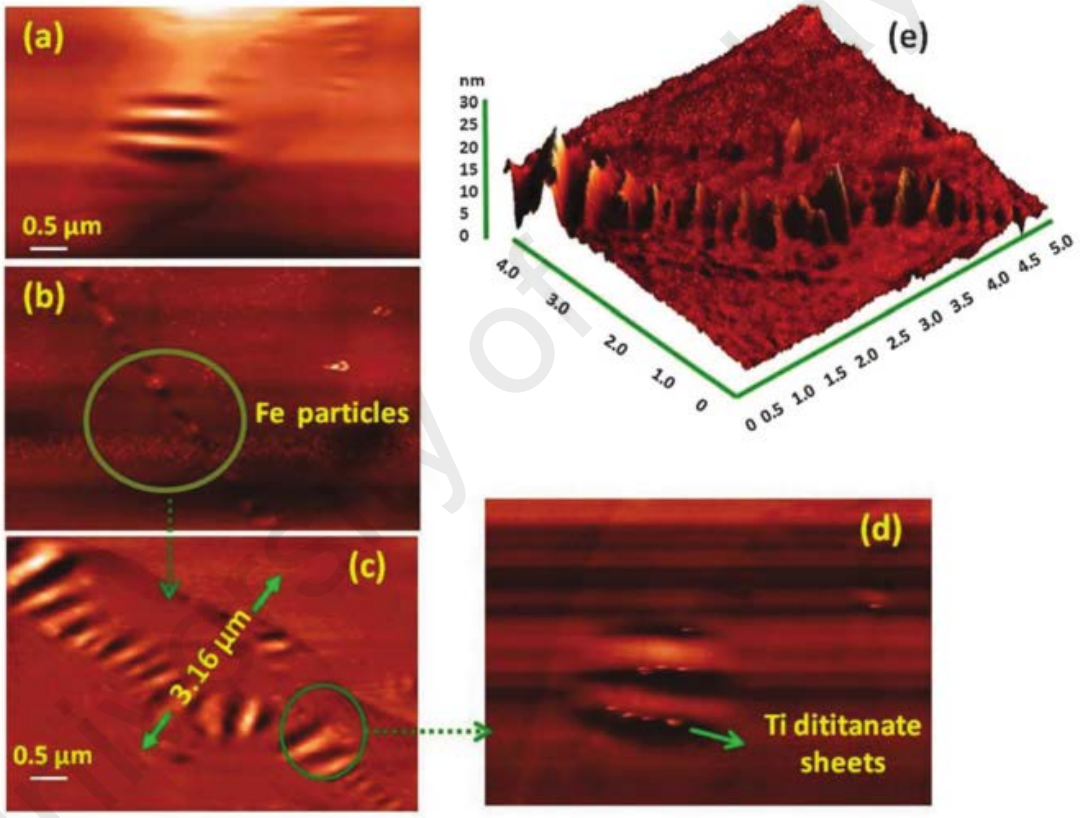


Figure 2.23: 2D and 3D atomic-force images of the DNA hybrid material: a) The DNA hybrid with a double stranded structure; b) Fe particles; c and d) Ti sheets in the periphery of the DNA material; e) Fe_3O_4 particles orderly arranged in the hybrid DNA (Mondal et al., 2014).

2.5.5 Pharmaceutical and biomedical applications

The pharmaceutical applications of DESs can substantially intermingle with their biotechnological applications. Therefore, this section will be focused entirely on the design, delivery and analysis of pharmaceutical/biomedical materials and some potential contributions of DESs in this area. The pharmaceutical materials in question are polymer-based materials and they are used either as biodegradable elastomers in biomedicine or as drug-solubilizing vehicles in pharmaceuticals. Bacterial infections are a major concern in biomedicine. These infections stem from the general procedures used in biomedicine, such as the implantation of medical devices and they generally trigger the formation of rapidly-evolving, organized bacteria communities (biofilms) that usually are resistant to common antibiotics. For these reasons, the development of an anti-fouling coating and bactericidal materials (biodegradable elastomers) is a priority (Carriazo et al., 2012). The tremendous versatility of biodegradable elastomers as polymeric networks for regenerative medicine stems from their success as effective agents for tissue-engineering scaffolds, gene-delivery, bio-imaging systems and shape-memory polymers for temperature-controlled drug delivery (Serrano et al., 2011; Yang et al., 2004). García-Argüelles et al. (2013) used DESs for the synthesis of biodegradable polyester with antibacterial activity. In order to synthesize a biodegradable poly octanediol-co-citrate elastomer (POC), the authors used 1,8-octanediol (polyester precursor) and halide salts (choline chloride, tetraethylammonium bromide, hexadecyltrimethylammonium bromide and methyltriphenylphosphonium bromide) to form a eutectic mixture in which citric acid was dissolved to act as a second polyester precursor. Condensation and cross-linking were possible because of the formation of ester bonds

between 1,8-octanediol and citric acid. Figure 2.24 provides a template of the reaction. Figure 2.25 shows that the resulting polymers exhibited remarkable cytocompatibility and antibacterial activity. The toxicity of the polyester to bacterial species was expected because quaternary ammonium salts are known to target bacteria's cytoplasmic membranes and cell walls by causing the loss of structural organization and integrity (McDonnell & Russell, 1999).

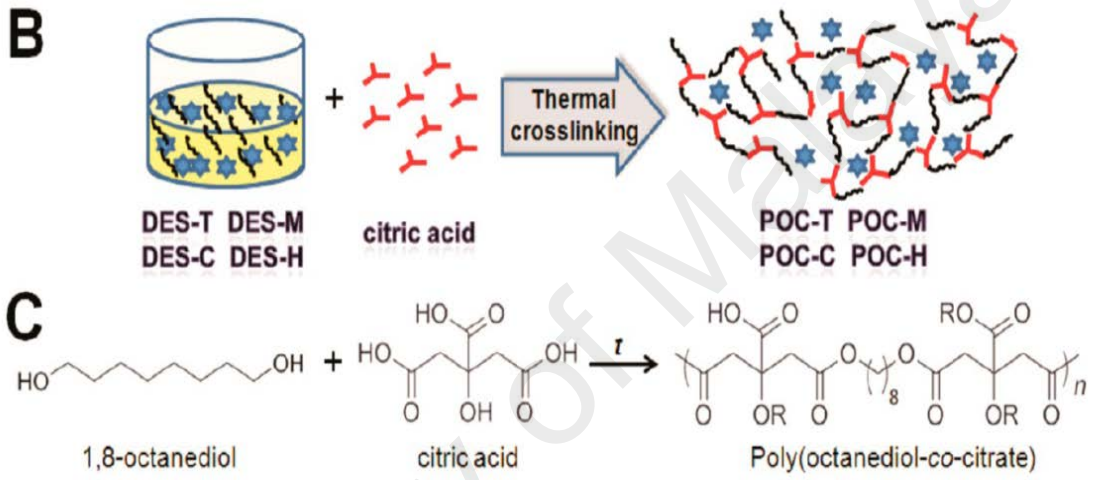


Figure 2.24: Preparation of poly (octanediol- co-citrate) polymer B) 1,8-octanediol molecules represented as black curled lines, antibacterial compounds as blue stars and citric acid as red trident figures; DES-T: [tetraethylammonium bromide]-[1,8-octanediol]; DES-M: [methyltriphenylphosphonium bromide]-[1,8-octanediol]; DES-C: [Choline chloride]-[1,8-octanediol]; DES-H: [hexadecyltrimethylammonium bromide]-[1,8-octanediol]; C) Chemical reaction of the synthesis of standard POC using the respective DESs; t: the curing temperature (García-Argüelles et al., 2013).

Elastomers with antibacterial activity are not the only benefit of DESs in this discipline, i.e., Park et al. (2013) demonstrated the formation of cotton fabrics with antibacterial properties. These fabrics were prepared via treatment with 3,3,4,4-benzophenone tetracarboxylic dianhydride (BPTCD) in a [ChCl]-based DES medium As a result, the fabrics exhibited a high level of antibacterial activity, before and after UV irradiation (Table 2.18).

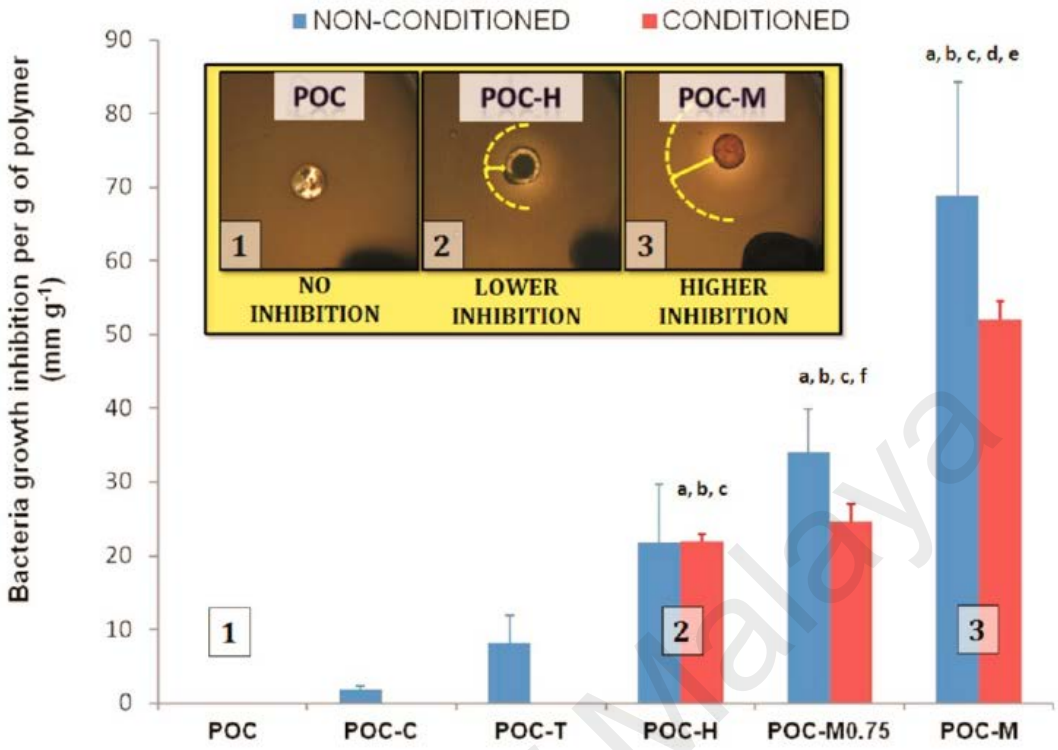


Figure 2.25: Inhibition of *E. coli* by different poly octanediol-co-citrate elastomer (POC)-based DESs. Inhibition of bacterial growth measured as the diameter of the zone of inhibition around polymer discs normalized by the grams of polymer. The graphs shows significant differences with respect to (a) POC, (b) POC-C, (c) POC-T, (d) POC-H, (e) POC-M075 and (f) POC-M; $p < 0.05$. The polymers discs were conditioned in culture medium to ensure the removal of acidic residues from the synthesis (García-Argüelles et al., 2013).

Hence, the addition of the DES improved the antibacterial activity of these cotton fabrics considerably (Park et al., 2013). Other issues plaguing the pharmaceutical industry are the failure to manufacture drugs that have adequate solubility and the matter of delivering them to the targeted organs. The composition of DESs, specifically NADES, is of natural origin; therefore it is expected that they will provide better media in which to dissolve drugs that are otherwise difficult to dissolve in water.

Table 2.18: Antibactericidal activity of cotton fabrics prepared in DESs (Park et al., 2013).

Specimen	Bacteria reduction (%)			
	S. aureus		*E. coli*	
	No UV	UV (2h)	No UV	UV (2h)
Pristine cotton	27.3	60.4	22.7	74.6
[ChCl]-[U]-BPTCD (5%)	99.3	99.9	99.4	99.9
[ChCl]-[U]-BPTCD (10%)	99.7	99.9	99.7	99.9
[ChCl]-[U]-BPTCD (15%)	99.2	99.9	87.4	99.9
[ChCl]-[EG]-BPTCD (15%)	99.6	99.9	99.6	99.9

U: Urea; **EG:** Ethylene glycol; **BPTCD:** 3,3,4,4-benzophenone tetracarboxylic dianhydride

Indeed, progress in that regard has already been achieved with DESs. Morrison et al. (2009) reported that several drugs (benzoic acid, griseofulvin, danazol, itraconazole and AMG517) exhibited a 5- to 22000-fold enhancement of their solubilities in DESs compared to water. Moreover, as shown in Table 2.19, a comparison of the solubilities of drugs in pure DESs versus their solubilities in DESs chief materials, in aqueous solutions of DESs and in water shows that the drugs were more soluble in pure DESs. This implies that the high solubility of the drugs in DESs is not due to the association of the drug with one of the individual components of the solvent; rather, the enhanced solubility is a result of the synergistic effect promoted by the eutectic mixture. The success of DESs in this field paves the way for the use of NADESs. The development of alternative routes for synthesizing drugs is urgently needed to combat the risks of polymorphisms and to enhance the bioavailability of drugs in biological fluids. The application of polymers to active pharmaceutical ingredients (APIs) to improve their dissolution and inhibit their crystallization seems to offer a potential course of action to meet this need (Marrucho et al., 2014).

Table 2.19: Comparison of the solubilities of several drugs in DESs, water and the individual components of the DESs (Morrison et al., 2009).

Solvent	Solubility (mg/ml)				
	Benzoic acid	**Danazol**	**Griseofulvin**	**AMG517**	**Itraconazole**
[ChCl]-[U] DES					
Pure DES	229	0.048	0.034	0.010	< 0.001
75:25 DES: water	23 (4.4)	0.0061 (9.4)	0.016 (9.2)	0.00022 (9.4)	< 0.001 (8.9)
50:50 DES: water	14 (4.1)	0.002 (9.4)	0.015 (8.7)	< 0.0001 (9.2)	< 0.001 (9.0)
Pure water	3 (3.8)	<0.0005 (8.9)	0.007 (8.9)	< 0.0001 (9.5)	< 0.001 (8.5)
[ChCl]-[MA] DES					
Pure DES	35	0.160	1.0	0.4727	22
75:25 DES: water	18 (0.2)	0.0044 (0.3)	0.1007 (0.3)	0.014 (0.4)	6.6 (0.6)
50:50 DES: water	11 (0.6)	0.002 (0.6)	0.043 (0.7)	0.002 (0.7)	1.2 (0.85)
Pure water	3 (3.8)	< 0.0005 (8.9)	0.007 (8.9)	< 0.0001 (9.5)	< 0.001 (8.5)
Individual components					
Aqueous [U]	11 (3.9)	0.01 (8.2)	0.053 (8.0)	< 0.0001 (8.7)	< 0.001 (5.2)
Aqueous [ChCl]	7 (3.1)	< 0.0005 (6.2)	0.005 (7.2)	< 0.0001 (8.0)	< 0.001 (5.8)
Aqueous [MA]	9 (0.9)	< 0.0005 (0.9)	0.084 (0.9)	0.005 (0.9)	12 (0.9)

U: Urea; **MA:** Malonic acid

To date, front polymerization (FP) has been one of the most efficient methods for polycondensation. FPs are more appealing than conventional polymerizations because their self-propagating nature provides rapid and high conversion rates and reduces the energy costs associated with the preparation of polymers. However, when FP is performed in conventional solvents, serious difficulties arise, mostly in terms of temperature control (Carriazo et al., 2012). This entails the possible degradation of the APIs and other precursors.

One solution to this ordeal is based on the use of thermally-stable solvents, such as ILs and DESs. Mota-Morales et al. (2013) described the formation of a drug vehicle for lidocaine hydrochloride (a common anesthetic) using DESs that were prepared using ammonium salts and acrylamides. The use of these DESs enhanced the conversion and release of the polymer at high temperatures (conversion rates above 90% in every case at temperatures between 80 and 120 °C). Sanchez-Leija et al. (2014) reported the synthesis of a polymer-API through free-radical FP using a DES system synthesized from lidocaine hydrochloride and acrylic/methacrylic acid, alongside precursors (1,1-bis(tert-butylperoxy)-3,3,5-trimethylcyclohexane) and cross-linkers (ethylene glycol dimethacrylate or pentaerythritol triacrylate). In solventless conditions and using minimal energy, the properties of the DES system allowed for a one-pot synthesis and full conversion, thereby preventing the degradation of the API. The use of the DES system further enhanced the design of pH-responsive polymer and copolymer complexes, which enabled the controlled release of the entrapped API. Thus, a controlled release of lidocaine hydrochloride was achieved by varying parameters, such as the pH, ionic strength and solubility of the drug in the medium. This control was enforced because of the swelling of the polymers and the already established interactions between the drug and the polymers in the DES precursor. Paiva et al. (2014b) reported the synthesis of a therapeutic DES using ibuprofen (i.e., a non-steroidal, anti-inflammatory agent) and *R*-(-)-mandelic acid, which is known for its antibacterial activity. This DES was used for the synthesis of a porous biopolymer that was used as a precursor for starch and poly-ε-caprolactone (SPCL) during supercritical foaming using CO_2. The porosity of the material was emphasized because this property is crucial for an effective and controlled release of the encapsulated biomaterial.

The results showed that the presence of a DES during such a procedure can enhance porosity, pore size and the interconnectivity of the samples. Ultimately, this process is dependent on the nature of the DESs used for SPCL doping. Also, a polymeric liquid eutectic vehicle for ibuprofen loading was developed using a liquid eutectic (containing 30% w/w eudragit EPO in 1:1 menthol:camphor). The results indicated that the hydrophobic character of this mixture was responsible for the sustainable release of ibuprofen that was observed over a period of more than seven days. This polymeric eutectic mixture represented a new controlled drug delivery system for ibuprofen, which, in the long term, may prove to be suitable for the treatment of periodontitis (Tuntarawongsa & Phaechamund, 2012).

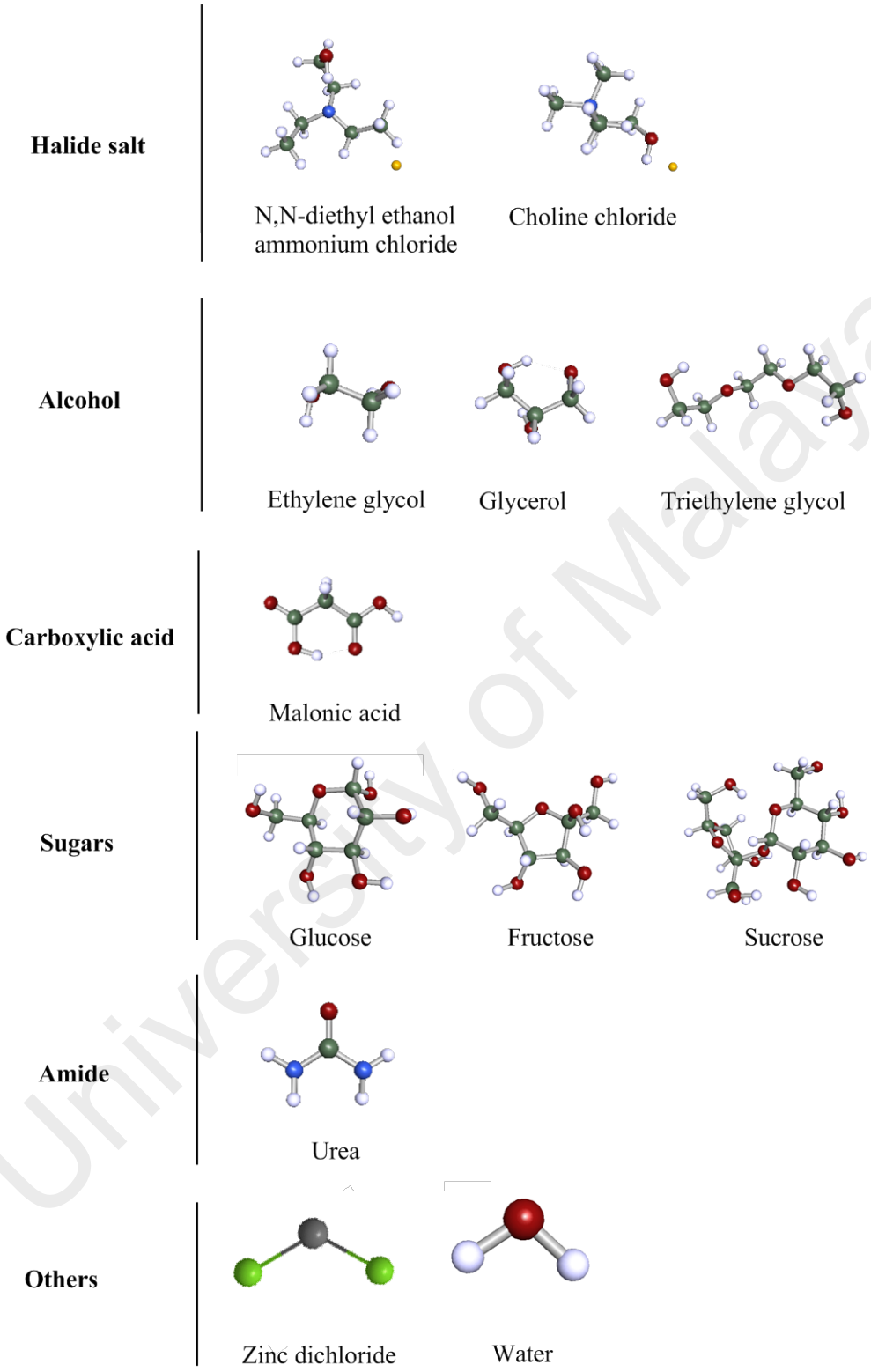
2.5.6 Applications in molecular biology

Potential applications of DESs in molecular cell biology were identified recently in a patent filed by Goldsborough & Bates (2014). This patent highlighted the ability of DESs to stabilize and preserve biomolecules as well as entire cells, tissues and biological samples. They found that RNA/DNA/protein-based compositions were stabilized in DES mixtures irrespective of their phases (solid, liquid, or gel). Another aspect of their findings was related to the ability of DES mixtures to stabilize whole cells' native morphology in several samples, i.e., blood, serum, plasma, urine, cerebral spinal fluid and human and animal tissues. The fixation of cells and the stability of their structures and morphologies entailed the use of DESs in various other molecular analytical methods, i.e., cell counting, immunohistochemistry, histochemistry, staining and coloration, flow cytometry, mass spectrometry, in situ hybridization and laser capture microdissection.

In addition, DESs could act as stabilizers of biomolecules; cells, e.g., cancer cells, white blood cells, viral cells and other microorganisms; subcellular organelles, e.g., mitochondria, exosomes and nuclei, in various samples, e.g., plasma, serum and blood, for subsequent diagnostic analysis. The stabilizing features of DESs only occurred with the solvent, not with its individual components. The use of the individual components of the DESs did not yield any noticeable effect. Once again, this gives credit to the synergistic effect (originating from complexation of the HBA and HBD) as the main reason for the neoteric properties of these solvents (Goldsborough & Bates, 2014). By considering these developments, it seems reasonable to forecast the future importance of DESs in the therapeutic, biotechnological and molecular cell biology areas.

CHAPTER 3: EXPERIMENTAL METHODOLOGY

The materials mentioned below were used throughout the study. The compounds depicted in Scheme 3.1 were used to prepare NADESs and DESs according to Table 3.1. N,N-diethylethanol ammonium chloride (DAC) (≥ 98%), glucose (≥ 99%), fructose (≥ 99%), triethylene glycol (≥ 99%), ethylene glycol (≥ 99%), glycerol (≥ 99%), urea (≥ 99%) and zinc chloride were purchased from Merck. Choline chloride (≥ 97%) and malonic acid (≥ 98%) was obtained from Sigma-Aldrich. The human cervical cancer cell line (HelaS3), the human prostate cancer cell line (PC3), the human gastric cancer cell line (AGS), the human skin malignant melanoma cancer cell line (A375), the human hepatocyte cell line (WRL-68), the human ovarian cancer cell line (CaOV3) and the mouse skin cancer (B16F10) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The human breast cancer cell line (MCF-7) was obtained from Cell Lines Service (300273; Eppelheim, Germany). Both the Dulbecco's Modified Eagle Medium (DMEM) and the Roswell Park Memorial Institute medium (RPMI 1640) were obtained from Life Technologies, Inc., Rockville, MD. Fetal bovine serum (FBS) was supplied by Sigma-Aldrich.



Scheme 3.1: Chemical structures of the individual components of the understudied NADESs and DESs.

Table 3.1: Composition of NADESs/DESs used throughout this study.

NADES	Salt	HBD	Third compound	Molar ratio (Salt/HBD)
NADES1	ChCl	Fru	-	2:1
NADES2	ChCl	Glu	-	2:1
NADES3	ChCl	Ethylene glycol	-	1:2
NADES4	ChCl	Glycerol	-	1:2
NADES5	ChCl	Urea	-	1:2
NADES6	ChCl	Fructose	Water	5:2:5
NADES7	ChCl	Glucose	Water	5:2:5
NADES8	ChCl	Sucrose	Water	4:1:4
NADES9	ChCl	Glycerol	Water	1:2
NADES10	ChCl	Malonic acid	-	1:1
DES1	DAC	TEG	-	1:4
DES2	DAC	Zinc chloride	-	1:2
DES3	DAC	Malonic acid	-	1:1
DES4	DAC	Ethylene glycol	-	1:2
DES5	DAC	Glycerol	-	1:2
DES6	DAC	Urea	-	1:2

3.1 Preparation of NADESs and DESs

Table 3.1 illustrates the composition, molar ratios and symbols of the NADES used throughout this study. The preparation method is similar to those previously described in the literature (Hayyan et al., 2013c). The NADESs were prepared according to the ratio listed in Table 3.1. The aqueous solutions were prepared using the same concentration of each component, in the DES, dissolved in distilled water individually (Gluaq, Fruaq, ChClaq, DACaq, etc.) or combined (i.e. ChCl+Glu+Water= NADES1aq).

3.2 *In vitro* profiling: MTT viability assay

The MTT cell viability assay was performed as previously described (Hayyan et al., 2015). The EC_{50} values were obtained from an average of at least three independent experiments. The standard error of the mean (SEM) derived from the repeated experiments were used to derive the variations from the average EC_{50} values. The statistical analysis was performed using Graph Pad Prism 5 software. Statistical significance was defined when $P \leq 0.05$.

3.3 Membrane Permeability assay

1×10^4 cells per well were seeded onto a 96-well plate for 16 h. The cells were then treated with DESs at EC_{50} concentrations and further incubated for 24 h at 37 °C in 5% CO_2. In order to examine plasma membrane permeability, cell permeability dye (Image-iT DEAD Green viability stain, Thermo Fisher) were added to live cells and incubated for 30 min, as previously described (Looi et al., 2013). It is an impermeant dye to healthy cells that becomes permeant when the plasma membrane of cells is compromised. Cells were washed two times with PBS before fixing with 3.7 % formaldehyde solution for 20 min and read under a fluorescent microplate reader at an excitation/emission wavelength of 488/515. The cells were kept overnight in PBS. The cells were visualized and images were captured the following day using Cellomics ArrayScan High content screening reader system (Thermo Scientific, PA, USA).

3.4 Oxidative stress assay

ROS assay was carried out to determine the influence of solvents on the production of ROS level in treated cells. 1×10^4 cells per well were seeded onto 96-well plate and incubated overnight at 37°C in 5% CO_2. The cells were then treated with EC_{50} concentrations of the solvents for 24 h, following which dihydroethidium (DHE) dye was added into live culture for 30 min. Cells were fixed and washed with PBS as described in previous study (Liew et al., 2014). The DHE dye probe is oxidized to ethidium in the presence of superoxides. The fluorescence intensity was measured using a fluorescent plate reader at an extension wavelength of 520 nm and an emission wavelength of 620 nm. The values are represented as means ± SD of three sets of experiments. The cells were visualized and images were captured the following day using Cellomics ArrayScan HCS reader (Thermo Scientific).

3.5 Western blot analysis

After incubation with NADES/DES (at EC_{50} concentrations) for 12hrs, AGS were washed twice with cold PBS. Cellular protein content was extracted using cell lysis buffer (50mM Tris–HCl pH 8.0, 120mM NaCl, 0.5%NP-40,1mM PMSF) and 25 µl of protein extract was separated by 10% SDS-PAGE. The extracted protein was transferred to a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad), blocked with 5% non-fat milk in TBS-Tween buffer (0.12M Tris–base, 1.5MNaCl, 0.1% Tween20) for 1 h at room temperature and incubated with the appropriate antibody (2:2000) overnight at 4 °C. The membrane was incubated with the anti-β-actin and anti-p53 antibodies. The next day, both membranes were incubated with an alkaline phosphatase conjugated anti-rabbit IgG (1:5000) for 1 h at room temperature.

The bound antibody was detected using a solution of staining buffer with NBT/BCIP (Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate). The antibodies were purchased from Santa Cruz Biotechnology, Inc., California, USA. NBT, BCIP and the lysis buffer were purchased from Sigma Aldrich.

3.6 *In vivo* assessment

The acute toxicity of the compounds was evaluated using six Imprinting Control Region (ICR) mice per groups at 8 to 12 weeks of age, with an average body weight of 25.6 g. The mice were assigned equally into 4 groups labeled as vehicle (dH2O), high dose (20 g/kg), medium dose (10 g/kg) and low dose (5 g/kg) of the compounds. Prior to administration, the mice were fasted (food but not water) overnight and for 3 to 4 h following compounds' administration in order to eliminate any residual food inside the gastrointestinal tract which might complicate absorption of the test substance. The mice were observed at 30 min, 2, 4, 24 and 48 h after administration for the onset of clinical or toxicological symptoms as well as mortality and behavioral changes following treatment. They were euthanized by CO_2 asphyxiation on 15th day and serum biochemical and histological (liver and kidney) parameters were determined using the standard method. The mortality of the mice were recorded within 14 days and used to calculate the LD_{50} for each compound. The sampled mice were housed in specific pathogen free facility at the University of Malaya. This work was designed to minimize animal suffering and the number of animals used. The study has been approved by the Faculty of Medicine Animal Care and Use Committee (FOMIACUC, Approval No: 2014-05-07/PHAR/R/CYL) at University of Malaya.

3.7 Physicochemical properties of NADESs and DESs

Prior to the measurement of the physical properties, the NADES samples were kept in vials tightly closed and stored in a desiccator to avoid humidity uptake. Viscosities were measured using a 127 Brookfield R/S plus Rheometer. The device was calibrated by a zero-calibration method. Viscosity values were taken at temperatures of 37 °C.

3.8 Computational methodology for COSMO-RS

3.8.1 Molecular geometry optimization

The geometry optimization of all species involved in this study was performed using the Turbomole program package. Using this program, the basic structure of the target molecule was drawn first. After which, geometry optimization was performed at the Hartree–Fock level and 6-31G* basis set. The generation of .cosmo file was then conducted through a single-point calculation by using DFT with Becke–Perdew and the Triple-ζ Zeta Valence Potential (TZVP) basis set. Finally, the .cosmo files were exported to the COSMOthermX program with parameterization BP_TZVP_C30_1301.ctd.

3.8.2 NADESs/DES representation in COSMOtherm-X

Since a single DES is composed of more than one molecule, employing its representation method in the COSMOtherm-X program is crucial. In this study, the electroneutral approach was adopted, whereby the DES was represented in COSMO-RS according to the mole composition of their constituents shown in Tables 3.1 and 3.2 (the salt cation, salt anion and hydrogen bond donor (HBD)). Membrane phospholipids were designed according to the same principle; that is using the most basic composition of their constituents (Table 3.2).

Table 3.2: Composition of cellular membrane and ratio (Harwood & Weselake, 2015).

Cell membrane elements	Composition and ratio			
	Fatty acid	**Alcohol**	**Metabolites**	**Functional group**
Phosphatidylcholine	Palmitic acid (2)	Glycerol (1)	Choline (1) Phosphate (1)	Phosphate (1)
Phosphatidylethanolamine	Linoleic acid (1); Palmitic acid (1)	Glycerol (1)	Ethanolamine (1) Phosphate (1)	Phosphate (1)
Phosphatidylserine	Stearic acid (1); Cervonic acid (1)	Glycerol (1)	Serine (1) Phosphate (1)	Phosphate (1)
Sphingomyelin	Oleic acid (1)	-	Choline (1) Phosphate (1)	Phosphate (1)
Glycolipids	Oleic acid (1)	-	Glucose (1) Sphingosine (1)	Phosphate (1)

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Binary natural deep eutectic solvents systems: *In vitro* and *in vivo* profiling

4.1.1 *In vitro* profiling:MTT viability assay

The MTT viability assay results in Table 4.1 depict NADESs as harmful materials, based on the EC_{50} values. The morphological assessment of HelasS3 and AGS, MCF-7 and WRL-68 cell lines following NADES1 and NADES2 treatment also demonstrated the lethal effects of these mixtures. Figure 4.1 shows that under NADES1, NADES2 and DES1 treatment, AGS and HelaS3 cell lines shrink considerably, with their shapes and morphologies no longer consistent with viable growth. Cell confluence was significantly affected and the culture medium was filled with dead cells and those approaching the necrotic state. The same effect was observed for MCF-7 and WRL-68 cell lines (Appendix A – Figure A1). The EC_{50} values of NADES1 and NADES2 ranged from 98 to 516 mM. They were obtained from dose-response curves shown in Appendix A (Figure A2). Those values were transformed from raw fluorescent data shown in Appendix B (Figure B1). The only other toxicological assessment of NADES ([ChCl]-[Glu]) on cancer cells had EC_{50} values $\geq$ 10 mM in MCF-7 cells (Radošević et al., 2015). Following the inclusion of DES1, the range of effective concentrations widened. Despite a lower endpoint, the final range of EC_{50} values ($34 \leq EC_{50} \leq 516$ mM) remained significantly higher than those reported. Across all cells, DES1 was the most toxic mixture (Table 4.1). The increase in solvent toxicity followed the same sequence between all cell lines: NADES2 < NADES1 < DES1. Cell line susceptibility varied between eutectics.

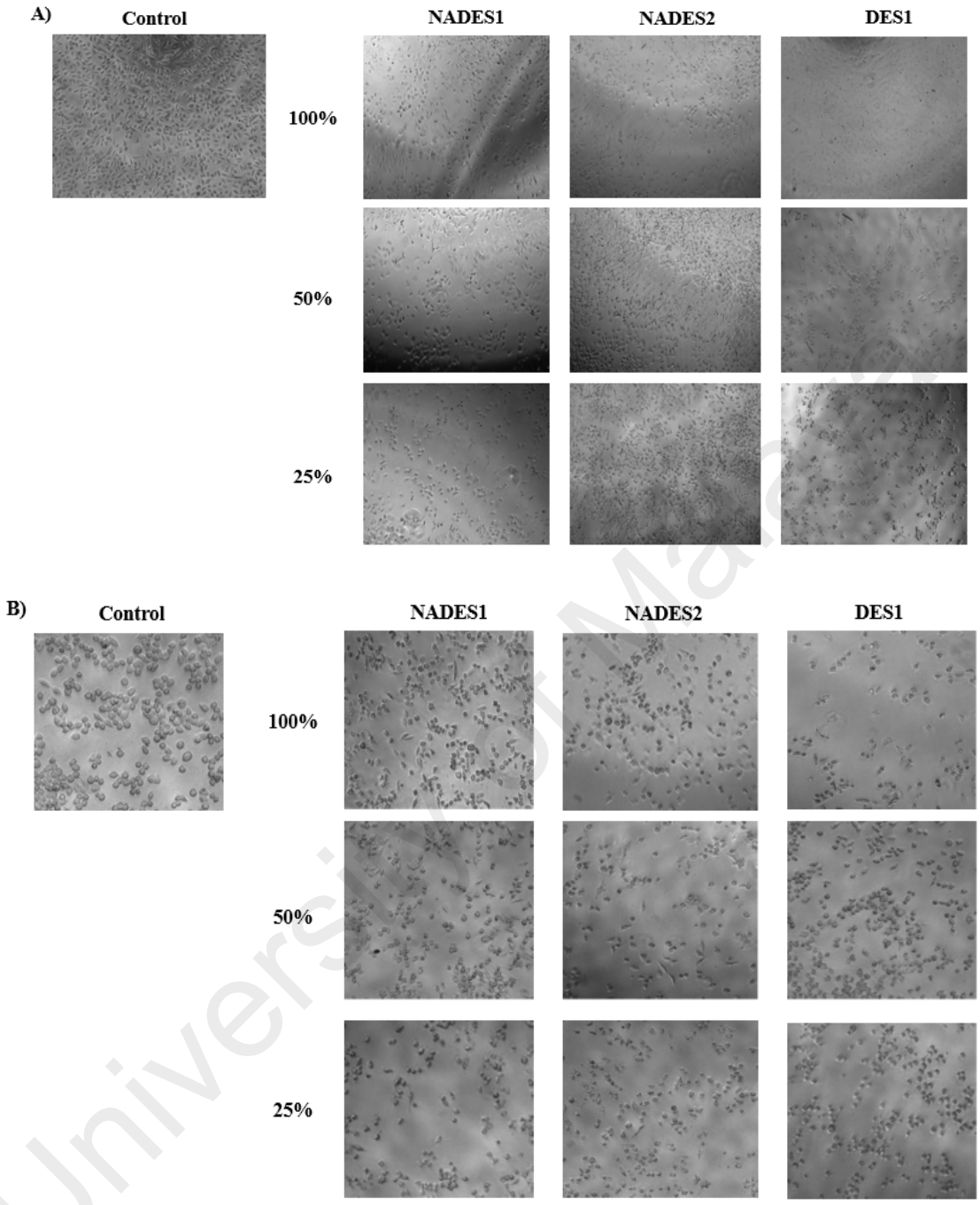
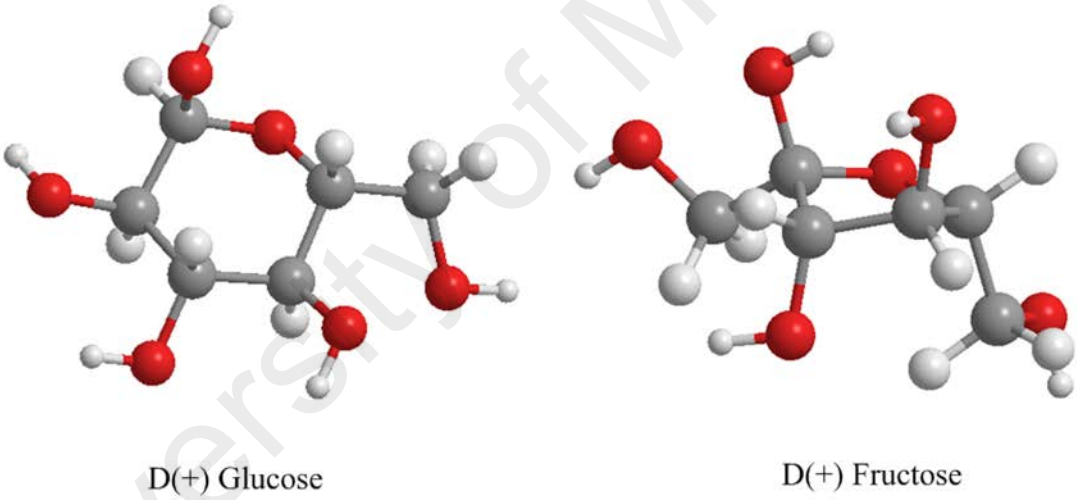


Figure 4.1: Light microscope images of A) AGS, B) HelaS3 cell lines submitted to NADESs/DES treatment. Control cells shown were not subjected to any treatment and represent the 100% growth. The other cells were treated with different concentrations (100%, 50% and 25%) of the solvents with 100% being 4.5 M.

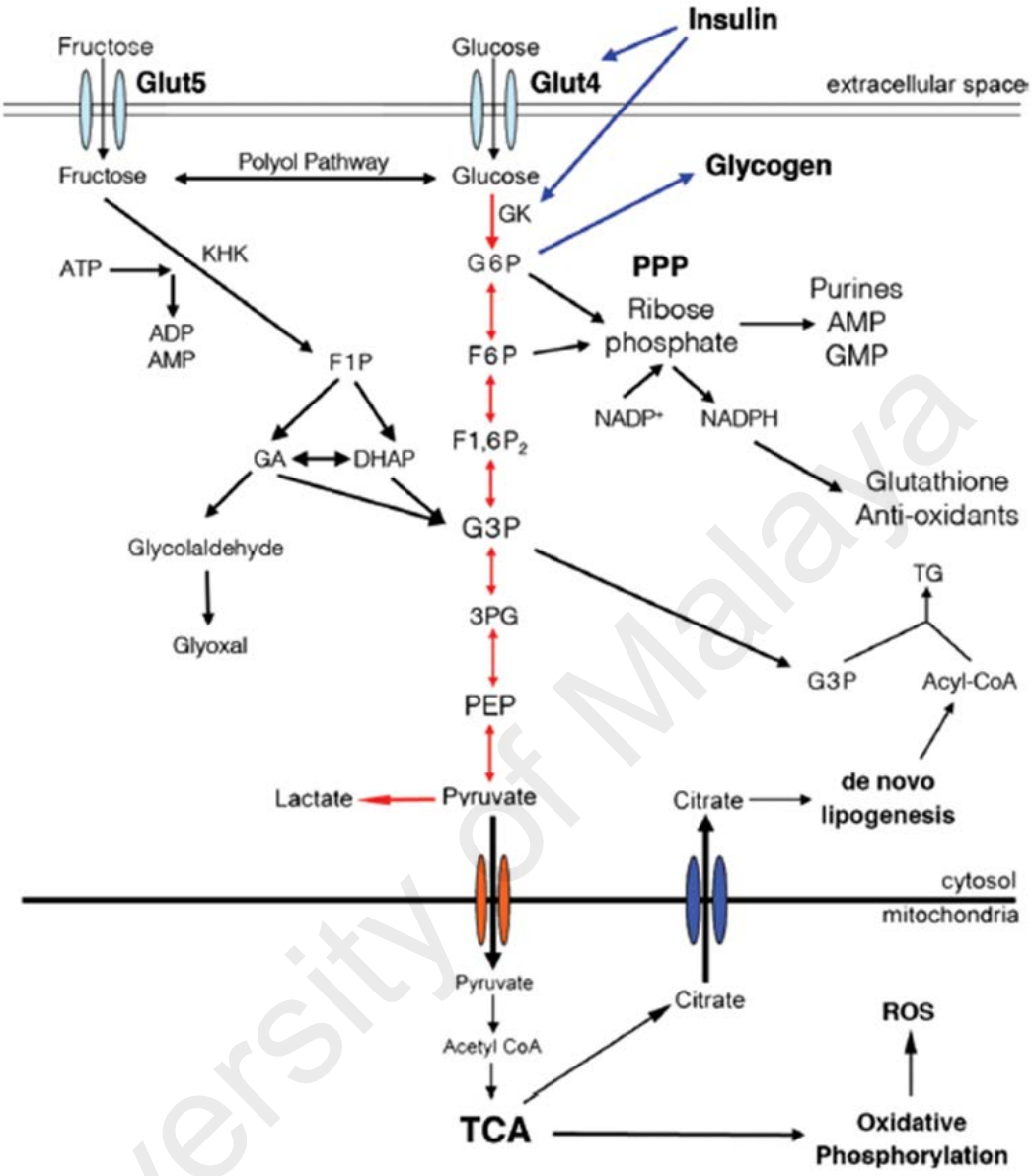
For NADES1, AGS cell line was the most susceptible, whereas HelaS3 cells were the least (AGS < MCF-7 < WRL-68 < A375 < PC3 < HelaS3). For NADES2, the trend was similar with one exception: WRL-86 cells were more susceptible than the breast cancer cell line (AGS < WRL-68 < MCF-7 < A375 < PC3 < HelaS3). DES1 was more toxic to the hepatic cell line than to any other cell line (WRL-68 < AGS < A375 < MCF-7 < PC3 < HelaS3). Overall, HelaS3 and PC3 cell lines were the least harmed, whereas the eutectic mixtures were increasingly lethal to AGS and WRL-68 cell lines, demonstrating that these mixtures harm normal (i.e., WRL-68) and cancerous cells, likely through a shared process in these cells. Thus, although greener than DES, NADESs can be used as potential anticancer agents but will require targeted delivery (e.g., nanoparticles) due to the risk that is posed to normal cells. Scheme 4.1 displays the chemical structures of glucose and fructose, which have the same chemical formula ($C_6H_{12}O_6$). In Table 4.1, NADES-based glucose (NADES2) was less toxic than its fructose (NADES1) counterpart across all cell lines. The metabolic pathways that each metabolite follows upon absorption explain this difference. Glucose and fructose are reducing sugars that represent an energy source for cells. Glucose is the primary energy source for all tissues, whereas fructose is metabolized primarily by the liver. Glucose metabolism is regulated by insulin, which determines the fate of the ingested glucose. When glucose is intended to produce energy, it enters aerobic glycolysis and is phosphorylated to glucose-6-phosphate (G-6-P) by the enzyme glucokinase (GK). G-6-P can be converted to NADH, antioxidants, nucleic acids and uric acid via the oxidative pentose phosphate pathway (PPP); to triglycerides and lipids by de novo lipogenesis; or to energy through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (electron transport chain) (Scheme 4.2).

Table 4.1: EC_{50} values of the understudied NADESs/DES across various cell lines.

Solvent	EC_{50} (mM)					
	Hela S3	**PC3**	**MCF-7**	**A375**	**AGS**	**WRL-68**
NADES1	436 ± 15	197 ± 17	102 ± 15	150 ± 28	98 ± 7.1	112 ± 7.8
NADES2	516 ± 40	340 ± 16	247 ± 8.1	265 ± 10	138 ± 14	185 ± 31
DES1	120 ± 15	116 ± 12	93 ± 18	91 ± 8.7	46 ± 0.75	34 ± 5.2



Scheme 4.1: Glucose and fructose 3D Models.



Scheme 4.2: Metabolic pathway of glucose and fructose (Charrez et al., 2015)[1].

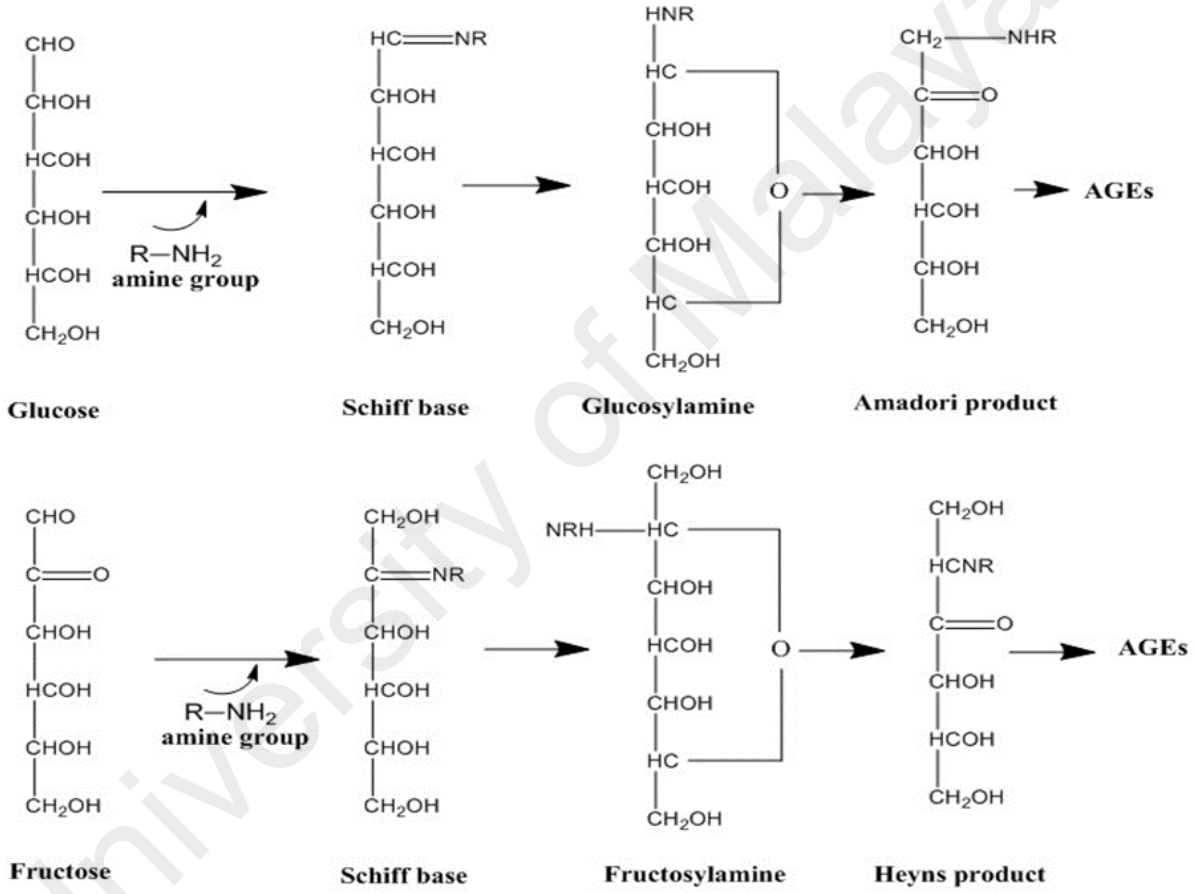
[1] Glucose intracellular transport occurs via the insulin-dependent transporter Glut4, whereas fructose enters the cell through Glut 5.

Insulin directly promote glucose metabolism through glycolysis or its storage as glycogen, by controlling the transcription of glucokinase (GK). Red arrows represent pathways used by glucose. Black arrows represent pathways used by glucose but preferentially by fructose. Metabolites and enzymes. Glut: glucose transporter; KHK: ketohexokinase; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; G6P: glucose-6-phosphate; F1P: fructose-1-phosphate; F6P: fructose-6-phosphate; $F1,6P_2$: fructose1,6 biphosphate; GA: glyceraldehyde; DHAP: dihydroxyacetone phosphate; G3P: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; TG: triglyceride.

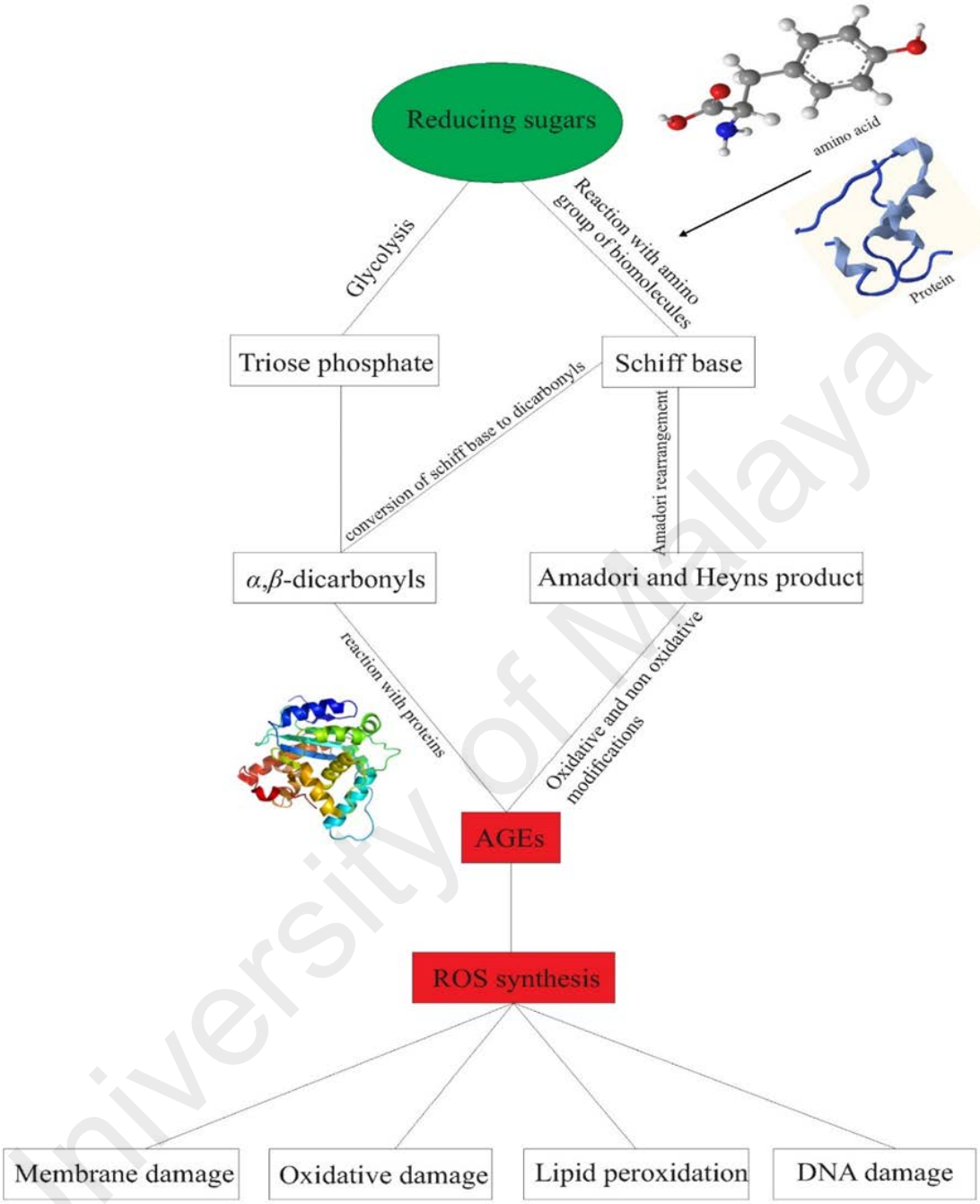
Fructose processing is not controlled by insulin secretion; thus, fructose can nearly continuously enter the glycolytic pathway (via polyol conversion) and undergo de novo lipogenesis (leading to fatty acid accumulation) (Ludwig, 2013). Under energy-deficient conditions, it can count as an effective beneficial measure. Under energy-deficient conditions, this pathway is an effective beneficial measure. However, at homeostasis, it can have disastrous intracellular effects. High-fructose diets increase the synthesis of advanced glycation end products (AGEs) faster than glucose-based diets. AGEs are obtained following glycation or through sugar intermediates that are produced during glycolysis.

Glycation refers to the non-enzymatic modification of biomolecules (amino acids, lipids, nucleotides) following the addition of a sugar, in a process that is known as the Maillard reaction (Scheme 4.3) (Helou et al., 2013). AGEs inflict serious damage to biomolecules by modifying their structure and subsequently enhancing ROS synthesis. Interactions of biomolecules with ROSs ultimately impair metabolic functions (Scheme 4.4). For instance, glycation of proteins results in biochemical dysfunction, protein crosslinks and alteration of protein structure (Pun & Murphy, 2012). Membrane interactions can also be affected by the formation of lipid glycation adducts through increased membrane fluidity, the promotion of lipid peroxidation and eventually oxidative damage (Bucala et al., 1993; Ravandi et al., 1996; Requena et al., 1997; Oak et al., 2000). Similarly, the effects of AGEs on DNA include strand breaks, unwinding of the double helix, mutations and formation of DNA-protein and nucleotide-nucleotide crosslinks (Kasai et al., 1998; Pischetsrieder et al., 1999; Chen & Chen, 2009). Fructose produces AGEs at least seven times faster than glucose, ultimately leading to over a 100-fold increase in ROS production compared to glucose-based AGEs. The cell is then unable to generate enough antioxidants to deal with the oxidative species (Bose & Chakraborti, 2008), (Bunn & Higgins, 1981).

The situation is worse for cancer cells, in which upregulation of glycolysis occurs because the cells require a higher amount of carbohydrates compared with normal cells for growth, proliferation, migration and invasion (Liu & Heaney, 2011). The higher carbohydrate requirement also stems from the fact that cancer cells initially use anaerobic glycolysis for glucose/fructose metabolism and thus, produce less total energy (energy output is higher for aerobic glycolysis than anaerobic glycolysis).



Scheme 4.3: Formation of AGEs from Amadori and Heyns products of glucose and fructose, respectively.



Scheme 4.4: Formation of AGE and ROS from reducing sugars (Fructose and glucose). Sugars use two strategies to synthesize AGEs intracellularly. The first one is through glycolysis and the second makes use of the Maillard reaction.

Accordingly, in cancer cells, high-glucose diets generate AGEs at a higher rate than normal cells, leading to oxidative damage, tissue damage and inflammation, due to the imbalance between AGEs and intracellular antioxidants. As a result, glucose, despite being a desired cellular metabolite, becomes cytotoxic. However, fructose is more cytotoxic, because the conversion and generation rates of AGEs and ROSs are significantly higher than those of glucose. Moreover, in cancer cells, fructose (converted to glucose intermediates via the polyol pathway; Scheme 4.2) is the preferred carbohydrate source for nucleic acid biosynthesis, whereas glucose is allocated primarily for glycolysis (Liu et al., 2010). Fructose-based nucleic acid synthesis via the non-oxidative PPP in cancer cells stimulates an increase in uric acid synthesis (Charrez et al., 2015). Overproduction of uric acid following high-fructose diets is often associated with endothelial dysfunction, cardiovascular disease and oxidative metabolism, with increased ROS concentrations and ultimately redox stress. This information is helpful in justifying the greater toxicity of NADES1 compared to NADES2. Other factors, such as the physical properties of the eutectic mixtures, can be used to explain the difference in EC_{50} values. Table 4.2 lists the viscosity and pH of NADESs at 25 °C. Both NADESs are neutral mixtures (pH ~7), but NADES1 (11733.1 mPa.s) is more viscous than NADES2 (8045.1 mPa.s) (Hayyan et al., 2012, 2013d). As highly viscous materials are more toxic than less viscous compounds, it may account for the greater toxicity of NADES1.However, in general, NADES1 and NADES2 had higher viscosity and less acidity than DES1 (71.3 mPa.s) but were less toxic, in contrast to the greater toxicity that is expected with highly viscous mixtures. Although the natural origin of the raw materials of the NADESs is one possible reason for this phenomenon, the acidity of the DES1 appears to influence this property.

According to Table 4.2, NADES1 and NADES2 are neutral mixtures (pH of 6.67 and 7.24, respectively).

Table 4.2: Viscosity and pH of NADESs at 25°C (Hayyan et al., 2012, 2013d).

Solvent	Viscosity (mPa.s)	pH
NADES1	11733.1	6.65
NADES2	8045.1	7.24

The intra-cytoplasmic pH is often found in a range that approximates that of NADES2 (7.1~7.2) (Damaghi et al., 2013), which also explains why it was the least detrimental eutectic mixture. Cancer cells often have a slightly more acidic extracellular environment due to lactate and H^+ overproduction from increased glycolysis, even in the presence of oxygen (The Warburg effect) (Wu & Zhao, 2013). The ensuing extracellular acidosis (5.8~7.6 in human and rodent tumors) (Wike-Hooley et al., 1984) and the resulting gradient difference can be toxic to cancer cells, which appears to be the case for NADES1 and DES1. Riemann et al. (2011) confirmed that induced extracellular acidosis promotes a sustained decrease in intracellular pH and the formation of ROSs. DES1 was more detrimental than NADES1, because its pH (~ 1) is significantly lower and thus, the influx of H^+ is higher. Hence, ROSs are generated at a higher rate compared with NADES1, causing DES1 to be more toxic than NADES1. This influx of acid into the cell also explains the higher toxicity of NADES1 versus NADES2. Moreover, the gradient difference that is created by the difference in pH contributes to the cellular absorption of ions (Na^+, K^+, Ca^+) and metabolites that are important for cancer progression, invasion and migration. The influx of NADES1 and NADES2 might be relevant to the cell for specific metabolic purposes, but import of DES1 differs, because DAC and triethylene glycol metabolites are not needed as much as glucose, fructose, or ChCl.

Following ingestion, TEG is metabolized by alcohol dehydrogenase to yield toxic diacid and hydroxy acid compounds, which advance metabolic acidosis (Herold et al., 1989; McKennis et al., 1962). Thus, their presence in high amounts promotes their cytotoxicity, rendering them lethal to the cell. As shown in Table 2, the EC_{50} values varied between cell lines, with the lowest value associated with AGS cell line and the highest corresponding to HelaS3 cell line. These findings suggested that a gastric cancer cell line was easily affected by DESs, whereas the opposite occurred for HelaS3 cell line. Given that cells are protected and compartmentalized primarily by their membranes, their interaction with NADESs must be a crucial factor for the ensuing cellular stress.

4.1.2 Assessment of membrane permeability

The cell permeability assessment following NADESs treatment showed a comparable effect with the DESs that were tested by Hayyan et al. (2015). Using a permeability dye (Image-iT DEAD Green viability stain) as an indicator of cell membrane damage, we evaluated the severity of NADES/DES cytotoxicity. Once the dye penetrates the cytoplasm, its detection of affected or dead cells by fluorescent imaging (green) establishes the effect of the applied material. As shown in Figure 4.2, the green fluorescence of NADESs and DES is more discernible in treated cells versus the control. In NADES-/DES-treated cells, this fluorescence stemmed from within the cells, implying increased porosity, whereas in control cells, only extracellular fluorescence of debris could be seen, suggesting that the dye did not penetrate the cells, allowing them to remain healthy. These results established the capacity of NADESs to perforate cellular membranes. However, DESs were more destructive than NADESs, based on the stronger emitted fluorescence of DES1-treated cells.

The increased porosity in DES1-treated cells versus NADES1 and NADES2 might be related to the complex interactions between these mixtures and the cell membrane. Membranes are composed of a matrix of lipids and proteins, the structures of which regulate their permeability. The composition of membrane lipid moieties depends on the cell type and its physiological requirements and functions (Escribá et al., 2008). One of the most important constituent of lipid bilayers are phospholipids, the distribution of which creates a unique membrane potential that regulates membrane permeability and the diffusion of ionic and molecular species (due to the nature of their various head groups and phosphate moiety densities). Phosphatidylcholine (choline head group) is the most abundant phospholipid in plasma membranes (Szachowicz-Petelska et al., 2012). It is synthesized from choline, primarily by exogenous routes to minimize energy expenditure. Choline is pivotal in the metabolism of phosphatidylcholine and other bilayer phospholipids, such as phosphatidylserine, phosphatidylethanolamine and sphingomyelin (Scheme 4.5). Thus, for normal and cancer cell lines, intracellular choline import is a crucial feature of their metabolism. Several protein families that are embedded in the membrane bilayer participate in the intracellular diffusion of choline. They consist of the polyspecific organic cation transporter (OCT) family for low-affinity facilitated diffusion, the choline transporter (CHT1) family for high-affinity Na+-dependent transport; and the choline transporter-like (CTL1) family for intermediate-affinity Na+-independent transport (Michel et al., 2006). Thus, similar to glucose and fructose moieties, the diffusion of choline moieties is assisted by membrane transporters. Consequently, the entry of choline or choline-based moieties is a natural phenomenon in eukaryotic cells.

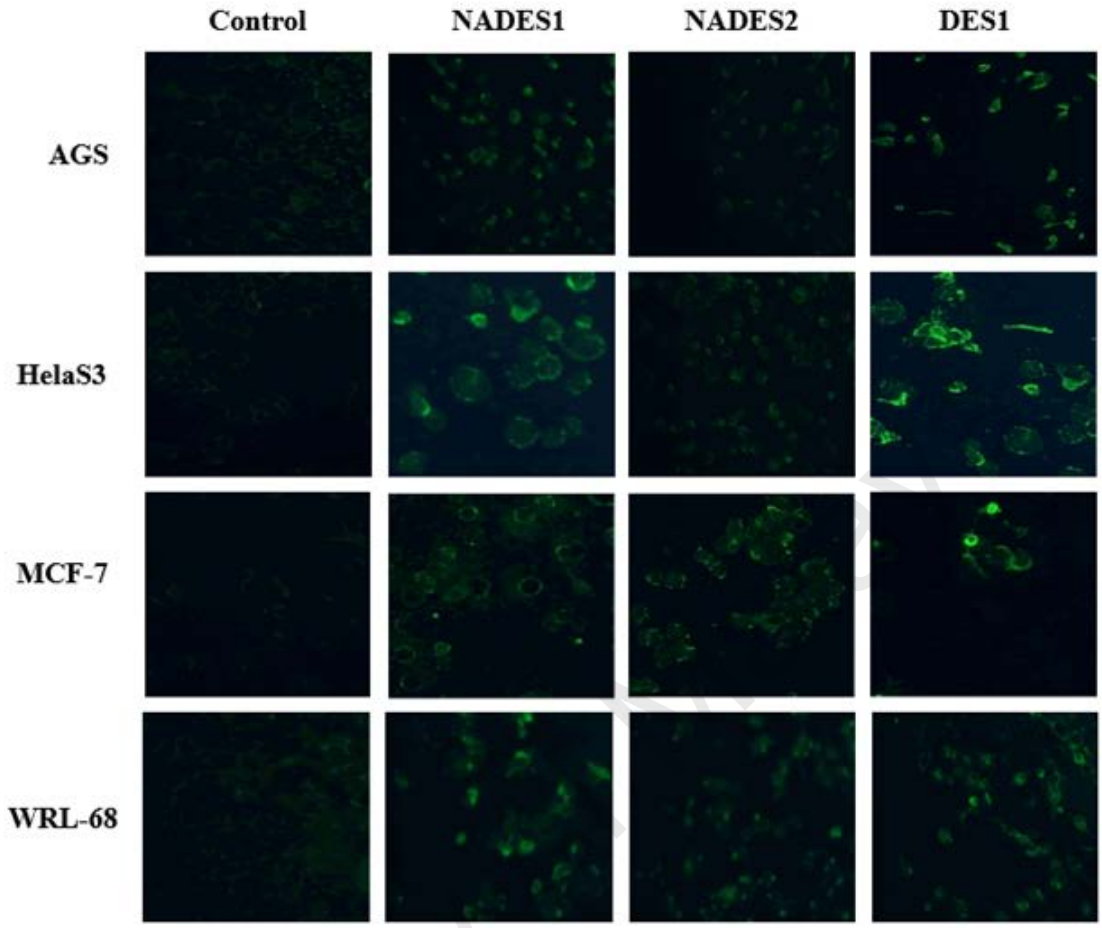
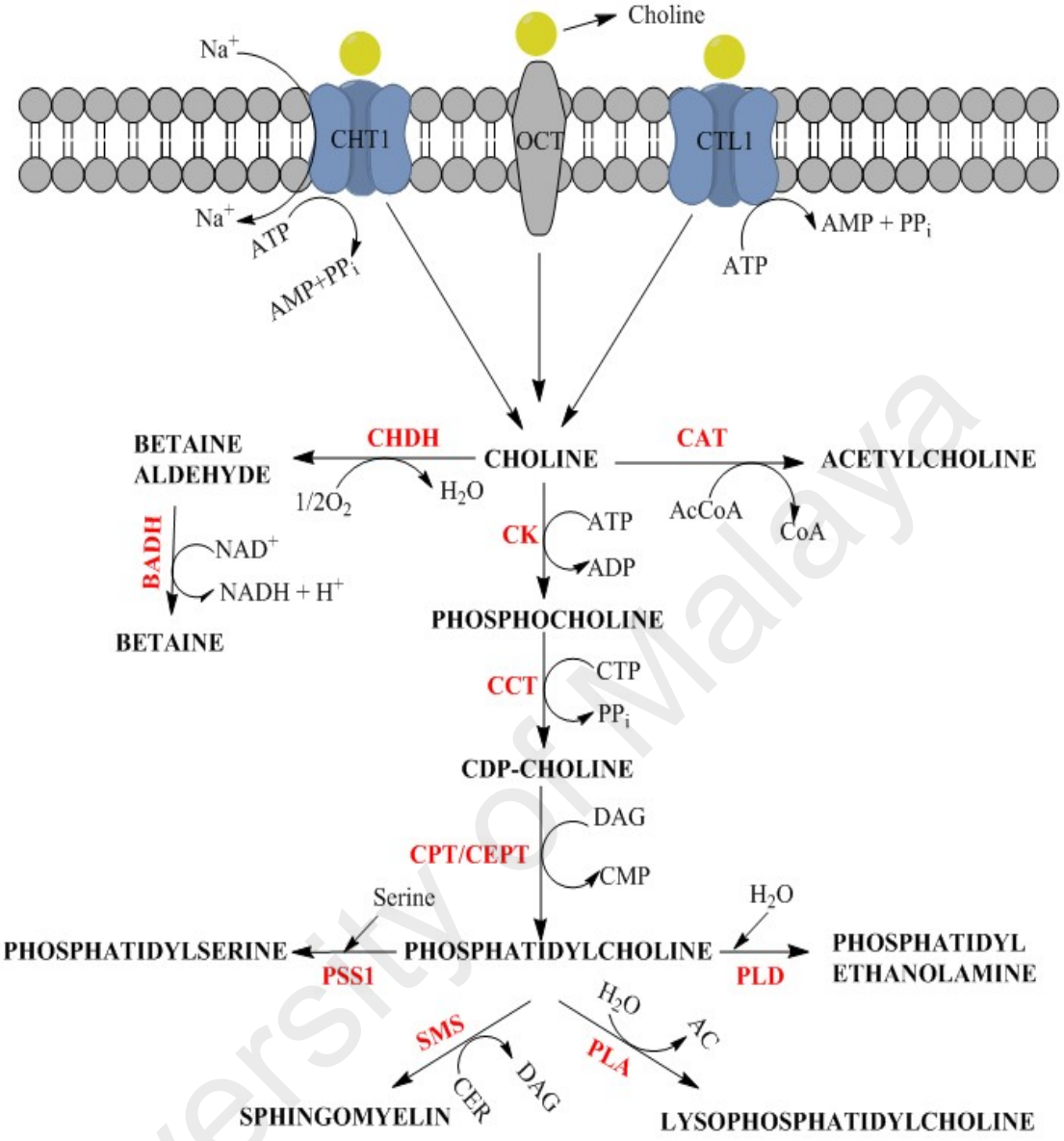


Figure 4.2: Effect of NADESs/DES on the permeability of various cell lines. Fluorescent images of AGS, HelaS3, MCF-7 and WRL-68 control and treated cells with EC_{50} concentrations of NADESs/DES.

The increased permeability of NADESs-treated cells could be an indirect consequence of the limiting uptake rate of choline species through embedded membrane transport proteins and/or the threshold concentrations of these mixtures—i.e., as the rate of choline transport in cells becomes limited (Plagemaen, 1971), extracellular moieties might begin to accumulate on the cell surface, thereby increasing porosity. In contrast to NADESs, DES1 species (i.e., DAC and TEG) are not indigenous to the cell or perhaps are not required in similar amounts. Hence, their intercellular diffusion is restricted.



Scheme 4.5: ChCl intracellular transport and metabolism[2].

2

Choline enters the cell either using low-affinity facilitated diffusion via OCT transporters; high affinity Na+-dependent transport via CHT1 transporters; or intermediate affinity Na+-independent transport via CTL1 transporters. Enzymes and metabolites. CHDH: choline dehydrogenase; CAT: choline acetyltransferase; AcCoA: acetyl-coenzyme A; CoA: coenzyme A; BADH: betaine aldehyde dehydrogenase; NAD+: nicotinamide adenine nucleotide; NADH: reduced nicotinamide adenine nucleotide; CK: choline kinase; CCT: citydylyltransferase; CTP: cytidine triphosphate; CDP: cytidine diphosphate; CPT: cytidine cholinephosphotransferase; CEPT: choline/ethanolaminephosphotransferase; DAG: diacylglycerol; CMP: cytidine monophosphate; SMS: sphingomyeline synthase; CER: ceramide; PLA: phospholipase A; AC: acyl chains; PPi: pyrophosphate.

As a result, they are instead primarily retained by the cell membrane, having a more pronounced deleterious effect. Previous studies established that the accumulation of ammonium cations (at a specific threshold concentration) on cellular membranes disrupts the lipid bilayer and induces cell death (Gal et al., 2012). The attractions and interactions of lipid bilayers with ionic species depend on the lipid organization and density charge, the latter of which is ultimately derived from the concentrations of acidic and basic functional groups and their average association constants with hydrogen or hydroxyl ions (all of which vary between cells) (Dobrzynska et al., 2006). For instance, the colorectal cancer cell line is associated with an overall increase in the concentration of all phospholipid types at the cell membrane, including phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (Szachowicz-Petelska et al., 2012). This property might account for the difference in EC_{50} values between cancer cell lines and the normal cell line (WRL-68). These inferences are subject to the transport modes and import of NADESs into the cell. It is pivotal to ascertain whether the cellular entry of NADESs occurs as dissociated components or as a single molecule. This mechanism can be determined by analyzing the cytotoxic values of aqueous solutions of the raw materials of NADESs and comparing them with the EC_{50} of the pure mixtures. In Table 4.3, the EC_{50} values of the aqueous solutions of NADESs and their chief materials are presented. NADES1aq, (101 mM) and NADES2aq (145 mM) were the least toxic mixtures, followed by glucoseaq (58 mM), fructoseaq (49 mM), DES1aq (54 mM), ChClaq (35 mM), DACaq (37 mM) and TEGaq (13 mM). The EC_{50} values of pure NADES1 (98 mM), NADES2 (138 mM) and DES1 (46 mM) were in a similar range as those of their aqueous solutions (NADES1aq -101 mM and NADES2aq -145 mM).

It is interesting to note that pure NADESs had significantly lower cytotoxic values than aqueous solutions of their individual elements (55 ≥Glucoseaq≤ 61mM; 43 ≥Fructoseaq≤ 55mM; 30 ≥ChClaq≤ 40mM), suggesting that NADESs did not dissociate after crossing the cellular membrane, in contrast to the assumptions of partial or complete dissociation in the literature. Based on the intersection between the ranges of EC_{50} values of pure NADESs (90.9 ≥NADES1≤ 105.1 mM; 124 ≥NADES2≤ 152 mM) and their aqueous solutions (92 ≥NADES1aq≤ 110 mM; 138 ≥NADES2aq≤ 152 mM), the inherent implication suggest that following cellular adsorption, the individual aqueous chief components of DESs form pure NADES mixtures intracellularly to reduce their cytotoxic effects on the cells.

Table 4.3: EC_{50} values of aqueous solutions of NADESs and their raw materials.

Solvent	EC_{50} in AGS cell line (mM)
$Fructose_{aq}$	49 ± 6
$Glucose_{aq}$	58 ± 3
$ChCl_{aq}$	35 ± 5
DAC_{aq}	37 ± 8
TEG_{aq}	13 ± 1.8
$NADES1_{aq}$	101 ± 9
$NADES2_{aq}$	145 ± 7
$DES1_{aq}$	54 ± 2.3

Although the dilution and solubility factors of the chief ingredients in aqueous solution as well as the type of cancer cell used (in this work, AGS) might have played a significant role, the previous suggestion would help explain the different profiles between pure NADESs and individual ingredients' aqueous solutions and the similarity between pure mixtures and their

aqueous solutions. This hypothesis would somewhat be consistent with Choi et al. (2011) concerning the synthesis of NADESs by plant cells, except that in this case it is extrapolated to mammalian cells. This assumption is based on the premise that if dissociation had occurred prior to absorption, then aqueous solutions of the individual raw materials would dictate the threshold concentrations at which cell death was induced. However, the pure solutions of NADESs had a distinct profile compared with aqueous solutions of their individual elements. With regard to DES1, the ranges of its aqueous solutions ($51.7 \geq$ DES1aq ≤ 56.3 mM) and pure mixtures ($45.25 \geq$ DES1 ≤ 46.75 mM) do not intersect. Moreover, the range of pure DES1 mixtures ($45.25 \geq$ DES1 ≤ 46.75 mM) approximates that of the aqueous solution of DAC ($29 \geq$ DACaq ≤ 45 mM), suggesting that DES1 dissociates in the cellular media prior to entry. The addition of water to the combination of ingredients merely reduced the toxicity of DAC, which was already determining the toxicity of the overall mixture. It can also be argued that the low EC_{50} values of DES1 are attributed to the lower requirements of DES1 species in AGS cells.

4.1.3 Assessment of redox stress

The results in this study present NADESs as architects of redox stress. Figure 4.3 shows the images of four treated cell lines (HelaS3, AGS, MCF-7 and WRL-68) that were stained with dihydroethidium (DHE) dye. DHE freely permeates cell membranes and is thus used to monitor superoxide production. DHE reacts with superoxide ions to form a red fluorescent product (ethidium) that intercalates into DNA (forming small red dots in the cell). Although recent studies have suggested that the end product is 2-hydroxyethidium, the result is that DHE is retained well by cells and helps detect superoxide radicals (Owusu-Ansah et al., 2008). The oxidized form of DHE gives off red fluorescence upon DNA intercalation.

As shown in Figure 4.3, the distinction between control and treated cells is notable, wherein the accentuated red fluorescence is visible among NADES-/DES-treated cells. Further, the intercalated DNA-DHE was strongly visible in NADES-/DES-treated cells. As expected, the fluorescence was more pronounced in cells that were treated with DES1 compared with NADESs, suggesting that DES1 stimulates the synthesis of ROS to a greater extent than NADESs (correlating with the hypothesis that NADESs are easier to deal with intracellularly) or that redox stress is merely one of the mechanisms by which eutectics induce toxicity, with NADESs operating through a disparate mode of action. Regardless of which hypothesis is correct, these NADESs are less strenuous on the cell than DESs.

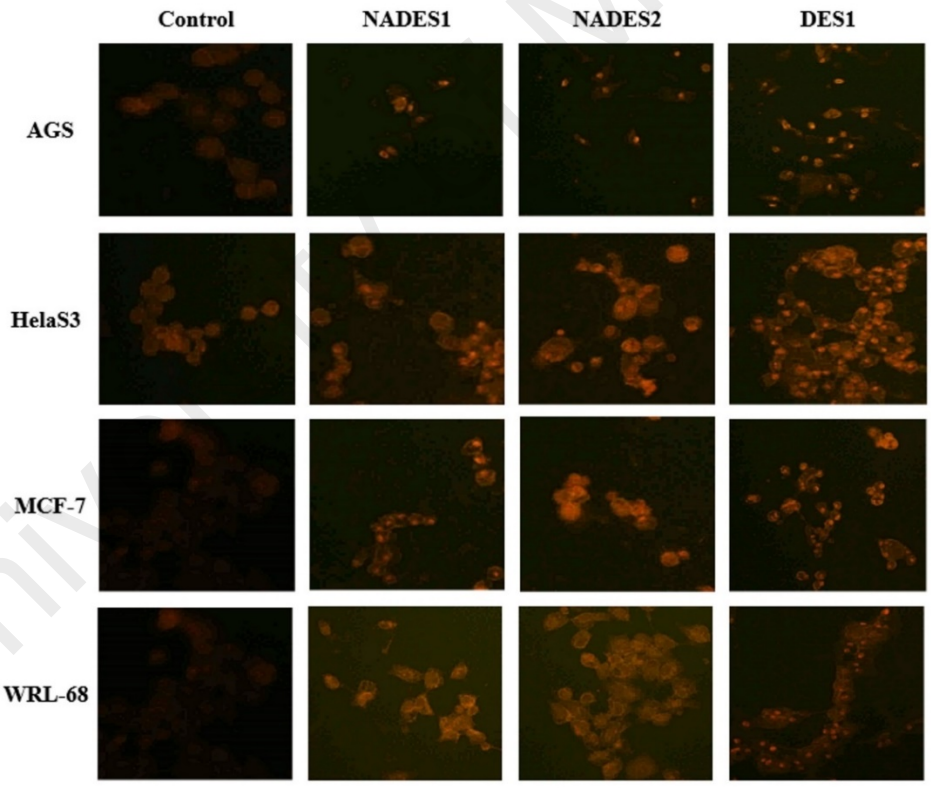


Figure 4.3: ROS production following NADESs/DES treatment. The cells were treated at each and every time with the corresponding EC_{50} values of the respective cells listed in Table 4.1.

4.1.4 Western blot analysis

Western blot analysis was performed to add further proof of the deleterious effect of NADES and DES and therefore solidify the findings observed across the above experiments. The death rate of cancer cells after treatment was appreciated via monitoring both β-actin and p53 protein levels. β-actin proteins are highly conserved and constitutively expressed in vertebrates' cells. They are an essential component of the cytoskeleton and they play critical roles in various cellular processes such as, cell migration, cell division and the regulation of gene expression (Bunnell et al., 2011). They are often used as a control during western blot to ensure the validity of other results. It is therefore important to record relatively similar expression levels of β-actin across both treated and control cells. Figure 4.4 shows the scan of the PDVF membrane following blotting with anti β-actin antibody. The band is located at approximately 42 kDa, characteristic of the size of the β-actin protein. Relatively similar levels of expression were recorded for non-treated and treated AGS cells; confirming constitutive expression among all cells. The next assessment was focused on p53's expression. p53 is a tumor suppressor protein, very active in the context of cellular proliferation. Its loss or inhibition often prevents apoptosis and contributes to uncontrolled cell growth. As such, more than half of all cancers are associated with a mutation or loss of function of this protein; that is the expression of an inactive/mutant form of p53 protein. The mutation of the p53 gene does not only entail protein' loss of function; it often give rise to a mutant protein with novel properties. In cancer cells, the mutant p53 is often expressed intracellularly at high concentrations, because it promotes crucial events of metastatic progression, such as cell migration and invasion.

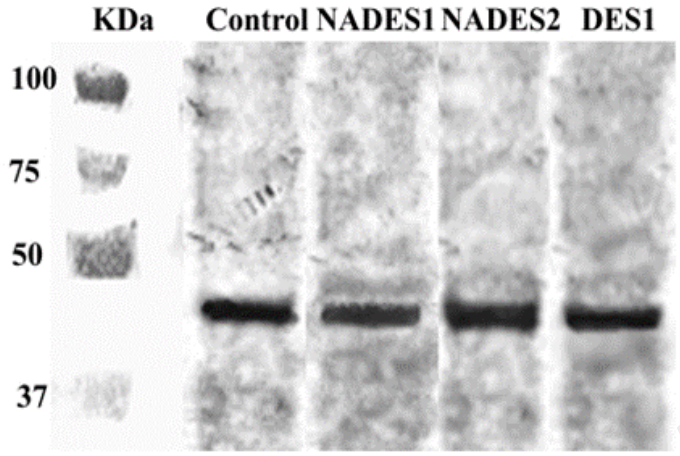


Figure 4.4: Western blot analysis revealed constitutive and similar levels of β-actin among both treated and untreated cells.

As such it represents a good measure of cellular stability and survival, especially in the context of cancer growth (Muller et al., 2011). Figure 4.5 depicts the results following membrane blotting with an anti-p53 antibody. The band is located at approximately 53kDa.

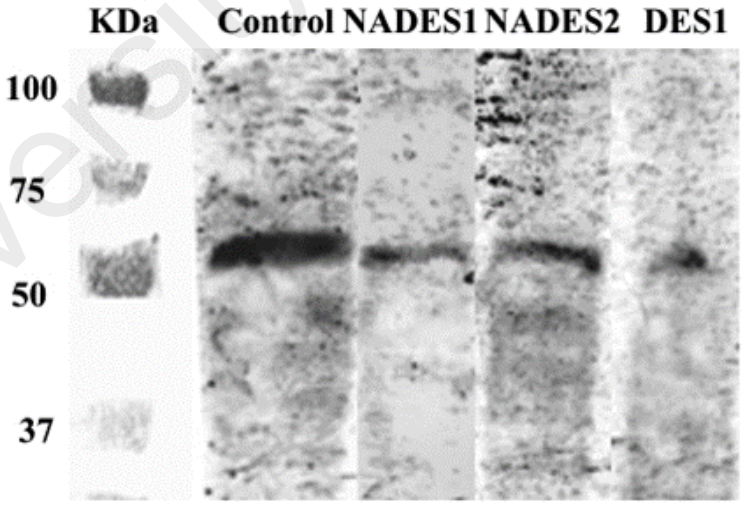


Figure 4.5: Western blot analysis of levels of p53 in control and treated cells. The concentrations of the latter are more pronounced in control and NADESs and less in DES1.

The untreated cells, as expected, expressed the protein at high levels, but for NADES/DES treated cells, the concentrations were much lower. The bands of NADES1 and NADES2 were of lower intensity than control. The protein levels decreased even further upon treatment with DES1. It reinforced the idea that DESs are more detrimental to cell growth than NADESs, although both exhibited anticancer activity.

4.1.5 *In vivo* analysis

In vivo profiling of NADES toxicity was performed in mice and biochemical variables were assessed in the liver and kidneys. As seen in the blood biochemical analysis in Table 4.4, the eutectics preferentially targeted the liver, which is the primary organ that metabolizes fructose and glucose. Thus, it is understandable that excess levels of these reducing sugars primarily injured hepatic cells. In Table 4.4, the parameters that varied the most were all affiliated with the liver: albumin, alkaline phosphatase (ALP), aspartate transaminase (AST) and G-glutamyl transferase. The fluctuations in the concentrations of these molecules are indicative of hepatocellular injury. Notably, the serum AST levels were 363 and 282 IU/L in NADES1- and NADES2-treated animals. The AST readings were approximately 10-fold higher than the normal range (15-37 IU/L). No increase in serum alanine aminotransferase (ALT) levels was observed. The AST/ALT ratios were greater than 5:1, implying that there was muscle or heart failure in addition to liver damage. Table 4.5 shows the recorded LD_{50} values. With regard to the discussion on EC_{50} above, the increasing trend of solvent toxicity in the cell lines was as follows: NADES2 < NADES1 < DES1. During the *in vivo* assessment, this trend was reversed (DES1 < NADES1 < NADES2). This difference was attributed to the viscosity of these solvents, because NADESs were more viscous than the DES and therefore were difficult to handle and might have failed to circulate properly in mice.

Table 4.4: Biochemical analysis of blood following NADESs/DES treatment.

Clinical Chemistry	NADES1	NADES2	DES1	Unit	Ref. Range	
Renal Function Test						
Sodium	146	152±5	150±7	mmol/L	136	145
Potassium	9.2	9.2±1.4	8.7±0.7	mmol/L	3.6	5.2
Chloride	105	111±7	110±8	mmol/L	100	108
Carbon dioxide	26.8	22.1±4	18.7±4.3	mmol/L	21	30
Anion Gap	23	28±1	27±3	mmol/L	10	20
Urea	9.7	9.0±2	8.9±1.1	mmol/L	2.5	6.4
Creatinine	-	18	11	umol/L		
Liver Function Test						
Total Protein	53	52±9	51±3	g/L	64	82
Albumin	14	13±4	13	g/L	35	50
Globulin	39	39±5	38±3	g/L	23	35
Total Bilirubin	2	3	2±2	umol/L	3	17
Conjugated Bilirubin	<1	1	<1	umol/L	0	3
Alkaline Phosphatase (ALP)	34	37±14	30±19	IU/L	50	136
Alanine Aminotransferase (ALT)	70	45±4	54±27	IU/L	12	78
Aspartate transaminase (AST)	363	282±18	349±23	IU/L	15	37
G-Glutamyl Transferase	<3	<3	<3	IU/L	15	85
Lipid Profile						
Triglyceride	1.2	1.1±0.6	0.7±1.1	mmol/L	<1.7	-
Total Cholesterol	2.0	3.1±.06	2.3±0.9	mmol/L	<5.2	-
HDL Cholesterol	2.01	2.85±0.57	2.24±0.79	mmol/L	<1.1	-

This may have blocked and halted blood flow. The injected solvents were not diluted with water; thus, they remained just as viscous as during their preparation. Dilution with water alters the physicochemical profiles of DESs (Dai et al., 2015) and can provide more manageable fluids, in terms of application and cytotoxic liability.

Table 4.5: LD_{50} values collected upon treatment of mice with the understudied NADESs/DES.

Solvent	LD_{50} (g/mL)
NADES1	1.84
NADES2	1.2433
DES1	4.46

4.2 Comparative assessment of binary deep eutectic solvents systems against binary natural deep eutectic solvent systems

4.2.1 Analyses of the cytotoxic values of binary DESs and NADESs

During the first part of the study, half-inhibitory concentrations of the understudied DESs were determined across the various cancer and normal cell lines. The results are shown in Table 4.6. They show a clear indication of toxicity from both types of salt based DESs. A brief outlook shows that binary NADESs are significantly less toxic than binary DESs. The EC_{50} values of the binary DESs interval ($19 \leq EC_{50} \geq 109$ mM) do not overlap those of the binary NADESs interval ($279 \leq EC_{50} \geq 1260$ mM); even when the comparison includes only those binary DESs ($37 \leq EC_{50} \geq 109$ mM) with similar HBDs as the binary NADESs. This implies that binary NADESs are significantly less toxic than binary DESs.

On average, across all cells, the DAC-based DESs adopted the following increasing trend of toxicity: DES5 < DES6 < DES2 < DES4 < DES3. Binary NADESs adopted the following trend: NADES4 < NADES3 < NADES5. The least toxic binary eutectics were prepared using glycerol, whereas the most toxic binary DES and NADESs were based on malonic acid and urea, respectively. It is not surprising to see a glycerol eutectic listed as the least toxic material in a group including ethylene glycol, urea, zinc dichloride and malonic acid. Glycerol is an important metabolite which plays important functions in various physiological processes in mammals. It is involved as an intermediate in carbohydrate and lipid metabolism as well as in glycolysis and eventually the Krebs cycle and oxidative phosphorylation (ATP synthesis) (Lin, 1977). The importance of glycerol is even more pronounced in cancer cells due to higher than normal requirements of energy to sustain their uncontrolled growth. On the other hand, ethylene glycol is not essential to mammals' metabolism as it possesses a low acute toxicity and has been reported to induce metabolic acidosis and central nervous system depression (Gomes et al., 2002). Urea plays important roles in regulating kidney function in mammals through renal nitrogen excretion and qualifies as the major circulating source of nitrogen-containing compounds excluding proteins. Hence, urea levels are widely monitored as they play prominent roles in determining normal and disease states (Weiner et al., 2015). However, several studies have reported that an excess level of urea (uremia) could induce protein carbamylation, which would disrupt the numerous pathways of a given protein (Weiner et al., 2015). Zinc chloride can be tolerated in minute amounts in mammals because ionic zinc is an essential trace element, which is required for several enzymes activities and also act as a stabilizer of cellular membrane. However, if administered at high concentrations, it could lead to acute cytotoxicity characterized by the inhibition of energy metabolism, the induction of redox stress, or the activation of pro-apoptotic molecules eventually resulting in

cellular necrosis (Plum et al., 2010). Finally, malonic acid is a known inhibitor of the citric cycle and oxidative phosphorylation in mammals; it also decreases the optimal pH for cell growth (Greene et al., 1993). Although DESs assume distinct and unique profiles from their chief ingredients, it is often assumed that DESs dissociate partially in cellular media or cellular environment. However, the intracellular partial dissociation suggests that the resulting toxicity might be a combination of the potency between the individual starting materials and the DESs.

Table 4.6: Inhibitory concentrations of the understudied binary NADESs and DESs across the various cell lines.

NADESs/DESs	EC_{50} (mM)			
	AGS	**HelaS3**	**MCF-7**	**WRL-68**
NADES3	432 ± 60	702 ± 23	834 ± 22	608 ± 91
NADES4	452 ± 72	731 ± 63	1260 ± 104	652 ± 69
NADES5	279 ± 29	382 ± 17	831 ± 25	405 ± 39
DES2	59 ± 10	70 ± 12	65 ± 5	63 ± 6
DES3	34 ± 2	19 ± 3	93 ± 5	25 ± 4
DES4	37 ± 3	76 ± 10	79 ± 10	68 ± 6
DES5	65 ± 5	109 ± 22	98 ± 11	109 ± 13
DES6	43 ± 3	74 ± 13	103 ± 9	76 ± 6

Therefore, it is important to account for the effect of the starting materials when analyzing DESs results; hence, the above descriptions of the major roles of these starting materials in mammalian systems. Using these descriptions, the lower toxicity of glycerol, urea and ethylene glycol eutectics in both binary NADESs/DESs was expected.

Concomitantly, the deleterious effect of malonic acid, zinc dichloride based on their natural properties was also expected.

4.3 Investigation of the effect of water on ternary NADESs solvent systems' toxicity and analysis of the interactions between lipid bilayer and the eutectics

4.3.1 *In vitro* assessment

The cytotoxicity of the five understudied NADESs was assessed on various human and mice cancer cell lines, namely, HelaS3, CaOV3, MCF-7 and B16F10. Table 4.7 illustrates the EC_{50} values obtained. The results indicate the following decreasing order of toxicity for HelaS3, MCF-7 and B16F10 cell lines: NADES7 > NADES5 > NADES3> NADES4 > NADES6. In CaOV3 case, NADES4 was more toxic than NADES3 resulting in a slightly different trend: NADES7 > NADES5 > NADES4> NADES6 > NADES3. However, if the SEM of the EC_{50} values are included, the resulting EC_{50} intervals of both NADES3 (198.5-213.5 mM) and NADES4 (185.5-200.5 mM) overlap, as the end values are close to one another. Overall, a trend between NADESs' cytotoxicity and various factors namely the cellular requirements of cancer cells, the physical properties of NADESs (especially viscosity); the addition of water; and the nature of NADESs' raw materials as well as their interactions with the different functional groups present on the cell surface was noticeable. The merits of most DESs stem from the qualifications of ChCl, with specific referral to the metabolism and function of choline in mammalian cells. Choline is the preferred cellular raw material used for the synthesis of cellular membranes phospholipids, namely phosphatidylcholine and sphingomyelin (Lodish et al., 2000; Plagemaen, 1971). Consequently, ChCl has been classified a salt of relatively safe profile (although high intake is associated with adverse conditions).

However, the DESs cytotoxic profiles obtained thus far do not share the low acute cytotoxic labeling of ChCl. This has prompted the examination of the role of the HBD in these profiles. From a cellular perspective, fructose, glucose, sucrose (50% glucose and 50% fructose) and glycerol are essential carbohydrates whose metabolism provide energy required for various cellular functions.

Table 4.7: EC_{50} of the studied ternary NADESs on various cell lines.

Solvent	EC_{50} (mM)				
	Hela S3	**CaOV3**	**MCF-7**	**B16F10**	**WRL-68**
NADES6	177±7.3	206±7.5	127±9.22	195±7.7	97.72±7.91
NADES7	182±7.6	193± 7.5	186 ± 7.9	211 ± 8	88.76±7.7
NADES8	166 ± 5.8	154 ± 5.6	150 ± 5.5	136 ± 5.7	64.43±5.56
NADES9	427 ± 11	483 ± 11	457 ± 11	340 ± 10.3	119±12.33
NADES10	20 ± 8.4	15 ± 8.2	35 ± 8.3	35 ± 8.8	8.28±2.4

Upon adsorption, fructose and glucose undergo glycolysis if energy is needed, or are stored as glycogen. The glycolytic pathway for these molecules can lead to either the pentose phosphate pathway (for nucleic acid synthesis), the mitochondrial tricarboxylic acid pathway (for energy production), or de novo lipogenesis (for fatty acids synthesis). Cancer cells, especially, require more energy than normal cells, given their abnormal and exponential growth features. Therefore, they use a higher amount of energy or energy sources (glucose and fructose) for metastasis, growth, invasion and migration purposes (Port et al., 2012; Santos & Schulze, 2012). Likewise, glycerol is the precursor of triglycerides and phospholipids. It is activated by a phosphorylation reaction and forms glycerol-3-phosphate (G-3-P), which is then involved in the carbohydrate and lipid metabolism.

Alternatively, glycerol also functions as a shuttle of electrons from cytosol to mitochondria by regenerating NAD^+ from NADH (Laforenza et al., 2016). In both normal and cancer cell lines, glycerol can be used for gluconeogenesis, although the main metabolite used for that purpose is different; probably glycogen. Nevertheless, there is evidence that in cancer cells, a higher than normal plasma concentration of glycerol (comparable in this case to NADES4 treatment) contributes to increased glycerol turnover for gluconeogenesis and lipogenesis (Liu et al., 1995; Lundholm et al., 1982). Judging from these facts, a higher cellular tolerance of these carbohydrates-based eutectics is expected and this is reflected by the EC_{50} values recorded for NADES3, NADES4, NADES5 and NADES6. In contrast, NADES7 which boasts an organic acid as raw material, is the most lethal mixture. Dai et al. (2013) listed NADES7 as a eutectic utilized by plants for developmental or metabolic purposes. Although this is valid for certain plants tissues, where malonic acid accounts for as much as 4% of the dry weight and up to 50% of the total acid- and may be actively used during nitrogen assisted symbiosis or abiotic stress as a defense chemical; the scenario might be slightly different for mammalian cells (Kim, 2002). Indeed, in mammalian systems, malonic acid is known to stall the Krebs cycle by inhibiting succinic dehydrogenase (mitochondria complex II); a crucial enzyme for the citric acid cycle and the electron transport chain (Hosoya & Kawada, 1958). Alternatively, this paralyzes ATP synthesis. Moreover, malonate is known to disrupt glycogenesis, lipid synthesis and carbon dioxide production during glycolysis (Hosoya et al., 1960). It comes as no surprise that calls have been made for malonate to be used as an anticancer agent. As a matter of fact, Fernandez-Gomez et al. (2005) showed that malonate causes SH-SY5Y neuroblastoma mitochondrial failure by inducing a rapid build-up of ROS, which overwhelms mitochondrial antioxidant capacity, ultimately leading to cellular apoptosis.

This shows that with regards to the HBD, the inclusion of organic acids seem to increase the overall toxicity of NADESs. This is consistent with the other cytotoxic reports on DESs/NADESs (Paiva et al., 2014a; Radošević et al., 2015; Zhao et al., 2015). Zhao et al. (2015) observed that NADESs with organic acids as HBDs had a low pH (less than 6.5); when the optimal growth range for mammalian cells is 7.0-7.4. This change in environmental conditions is partially responsible for the high toxicity of NADES7. DESs investigations led to a similar observation. For instance, Radošević et al. (2015) observed the formation of intracellular calcium oxalate crystals following [ChCl]-[Oxalic acid] DES treatment of CCO and MCF-7 cell lines. Another perspective on organic acids as HBDs was shown by Paiva et al. (2014a). The authors examined NADESs toxicity towards fibroblasts like-cells (L929) and reported that the most lethal solvents also had organic acids as HBDs (i.e. tartaric acid and citric acid). However, it must be noted that the solvents with the highest viability, also had organic acids as ingredients, although the remaining constituent was another HBD (sucrose) and not a salt (ChCl) (Paiva et al., 2014a). It might be that the devastating effect of organic acids in NADESs is better countered with the use of biomaterials (e.g. sugars). The arguments above do not presume to provide a complete understanding of the reasons behind the variation in EC_{50} values; but serve to highlight that safer NADESs can be obtained by using biomaterials of cellular necessity. Of course, the interactions of these mixtures and their aggregation on cellular membranes as well as the neoteric properties of NADESs, remain aspects to be investigated. Meanwhile, physical properties of NADESs can also be used to better appreciate the obtained cytotoxic values. Table 4.8 lists the known viscosities values at 30 °C of the understudied NADESs. Sorting out viscosities in a decreasing order (NADES5 > NADES7 > NADES4> NADES3 > NADES6) reveals that they form a series almost similar to the cytotoxicity trend above.

Table 4.8: Viscosities of the understudied ternary NADESs (Zhao et al., 2015).

NADES	Molar ratio	Viscosity (mPa.s)
NADES6	5:2:5	545
NADES7	5:2:5	579
NADES8	4:1:4	1150
NADES9	1:2:1	36
NADES10	1:1	913.80

According to Table 4.8, NADES8 and NADES10 possess the highest viscosities at 30 °C (respectively 913.80 and 1150 mPa.s). It is no surprise that they also possess the highest EC_{50} values across all examined cells, as high viscosity is often associated with increased lethality. Despite being less viscous than NADES8, NADES10 was identified as the most toxic material tested, with an EC_{50} interval ($15 \leq EC_{50} \geq 35$ mM) at least three times lower than NADES3' interval ($136 \leq EC_{50} \geq 166$ mM). In a separate study, upon testing numerous DESs and NADESs (including similar NADES6, NADES7 and NADES10 used in this study) on several bacteria species (i.e. *Escherichia coli*, *Staphylococcus aureus, Salmonella enteritidis*, *Listeria monocytogenes*), Zhao et al. (2015) also identified NADES10 as one of the most toxic mixture. In contrast, NADES6 and NADES7 were found to be non-inhibitory to all studied bacteria. Moreover, the most toxic DESs and NADESs ([ChCl]-[Toluenesulfonic acid], [ChCl]-[Oxalic acid], [ChCl]-[Levulinic acid], [ChCl]-[Malonic acid], [ChCl]-[Malic acid], [ChCl]-[Citric acid], [ChCl]-[Tartaric acid] included organic acids as HBDs (Zhao et al., 2015). This is similar to this study's findings, in which the malonic acid based NADES was the most toxic.

This stipulates that despite the intrinsic high viscosities (compared to water and organic solvents), NADESs composed of sugars are relatively less dangerous to biological machinery than those composed of organic acids. On the other hand, NADES9 exhibited the lowest viscosity at 30 °C (36 mPa.s) as well as the lowest cytotoxicity values. With reference to Table 4.8, NADES7 is slightly more viscous than NADES6. Viscosity or micro-viscosity (in cellular terms) is an important property to consider in intracellular activities. Not only does it affect diffusion within biological systems, but it is also involved during processes such as protein–protein interactions, transportation of small solutes, macromolecules and signal transduction in living cells. The local micro-viscosity in cells ranges from 1 to 400 mPa.s (Liu et al., 2014). The highest values (≥ 200 mPa.s) usually correspond to the microviscosity in the hydrophobic domains of living cells (lipid bilayers of cell surfaces); whereas, values between 1-3 mPa.s are attributed to the aqueous phases of the cellular cytoplasm (Juneidi et al., 2015; Kuimova et al., 2009). A variation or a disturbance of these homeostatic values leads to the onset of various diseases (atherosclerosis, diabetes) as well as cell death (Deliconstantinos et al., 1995; Nadiv et al., 1994). The understudied NADESs possess viscosities higher than 500 mPa.s; with the exception of NADES9 (36 mPa.s), which happens to be the least toxic mixture tested (with EC_{50} values 1.6 to 32 times lower than the other NADESs). Hence, it is not difficult to perceive the substantial influence that these high viscous materials may incur on cells. Just like DESs perforate cellular membranes (Hayyan et al., 2015), NADESs can probably enhance cellular membrane permeability. As such, the introduction of such viscous substances in cellular medium can result in a major variation of cytoplasmic microviscosity and eventually lead to cell death. Based on the available knowledge of DESs, the viscosities of NADESs are assumed to originate from the rigidity of their supramolecular complexes reposing on a strong hydrogen bond network.

It entails that any disruptive action on this network will affect the viscosity of NADESs. In fact, Dai et al. (2015) recently provided evidence of the progressive rupture of this hydrogen bond network upon addition of water. The authors also showed that the supramolecular complexes of NADESs remain intact if the volume of added water is less than 50%. Pass this threshold, the resulting mixture consists merely of dissociated NADESs ingredients. This is a consequence of the complete rupture of hydrogen bonds stabilizing NADESs. The fact that the entire NADES structure repose on hydrogen bonds means that their progressive breakdown simultaneously induces a change in their physicochemical properties. Accordingly, Dai et al. (2015) reported a decrease in viscosity from 397 to 7.2 mPa.s, following the addition of 25% of water to [ChCl]-[Glucose]-[Water] (in this case NADES7). This argument is further justified by the fact that a measurement of NADES9 ([ChCl]-[Glycerol]-[Water]) viscosity recorded a value of 36 mPa.s whereas Zhao et al. (2015) recorded a value of 177 mPa.s for the [ChCl]-[Glycerol] DES (both at 30 °C). The effects of water can also be acknowledged through the observation that of all the understudied NADESs, the most toxic and most viscous was the one prepared without the use of water (NADES7). Moreover, Dai et al. (2015) stated that the water activity of NADESs increase with increasing water content (or after water addition). Consequently, the polarity of the eutectics after addition of water may mimic that of water itself. This may influence the interactions of these solvents with cell surfaces. The importance of these interactions must be dully underscored, as DESs have shown that they promote cellular failure through an increase in membrane porosity (Hayyan et al., 2015).

4.4 Cytotoxic mechanism and anticancer potential of deep eutectic solvents

4.4.1 COSMO-RS interpretation

The interactions of solvents with their immediate environment (solute) can be an important descriptive parameter for toxicity. These interactions are dictated by various thermodynamic properties, such as activity coefficients and solubilities which in turn deliver crucial information related to reaction medium, separation processes and other applications. Foreknowledge of these fluid phase properties help scientists choose the adequate solvent for a particular application. For instance, activity coefficients can be used to analyze the interaction patterns between solvent and solute. Numerous predictive models based on empirical equations using local composition and group contribution concept have been developed to predict the thermodynamic properties of various materials, e.g. the Universal Quasi-chemical Functional Group Activity Coefficient (UNIFAC), the Universal Quasi-chemical (UNIQUAC), the Non Random Two Liquids (NRTL), the Quantitative Structure-Property Relationship (QSPR) models. Although these models have been successful with traditional solvents, two particular issues limit their applications with ILs and DESs. First, as novel mixtures, the group interactions parameters of ILs are inexistent in the databases of the above models; further, some of these models, such as UNIFAC, are not suitable for non-molecular mixtures. Hence, using the conventional approaches have proven to be quite challenging (Diedenhofen & Klamt, 2010). Recently, the application of the Conductor-like Screening Model for Real Solvent (COSMO-RS) model enabled the efficient prediction of thermodynamic behavior for ILs. COSMO-RS is a very useful and fast tool for the prediction of thermophysical and chemical properties of fluid mixtures (Klamt, 2005). COSMO-RS' quantum chemical basis is a dielectric continuum model called COSMO (conductor-like

screening model). In a COSMO module, the solute (molecule) is placed inside a molecular shaped cavity and the surrounding solvent is described by a continuum (Diedenhofen & Klamt, 2010). The molecule‘s inherent moments then draw charges from the surroundings to the surface of the cavity so as to cancel the resulting electric field within the conductor and tangential to it. The charge induced at the surface is then calculated and is termed as the screening charge (σ). The screening charge or the local polarization charge density is one of the most important asset of COSMO-RS analysis. Figure 4.6 describes the continuum-like screening process. Each molecule (solute) possesses a screening charge σ, from which polarities and hydrogen bond donor or acceptors capabilities can be inferred. With this information, interactions between solute and solvent can be predicted. Charge densities information obtained from the cavity surface are best described in histograms typically referred to as σ-profile and σ-potential. COSMO-RS can thus be used to study the possible thermodynamic behavior of an individual component in a mixture and its affinity or interactions with the other components through the σ-profile and σ-potential, respectively. The σ-profile describes the molecule polarity properties. Each peak in the σ-profile plot for a molecule corresponds to its constituent atoms depending on their screening charge densities. The negative partial charges of atoms cause positive screening charge densities and vice-versa.

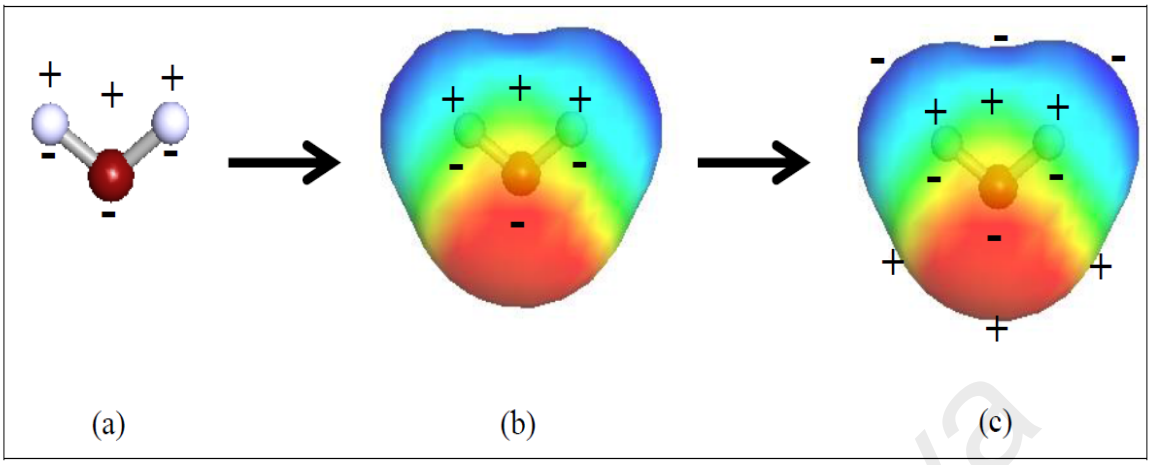


Figure 4.6: Continuum like screening model. a) Original molecule; b) Molecule imbedded in the cavity surrounded by the continuum; c) Charge densities on the cavity's surface.

COSMO calculations in COSMO-RS applications are performed in an ideal perfect conductor. The solute electron density and geometry are thus converged to an energetically optimal state in the conductor, which acts as the reference state for any COSMO-RS calculations. The resulting geometries, energies and surface screening charge densities are stored in COSMO files. COSMO-RS calculations need to be done only once for each molecule, regardless of the mixture in which the molecule appears. Although originally developed to assist with the prediction of environmental distribution and toxicity, COSMO-RS can also be applied to life sciences and molecular studies. Examples include the prediction of drug's partition coefficients and the computation of proteins pKa (Andersson et al., 2013; Buggert et al., 2009). With regards to ILs, three approaches can be used to describe ILs in COSMO-RS: (i) the electro-neutral approach, (ii) the ion pair approach and (iii) the meta-file approach. The electro-neutral approach is considered the most reliable as it assume that the cations and anions of ILs behave as separate species in a continuum liquid mixture (Mulyono et al., 2014).

Therefore, this approach was also extended to DESs as both classes of solvents are considered analogues. DESs is described according to the salts' cation and anion as well as the HBD. Using the elements in Table 3.2, the listed phospholipids were modelled in order to examine their interactions with the NADESs. The sigma profiles and potentials of phospholipids, binary DESs, binary NADESs and ternary NADESs systems are shown in Figure 4.7 and 4.8, respectively. In the σ-profile, when the screening charge density is lower than -0.0084 eÅ^{-2} or exceeds +0.0084 eÅ^{-2}; the molecule is considered sufficiently polar to induce hydrogen bonding. Figure 4.7 is divided into three quadrants with their corresponding σ values; the HBD region (σ < -0.0084 eÅ^{-2}), the nonpolar region (-0.0084 ≤ σ ≥ 0.0084 eÅ^{-2}) and the HBA region (σ > 0.0084 eÅ^{-2}). Negative values represent positive polarities and vice-versa. Hence, the elements in the HBD region of a molecule interact or attract elements in the HBA region (another molecule) since they are of opposite polarities. In Figure 4.7, the σ-profiles vary according to the molecular species involved. The similarities between Figures 4.7A and 4.7C; and between 4.7B and 4.7D are striking especially with regards to the position and frequency of the peaks. In Figures 4.7 A and C, the bulk of the peaks reside between the HBD and HBA region; thereby implying a higher propensity for hydrogen bonding for both ternary and binary NADES solvent systems and thus, greater polarity. However, the majority of peaks in Figures 4.7B and D are found in the non-polar region; thus, indicating that DES and phospholipids are characteristically less polar than NADESs. The long hydrophobic fatty acid chains in DAC salts and phospholipids account for the recorded hydrophobicity. Compared to NADESs, phospholipids and DESs σ-profiles' breadth is narrower; this is usually indicative of a less polar character (Figure 4.7B, D) (Mulyono et al., 2014).

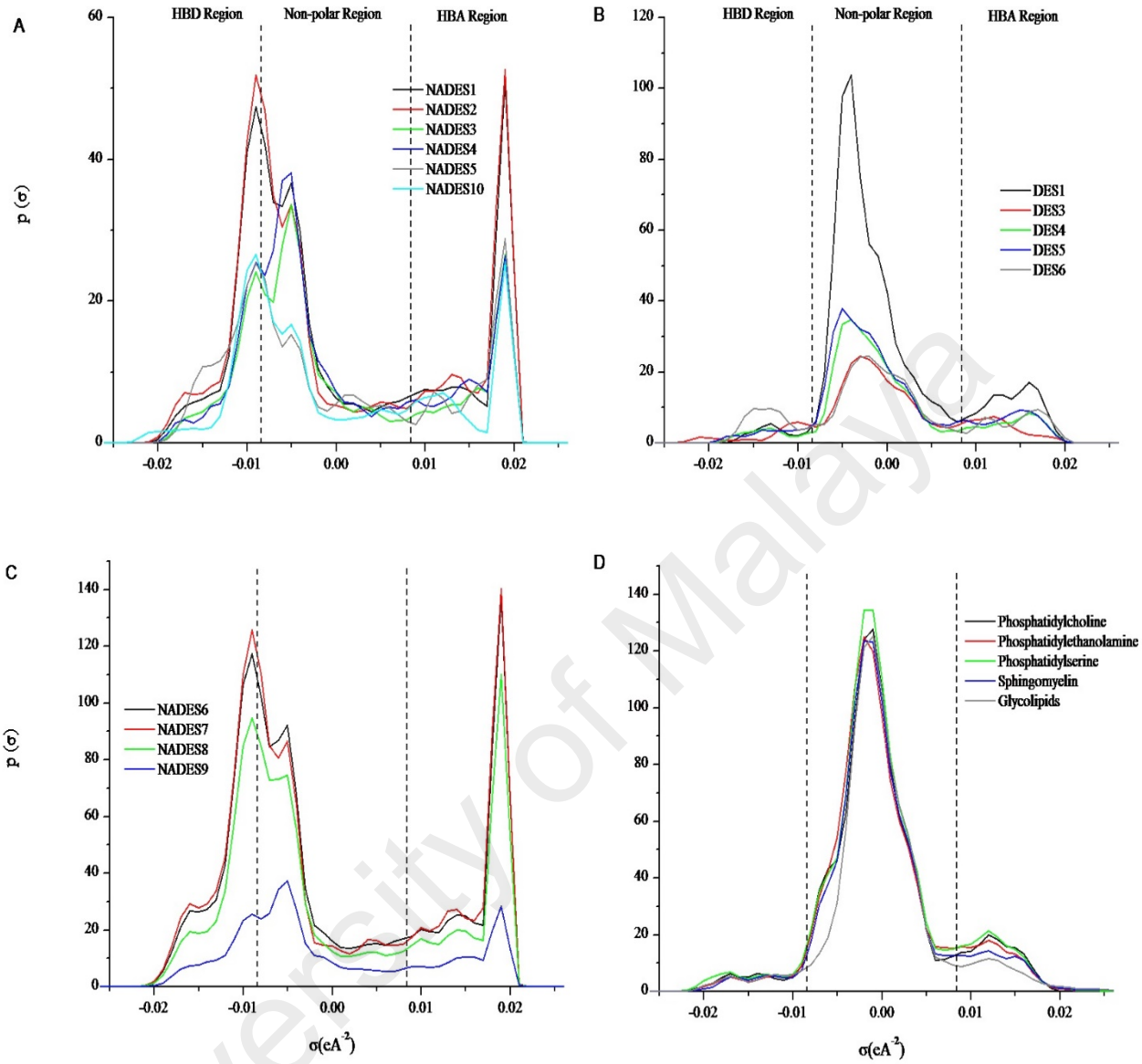


Figure 4.7: σ-profiles of phospholipids NADESs and DESs. A-) Binary NADESs; B-) Binary DESs; C-) Ternary NADESs; D-) Membrane phospholipids.

In σ-profile histograms, peaks indicate the presence of a specific molecule. Herein, only those of relevance will be discussed. For NADES and DES solvent systems alike, the first peaks around -0.015 eÅ^{-2} are associated with the presence of an H^+ atom donor (Figure 4.7A, B and C). This electropositive character stems from the hydroxyl groups of cholinium, ethylene glycol, urea, glycerol, fructose, glucose and malonic acid. The electronegative character detected with the low intensity peaks located between $+0.01 \leq \sigma \leq +0.015$ eÅ^{-2} are associated with the hydrogen bond acceptor capabilities of oxygen atoms, most likely originating from the hydroxyl groups of the HBDs. The third high/low intensity peaks located at +0.019 eÅ^{-2} are associated with Cl^- anions (high for NADESs and low for DES). The final fourth high intensity peaks are located in the non-polar region. They most likely correspond to aliphatic species in the cation alkyl chains, as they are more prominent in DESs' histograms. Compared to NADESs and DESs' profiles, phospholipids (Figure 4.7D) exhibit a significant non-polar character, most likely originating from their long aliphatic chains. This indicates major non-polar attributes. The broad HBD region of the phospholipids between $-0.01 \leq \sigma \leq -0.02$ eÅ^{-2} represent H atoms in the glycol groups of glycerol or in the functional group of the acids. These may interact with the elements in the HBA region (positively charged N^+ atom of ChCl at +0.02 eÅ^{-2}, or the Cl^- atoms of ChCl at +0.015 eÅ^{-2}) of NADESs and DESs (Figure 4.7A, B, C) to form hydrogen bonds. The peaks located between $+0.01 \leq \sigma \leq +0.02$ eÅ^{-2} (Figure 4.7D) represent the negative charge of the O atoms present in the hydroxyl groups of the phospholipids ingredients, namely, phosphate, glycerol, fatty acid. These O atoms may interact to form hydrogen bonds with the H atoms in NADESs that produce the peaks shown at -0.015 eÅ^{-2}. The importance of these interactions is perhaps best reflected with the σ-potentials of Figure 4.8.

The σ-potential represents the interaction behavior and affinities between molecules in a system. On the σ-potential plot, a lower negative value of μ (σ) indicates higher affinity and vice-versa. In Figure 4.8A, the bulk of the histogram is located in the HBD donor region, indicating their preferred affinities. NADES1 and 2 are visibly the strongest nucleophiles among the binary NADESs, thus, they will easily donate protons to electrophiles thereby forming hydrogen bonds far more easily than the other binary NADESs. NADES3, 4 and 5 hold moderate σ values. Hence, their propensity to act as donors is somewhat moderate. However, NADES10 graph is not entirely skewed towards the HBD region. The highest σ registered for NADES10 is ~2. Moreover, non-polar region affinities considerably define this NADES as the most hydrophobic of the binary NADESs and the one least likely to act as a HBD. In Figure 4.8C, binary DESs show a more pronounced affinity for hydrogen bond donor and moderate to low affinities for hydrogen bond acceptors and non-polar regions. Although, the DESs possess comparable negative potentials, DES1 hold the most negative σ value in the HBD region; hence its ability to interact with hydrogen donors is the highest. This implies that DES1 possess the least hydrophobic character of all the tested DESs. The least negative potential in the HBD region belonged to the DES3. Alternatively, DES3 also possessed one of the highest σ value within the non-polar region; thus, this mixture was the most hydrophobic among DAC-based DESs. Ternary NADESs, in contrast, differ slightly from the other systems in their σ-potentials. Judging from the σ values in Figure 4.8B, NADES 6 and 7 possess the highest HBD attributes followed in intensity by NADES8 and then NADES9. The addition of water during synthesis is one of the main difference between the NADES6, 7, 8 and NADES 9. Notwithstanding the individual merits of NADES' unique HBDs, water is known to strengthen DESs' supramolecular structure by inducing stronger hydrogen bond network.

Thus, its addition augments the capabilities of NADES6, 7 and 8 as HBD. When compared to phospholipids (Figure 4.8D), NADES5, NADES6, NADES7 and NADES 8 affinities for HBD (highest σ ~15); although relatively similar for HBA and lower for non-polar regions. It entails that ternary NADESs potential as HBD is higher compared to binary NADESs. The ability to favor hydrogen bonding between NADESs and the bilayer might explain their cellular permeability and thus, their lower cytotoxicity. Figure 4.8D shows the σ-potentials of modelled phospholipids. The phospholipids possess clear and strong affinities for HBD and HBA given the distribution of their histograms. As expected of an amphipathic bilayer, phospholipids can act as both nucleophiles and electrophiles. However, they possess positive high σ and are thus, mostly hydrophobic. In their nonpolar regions, less negative σ values compared with binary NADES were recorded and this results in high interactions with other molecules' nonpolar surfaces. A comparison between DES and NADES' COSMO results requires some basic similarities i.e. number of HBD, chemical nature of HBD. Therefore, a logical comparison should be focused between binary DES and binary NADES prepared using the same HBDs, i.e., urea, glycerol, ethylene glycol and malonic acid. In Figure 4.8, binary NADESs show less negative values than binary DESs, inferring the former's higher hydrophilicity. Interestingly, the increasing trend in σ-values was similar between NADES ([ChCl]-[Urea] < [ChCl]-[ethylene glycol] < [ChCl]-[glycerol] < [ChCl]-[malonic acid]) and DES in most of the HBD region ([DAC]-[urea] < [DAC]-[ethylene glycol] < [DAC]-[glycerol] < [DAC]-[malonic acid]).

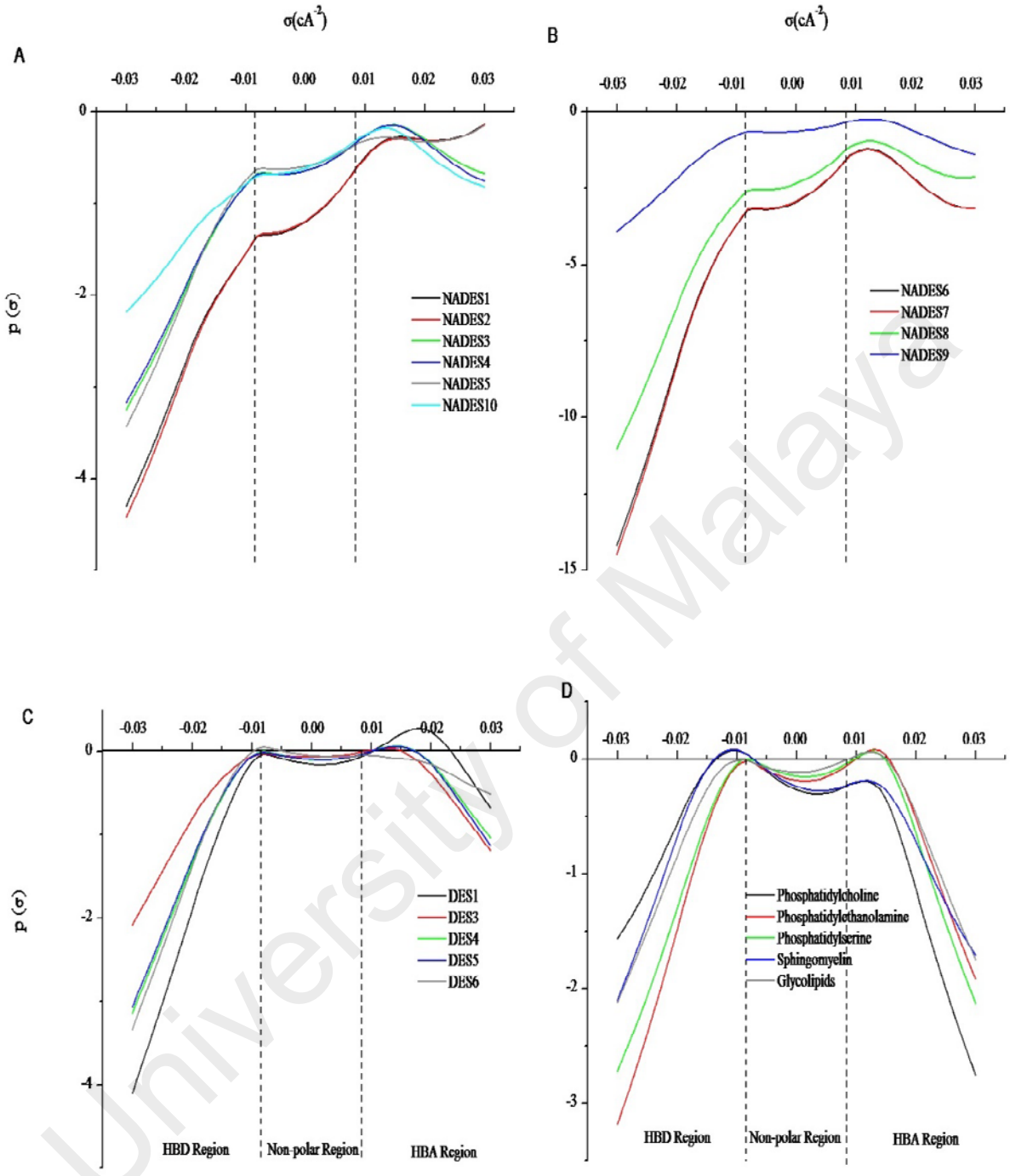


Figure 4.8: σ-potentials of phospholipids NADESs and DESs. A-) Binary NADESs; B-) Ternary NADESs; C-) Binary DESs; D-) Membrane phospholipids.

This suggest that the σ-values, hydrophobicity and affinities of urea, glycerol, ethylene glycol and malonic acid-based NADESs and DESs were also influenced to a great extent by the salt species. Further, the hydrophobic character of DAC-based DESs is evident as the attraction and affinities for non-polar regions are superior to those of ChCl-based NADESs. Several authors confirmed that the increasing permeability of cell membranes following treatment with ILs is mainly a consequence of the intercalation of the latter within the lipid bilayer (Bingham & Ballone, 2012; Hartmann et al., 2015). According to Jing et al. (2016) the hydrophobicity of ILs' ionic species induces the swelling of the lipid bilayer. Although the effect is obvious with long alkyl chain cations, it is more subtle with less hydrophilic anions, such as Cl^-. In fact, Cl^- anions freely disperse in solution and do not interact with the lipid bilayer. In contrast, the swelling becomes critical with increasing alkyl chain cations.

Yoo et al. (2014) study showed that these interactions, dictated by coulomb forces, ensure that the cation tail intercalates with the hydrophobic portion of the membrane whereas the cationic head groups stay near the hydrophilic surface of the bilayer. These aggregations ultimately cause bilayer damage. It appears that the more hydrophobic salt DAC is the most toxic according to the EC_{50} values as it displays the most hydrophobic character from COSMO-RS analysis. Hence, the link between hydrophobicity and cytotoxicity is herein reinforced. According to several reports, ILs exhibit a phenomenon known as "side chain effect" whereby an increase in cationic alkyl chain correlates with higher cytotoxicity (Frade & Afonso, 2010; Latała et al., 2009; Pham et al., 2009; Ranke et al., 2004). "The side chain effect" is the result of the interactions between ILs and cellular membranes, which ultimately culminate in the recorded cytotoxicity. Assumedly, two predominant factors are responsible for ILs cytotoxicity, namely the insertion of ILs ionic species in the lipid bilayer and a threshold ILs dosage concentration (Jeong et al., 2012; Jing et al., 2016).

The incorporation of ILs is dictated by the respective electric charges of ILs ions and the elements of the lipid bilayer. ILs hydrophobic cationic regions insert deep in the bilayer to reach their hydrophobic whereas ILs hydrophilic regions remain close to the bilayer surface where corresponding hydrophilic regions are encountered. This effect is more pronounced for longer cation alkyl chain ILs. ILs anionic species, depending on their hydrophobicity, are located deeper in the hydrophobic regions of the cell membranes. For instance, hydrophobic anions such as NTf^{-2} interact strongly with biological membranes than Cl^- because of their stronger electrostatic attractions. Consequently, a weak hydrophobic anion such as Cl^- typically does not insert in the bilayer (Yoo et al., 2014). ILs accumulation results in the deformation of the bilayer thickness; this can increase the porosity of the membrane and hence ILs' cytotoxicity (Hill et al., 2012).

In DESs, the "side chain effect" is predominant, as the longer alkyl chain ammonium salt was the most toxic when compared to ChCl-based DESs of similar HBDs. Table 4.9 displays the cytotoxic values of the salts' aqueous solutions. As expected, across AGS and MCF-7 cancer cell lines, DAC is the most toxic. Parallels with ILs longer alkyl chain effects are straightforward. Further, although in-depth work in this area is rare, it is hypothesized that DESs species aggregate on the bilayer. In doing so, they perforate cellular membranes, as highlighted by Hayyan et al. (2015) who showed that DESs increase cellular membrane permeability. The findings obtained in this study using COSMO-RS only allow to hypothesize the very basic interactions between DESs and the bilayer because this model is not an exact replica of what is found in membranes, especially in terms of ratio and functional groups occurrence. The various ratio of salts, water and HBDs in both NADESs and DESs as well as the hypothetical ratio of cell membrane phospholipids also account for the resulting fluctuating affinities.

Table 4.9: EC_{50} values of DAC and ChCl salts.

Salt (aqueous)	EC_{50} (mM)	
	AGS	**MCF-7**
DAC	356.7	358.1
ChCl	1957	1478

Phospholipids elements especially consist of a set ratio of functional groups on the cell surface, i.e., carboxyl, phosphate and amino groups. The ratio of these functional groups dictates the entry and the rate of passage of extracellular materials, such as NADESs' species in the intracellular medium as their proportions vary among different cell type. These proportions regulate the diffusion of NADESs and indirectly affect their effect on the cellular machinery. These interactions may correlate with solvent accumulation and aggregation on the cell surface, which ultimately leads to cellular demise through reduced growth. An example of such critical interactions between groups of opposite polarities and affinities was shown by Cornmell et al. (2008). The authors emphasized that the interactions taking place between aqueous quaternary ammonium salts cations, such as cholinium cations and the negatively charged groups present on cell surfaces may lead to the penetration of the latter in the cytoplasm. The consequences range from the loss of membrane integrity to a subsequent demise of the cell through an increased permeability of the cell membrane to exogenous species (Cornmell et al., 2008). The propensity of NADESs/DESs species to permeate cellular membranes allegedly obeys a principle of colloidal biology based on the Hofmeister phenomenon (Vlachy et al., 2009).

An elucidation of the specifics of the principle of affinities between chaotropic and kosmotropic DESs/NADES species and cell surface groups would provide a strong tool for the prediction of the toxicity of these mixtures.

4.4.2 Anticancer potential of NADESs/DESs

The investigation of alternative strategies to combat cancer is one of the current goals of the pharmaceutical industry. The extensive research focused on ILs portrayed mixed reviews with some labeling them as toxic materials. Paracelsus, the father of modern toxicology, once famously said that “the poison is in the dose”, thus suggesting that toxicity could be a desirable feature given that many toxins initially considered poisonous were found to be medically important. The extrapolation towards ILs accentuated research in their potential as anticancer, anti-viral, antimicrobial and as other types of biomedical agents (Kumar & Malhotra, 2009). For instance, some studies emphasized that the chemical nature of ILs predispose their usage as constituents of active pharmaceutical ingredients (APIs) (Ferraz et al., 2011). ILs can also be used to increase the low solubility and bioavailability of drugs, as well as alleviate drug polymorphism (Marrucho et al., 2014). Their application as anticancer materials revealed interesting results. Kumar & Malhotra (2009) screened representative phosphonium and ammonium-based ILs using leukemia, melanoma and cancers from diverse histologies: lung, colon, kidney, ovary, breast, prostate and central nervous system. The authors selected only the compounds which showed more than 60% of growth inhibition in at least 8 tumor cell lines for further testing. The authors showed that phosphonium-based ILs were more active than ammonium-based ILs. Hence, mixtures designed with quaternary ammonium salts are less efficient as anticancer therapeutic agents. Further, increase in alkyl chain length correlated with higher anti-tumor activity.

A similar conclusion was reached by Bachowska et al. (2012) when they screened the activity of quaternary ammonium and phosphonium salts for their toxic effect on Hela and K562 cancer cell lines, as well as on normal HUVEC cells. Their results showed that phosphonium salts were more potent than ammonium salts against Hela, K562 and HUVEC cells. That is, alkyl-tri-n-butylphosphonium and alkyltriphenylphosphonium halides (Br, I), possessing different lengths of alkyl chains (C1–16), were found to be very active against HeLa and K562 cells, but showed a complementary activity. One of the phosphonium salts used, Tri-n-butyl-n-hexadecylphosphonium bromide, showed an EC_{50} value of approximately 5 µm, whereas cisplatin, a reference compound, had an EC_{50} value of 55 µm. That is the potency of the ILs was 10 times more than the reference drug. The authors also showed that ILs do not induce cell death through apoptosis but rather via an alternative pathway as the level of caspase 3 and 7 activity in Hela cells did not increase compared to control cells or cells treated with staurosporine, an inducer of apoptosis, (Bachowska et al., 2012). As analogues of ILs, only limited work has established DES potential as anticancer materials (Hayyan et al., 2015). Virtually no analysis of the kind has yet been performed on NADESs. The selectivity indexes were calculated using the human hepatic cell line as a normal cell line (WRL-68) according to the following formula:

$$SI = \frac{EC_{50} \text{ for normal cell line}}{EC_{50} \text{ for cancer cell line}}$$

In this study, NADESs anticancer potential was assessed and compared to DESs. The results are shown in Table 4.10.The SI values in Table 4.10 range between $0.24 \leq SI \geq 1.061$ for NADESs and $0.269 \leq SI \geq 1.315$ for DESs. The highest SI value recorded among NADESs belonged to a binary NADES5 ([ChCl]-[Urea]) whereas DES3 ([DAC]-[Malonic acid]) possessed the highest DESs' SI.

Ternary NADESs had on average the lowest SI values. Badisa et al. (2009) stated a SI value of less than 2 indicates general toxicity for a compound. In other words, the cytotoxicity of the examined compound is not specific to any metabolic process in the cell line under scrutiny. Hence, at this molar ratio, none of these mixtures would qualify for further assessment. The cytotoxic values obtained from both NADESs and DESs alike were in the millimolar range whereas cisplatin's inhibitory/effective concentration on Hela cells is around 55 µm. Although the potential of these mixtures cannot be judged from their cytotoxic concentrations, it nonetheless serves as an indication to predict their efficacy compared to authorized drugs. In that regard, the tuneability of DESs/NADESs remains their greatest asset as it opens the door for modifications, i.e., increased lipophilicity imperative to turn these eutectics into acceptable anticancer agents. Interestingly, NADESs showed less potential than DESs given their lower average SI values. Based on their lower SI values, NADESs hold far less promise as anticancer mixtures; possibly because they are material of cellular necessity, thus, they would be primarily beneficial and essential for cell growth. All the more so, if they are synthesized intracellularly as hypothesized earlier. This also provides further arguments, demonstrating that NADESs are less toxic than DESs. On the other hand, DESs are expected to have higher SI values given their non-negligible cytotoxicities.

Although the values in this study do not exceed 1.5, in a previous work, Hayyan et al. (2015) recorded average NADESs and DESs SI values higher than 1.5. The authors used similar ingredients to this study (ChCl, ethylene glycol, glycerol and triethylene glycol), albeit at a different molar ratio for DES mixtures (1:3). They determined the EC_{50} values of different DESs on several cancer cell lines. The DES exhibited significant toxicity to the cancer cells (Table 4.11). The authors ascribed the primary cytotoxic mechanism of DESs to the increased permeability of the cellular membrane after treatment.

Table 4.10: Selectivity index of NADESs/DESs.

DES	Selectivity Index	
	HelaS3	MCF-7
NADES1	0.26	1.05
NADES2	0.36	0.75
NADES3	0.867	0.730
NADES4	0.892	0.520
NADES5	1.061	0.490
NADES6	0.55	0.77
NADES7	0.49	0.48
NADES8	0.39	0.43
NADES9	0.28	0.26
NADES10	0.41	0.24
DES1	0.867	0.730
DES2	0.907	0.965
DES3	1.315	0.269
DES4	0.902	0.860
DES5	1.000	1.110
DES6	1.030	0.740

MCF-7 cells were treated with the DESs and stained with a green membrane permeability dye and the nucleus was stained with the blue Hoechst 333258 dye (Figure 4.9). The control cells that were unaffected by the DESs showed less permeability to the dye in the cytosol and, hence, less green fluorescence in Figure 4.9. In contrast, cells treated with the DESs showed increasing green fluorescence, indicative of extended damage to the cell membranes.

Table 4.11: EC_{50} values of DESs for normal and cancer cell lines (Hayyan et al., 2015).

Solvent	EC_{50} (μg mL^{-1})						
	OKF 6	**MCF-7**	**PC3**	**A375**	**HepG2**	**HT29**	**H413**
[ChCl]-[Gly]	47.26	21.86	30.65	18.07	36.08	28.44	54.67
[ChCl]-[EG]	69.71	27.02	32.88	35.23	24.74	30.54	56.60
[ChCl]-[U]	81.93	29.37	27.78	59.61	37.71	36.21	68.02
[ChCl]-[TEG]	34.38	16.09	20.32	12.29	18.07	17.42	19.29

Gly: Glycerol; **EG:** Ethylene glycol; **U:** Urea; **TEG:** Triethylene glycol

The cells also experienced necrosis, as highlighted by the blue fluorescence in Figure 4.9. That is, the control cells showed a higher blue fluorescence characteristic of cell fluency whereas the treated cells diminished in number, as highlighted by the lower blue fluorescence. Furthermore, this increasing permeability delivered DESs species to the cytosol and triggered a massive redox imbalance, as shown in Figure 4.9. MCF-7 cells stained with the dihydroethidium dye emitted higher-intensity fluorescence for treated cells than for control cells. This implied that the DESs preferentially exerted their cytotoxicity by triggering redox stress. Another cytotoxic mechanism for DES or NADESs could be based on lipid bilayer aggregation, as suggested earlier. The DESs also exhibited a potential as anti-cancer agents. An examination of the selectivity index of these DESs, as shown in Table 4.12, revealed slightly higher EC_{50} values for normal cell lines, indicating that the DESs were more toxic to cancer cells than to normal cells. On average, the selectivity index was greater than 1.5. In Table 4.12, the NADESs consisting of glycerol, urea and ethylene glycol all possess SI values higher than 2, implying specific toxicity to MCF-7 or to an inherent metabolic process indigenous to that particular cell.

Table 4.12: Selectivity index of various DESs for cancer cells using the human oral keratinocyte cell line (Hayyan et al., 2015).

Solvent (1:3)	Selectivity Index					
	MCF-7	PC3	A375	HepG2	HT29	H413
[ChCl]-[Gly]	2.162	1.542	2.615	1.309	1.662	0.864
[ChCl]-[EG]	2.579	2.120	1.979	2.812	2.282	1.232
[ChCl]-[U]	2.789	2.949	1.374	2.172	2.263	1.204
[ChCl]-[TEG]	2.137	1.692	2.797	1.902	1.974	1.782

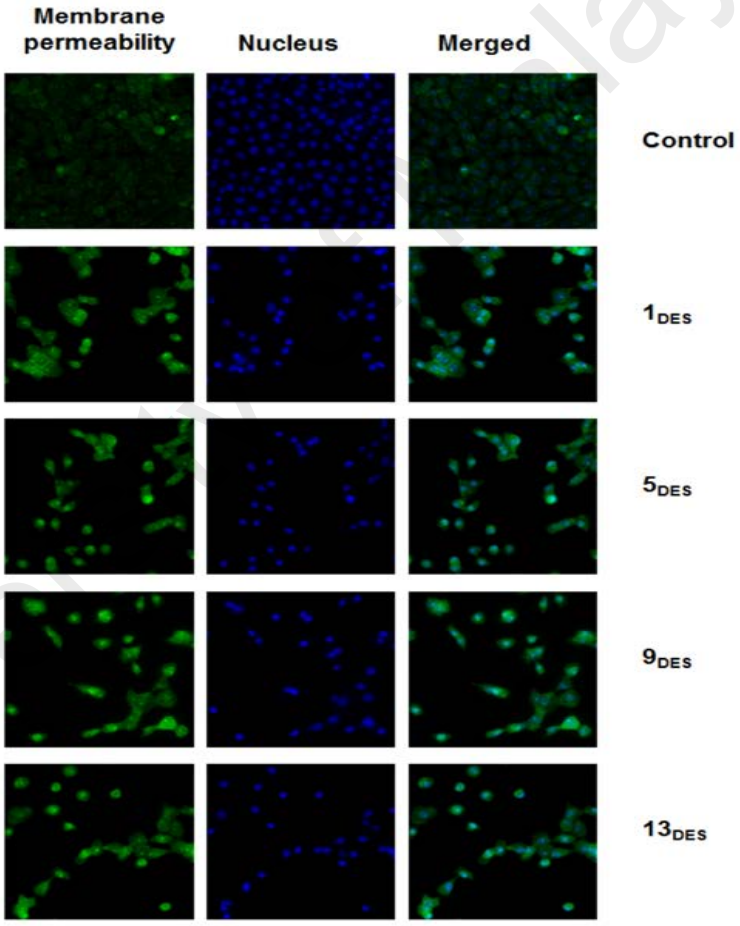


Figure 4.9: Membrane permeability assessment of MCF-7 cells following DESs treatment. Cells were stained with green membrane permeability dye and nucleus were stained with blue Hoechst 333258 dye. 1DES: [ChCl]-[Gly]; 2DES: [ChCl]-[EG]; 9DES: [ChCl]-[U]; 13DES: [ChCl]-[TEG]. ChCl: Choline chloride; Gly: Glycerol; EG: Ethylene glycol; U: Urea; TEG: Triethylene glycol (Hayyan et al., 2015).

Perhaps, an analysis of the effect of the DESs on cancer-enhancing factors will provide a better route for the design of solvents with potent activity against these types of cells. Nevertheless, the investigation of novel mixtures of NADESs/DESs is needed because, based on the results in Table 4.10 and 4.12, discovering such DESs is a possibility.

CHAPTER 5: CONCLUSION & RECOMMENDATIONS

5.1 Conclusion

Generally, the characteristics of NADESs/DESs are superior to those of traditional solvents. The complexation that results in the formation of these neoteric solvents has the ability to enhance features that are desired and required for the 21st century green processes. In coupling the significant characteristics of NADESs/DESs with the success achieved in recent studies in which they have been used as media, solvents/co-solvents or as catalysts for various biological processes, it seems clear that they possess enormous potential for beneficial applications in the future. Their low melting points and thermal stability foreshadows their upcoming importance as solvents for green industries. Their stabilizing effect on biomaterials implies that they can be incorporated in various biomolecular kits. Their potential as antibactericidal agents shows promise for their use as novel therapeutic agents. These solvents can be used as common fluids in molecular biology laboratories because they are easy to prepare and easy to store. DESs and NADESs are also expected to play a pivotal role in nano-medicine. That said, they still carry a lot of uncertainty regarding their toxicity, their chemical inertness and their non-requirement for purification. Numerous publications have enthusiastically provided a not-so-accurate description of DESs by promoting their greenness and safe usage. Although this might not be completely inaccurate, the fact that reports pointing to their toxicity have emerged should accentuate the pressure on the scientific community to engage in a full and comprehensive characterization of these promising mixtures. The safe label imposed on DESs has recently been extrapolated to NADESs, in spite of the rarity of cytotoxic analyses on the latter.

Ultimately, the status of DESs and NADESs as green solvents depends on an extensive characterization of their intrinsic properties. This study represents one of the very first attempts to fully characterize the cytotoxicity of NADESs *in vitro and in vivo*. It also demonstrates the importance of viscosity for NADESs' cytotoxic profile. Further, it represents the first attempt to dwell in the previously unexplored aspects of colloidal biology in which were theorized the cellular interactions between NADESs species and the lipid bilayer.

- Across all understudied cells, both ternary and binary NADESs proved to be less cytotoxic than the examined binary DESs. This was most likely due to the nature of their raw materials. Judging by their high cellular tolerance, the synthesis of NADESs intracellularly must be thoroughly investigated in order to understand their purposes. As it stands, at the very least for gastric cell lines - AGS, NADESs are not just synthetic materials but rather innate liquid phases, essential for metabolic activities.

- In terms of mechanism, an increasing influx of oxidant species is seemingly not the only pathway by which NADESs instill cellular necrosis. Nevertheless, *in vivo*, the higher toxicity of NADESs relative to the DES can be attributed to the viscosity of the material as well to the overall threshold concentrations, which are often lethal. Designing materials of lower viscosity might help solve this conundrum. Viscosities can be adjusted by adding water and/or co-solvents as well as by shuffling HBD species. In terms of physical characteristics, NADESs are similar to DESs. They exhibit high viscosities, poor conductivities and malleable densities at room

temperature. These characteristics are determined by the strong hydrogen networks holding together their supramolecular structures.

- Loosening this network brings about ideal conditions for the industrial use of these solvents. Changes in temperatures alter this network but so does the inclusion of water as a tertiary component.

- This study also emphasized the significant role of HBDs with regards to NADESs cytotoxic profiles. The use of biomaterials appears to be an important asset for lowering their cytotoxicity as organic acids should be used with caution given that they enhance the deleterious attributes of NADESs.

- Comparing NADESs to DESs established NADESs' negligible cytotoxicity. This cytotoxicity was influenced notably by the length of the salt's cation. This is significant not only because it highlights the importance of NADESs' natural constituents, but this confirms that cells tolerate NADESs to a far more extent than DESs. Thus, it adds more weight to the suggestion that NADESs are intracellularly synthesized. Moreover, it also explains the high solubility of various drugs and other cellular metabolite in NADESs recorded in previous work.

- The first-time application of COSMO-RS computational approach to cytotoxic studies proposed a hypothetical cytotoxic mechanism for NADESs mostly based on cellular aggregation. NADESs preferentially enter the cytosol following polar interactions with phospholipids head-groups; whereas, as a result of the "side chain

effect" and the random origin of their constituents, DESs immerse themselves in tail to tail hydrophobic interactions which leads to aggregation and subsequent necrosis. Thus, NADESs once again appear to be less toxic than DESs and the cytotoxicity recorded following their use might just be a case of threshold concentrations.

Although further assessment is needed to draw a comprehensive picture of the cytotoxicity mechanism of these neoteric mixtures, the results obtained in this work are encouraging with regards to their safety.

5.2 Recommendations

The prospects of NADESs in this study are encouraging with respect to several other applications and thus deserve further exploration.

- It would be relevant in the near future to investigate the full potential of NADESs in the study of metabolic and physiological processes. The evidence points to a natural synthesis of these materials in mammalian cells. If that is indeed confirmed, NADESs would go a long way to explain metabolic processes otherwise mysterious, such as the intracellular dissolution of weakly polar substances. Hence, it is imperative to shed the light on their importance in the cell as well as their intracellular functions.
- The anticancer potential of NADESs presented with opportunities which can be translated into reality if further screening continues. Therefore, it is important to continue to evaluate the potential of these mixtures by using their versatility to devise new mixtures. Further, the potential of NADESs as antiviral, or antibacterial or as therapeutic agents for other diseases should be investigated.

- The cytotoxicity studies of NADESs proved that they affect the cellular machinery by inducing redox stress and that their entry is facilitated by an increase in membrane permeability. The effect of these mixtures was only evaluated on fully grown cells.
- An assessment of NADESs effect on different mitotic stages as well as determining if they possess cytostatic potential.
- The stabilizing effect on NADESs on biomaterials must also be investigated as it could determine their use in various biomolecular kits.

REFERENCES

Abbott, A. P., Barron, J. C., Ryder, K. S., & Wilson, D. (2007). Eutectic-Based Ionic Liquids with Metal-Containing Anions and Cations. Chemistry – A European Journal, 13(22), 6495-6501.

Abbott, A. P., Boothby, D., Capper, G., Davies, D. L., & Rasheed, R. K. (2004a). Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids. Journal of American Chemical Society, 126(29), 9142–9147.

Abbott, A. P., Capper, G., Davies, D. L., & Rasheed, R. K. (2004b). Ionic Liquid Analogues Formed from Hydrated Metal Salts. Chemistry – A European Journal, 10(15), 3769-3774.

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Tambyrajah, V. (2003). Novel solvent properties of choline chloride/urea mixtures. Chemical Communications (1), 70-71.

Abbott, A. P., Frisch, G., Garrett, H., & Hartley, J. (2011). Ionic liquids form ideal solutions. Chemical Communications, 47, 11876-11878.

Anastas, P., & Eghbali, N. (2010). Green Chemistry: Principles and Practice. Chemical Society Reviews, 39(1), 301-312.

Andersson, M. P., Jensen, J. H., & Stipp, S. L. S. (2013). Predicting pKa for proteins using COSMO-RS. PeerJ, 1, e198.

Anna, & Wypych, G. (2014). 3.3 - Deep eutectic solvents (range). In Anna & G. Wypych (Eds.), Databook of Green Solvents (pp. 85): William Andrew Publishing.

Bachowska, B., Kazmierczak-Baranska, J., Cieslak, M., Nawrot, B., Szczęsna, D., Skalik, J., & Bałczewski, P. (2012). High Cytotoxic Activity of Phosphonium Salts and Their Complementary Selectivity towards HeLa and K562 Cancer Cells: Identification of Tri-n-butyl-n-hexadecylphosphonium bromide as a Highly Potent Anti-HeLa Phosphonium Salt. ChemistryOpen, *1*(1), 33-38.

Badisa, R. B., Darling-Reed, S. F., Joseph, P., Cooperwood, J. S., Latinwo, L. M., & Goodman, C. B. (2009). Selective Cytotoxic Activities of Two Novel Synthetic Drugs on Human Breast Carcinoma MCF-7 Cells. Anticancer Research, *29*(8), 2993-2996.

Bingham, R. J., & Ballone, P. (2012). Computational Study of Room-Temperature Ionic Liquids Interacting with a POPC Phospholipid Bilayer. The Journal of Physical Chemistry B, 116(36), 11205-11216.

Bose, T., & Chakraborti, A. S. (2008). Fructose-induced structural and functional modifications of hemoglobin: Implication for oxidative stress in diabetes mellitus. Biochimica et Biophysica Acta (BBA) - General Subjects, 1780(5), 800-808.

Brennecke, J. F., & Maginn, E. J. (2001). Ionic Liquids: Innovative Fluids for Chemical Processing. AIChE, 47(11), 2384 - 2389.

Broering, J. M., & Bommarius, A. S. (2005). Evaluation of Hofmeister Effects on the Kinetic Stability of Proteins. The Journal of Physical Chemistry B, 109(43), 20612-20619.

Bucala, R., Makita, Z., Koschinsky, T., Cerami, A., & Vlassara, H. (1993). Lipid advanced glycosylation: pathway for lipid oxidation in vivo. Proceedings of the National Academy of Sciences, 90(14), 6434-6438.

Buggert, M., Cadena, C., Mokrushina, L., Smirnova, I., Maginn, E. J., & Arlt, W. (2009). COSMO-RS Calculations of Partition Coefficients: Different Tools for Conformation Search. Chemical Engineering & Technology, 32(6), 977-986.

Bunn, H. F., & Higgins, P. J. (1981). Reaction of monosaccharides with proteins: possible evolutionary significance. Science, 213(4504), 222-224.

Bunnell, T. M., Burbach, B. J., Shimizu, Y., & Ervasti, J. M. (2011). β-Actin specifically controls cell growth, migration, and the G-actin pool. Molecular Biology of the Cell, 22(21), 4047-4058.

Byrne, F. P., Jin, S., Paggiola, G., Petchey, T. H. M., Clark, J. H., Farmer, T. J., Sherwood, J. (2016). Tools and techniques for solvent selection: green solvent selection guides. Sustainable Chemical Processes, 4(1), 1-24.

Cardellini, F., Germani, R., Cardinali, G., Corte, L., Roscini, L., Spreti, N., & Tiecco, M. (2015). Room temperature deep eutectic solvents of (1S)-(+)-10-camphorsulfonic acid and sulfobetaines: hydrogen bond-based mixtures with low ionicity and structure-dependent toxicity. RSC Advances, 5(40), 31772-31786.

Cardellini, F., Tiecco, M., Germani, R., Cardinali, G., Corte, L., Roscini, L., & Spreti, N. (2014). Novel zwitterionic deep eutectic solvents from trimethylglycine and carboxylic acids: characterization of their properties and their toxicity. RSC Advances, 4(99), 55990-56002.

Charrez, B., Qiao, L., & Hebbard, L. (2015). The role of fructose in metabolism and cancer. Hormone Molecular Biology and Clinical Investigation, (22), 79-90.

Chauvin, Y., Mussmann, L., & Olivier, H. (1996). A Novel Class of Versatile Solvents for Two-Phase Catalysis: Hydrogenation, Isomerization, and Hydroformylation of Alkenes Catalyzed by Rhodium Complexes in Liquid 1,3-Dialkylimidazolium Salts. Angewandte Chemie International Edition in English, 34(23-24), 2698-2700.

Chen, H.-J. C., & Chen, Y.-C. (2009). Analysis of glyoxal-induced DNA cross-links by capillary liquid chromatography nanospray ionization tandem mass spectrometry. Chemical Research in Toxicology, 22(7), 1334-1341.

Chen, L., Sharifzadeh, M., Mac Dowell, N., Welton, T., Shah, N., & Hallett, J. P. (2014). Inexpensive ionic liquids: [HSO_4]-based solvent production at bulk scale. Green Chemistry, 16(6), 3098-3106.

Choi, Y. H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I. W. C. E., Verpoorte, R. (2011). Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology? Plant Physiology, 156(4), 1701-1705.

Clark, J. H., & Tavener, S. J. (2007). Alternative Solvents: Shades of Green. Organic Process Research & Development, 11(1), 149-155.

Coleman, D., & Gathergood, N. (2010). Biodegrability of Ionic Liquids. Chemical Society Reviews, 39, 600-637.

Cornmell, R. J., Winder, C. L., Tiddy, G. J. T., Goodacre, R., & Stephens, G. (2008). Accumulation of ionic liquids in Escherichia colicells. Greem Chemistry, 10(8), 836-841.

Crawford, D. E., Wright, L. A., James, S. L., & Abbott, A. P. (2016). Efficient continuous synthesis of high purity deep eutectic solvents by twin screw extrusion. Chemical Communications, 52(22), 4215-4218.

Curzons, A. D., Constable, D. C., & Cunningham, V. L. (1999). Solvent selection guide: a guide to the integration of environmental, health and safety criteria into the selection of solvents. Clean Products and Processes, 1(2), 82-90.

Cvjetko Bubalo, M., Vidović, S., Radojčić Redovniković, I., & Jokić, S. (2015). Green solvents for green technologies. Journal of Chemical Technology and Biotechnology, 90(9), 1631-1639.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Ionic Liquids and Deep Eutectic Solvents in Natural Products Research: Mixtures of Solids as Extraction Solvents. Journal of Natural Products, 76, 10400-10409.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. Analytica Chimica Acta, 766(0), 61-68.

Dai, Y., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2015). Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. Food Chemistry, 187(0), 14-19.

Damaghi, M., Wojtkowiak, J. W., & Gillies, R. J. (2013). pH sensing and regulation in cancer. Frontiers in Physiology, 4, 370-380.

Davey, R. J., Garside, J., Hilton, A. M., McEwan, D., & Morrison, J. W. (1995). Purification of molecular mixtures below the eutectic by emulsion crystallization. Nature, 375(6533) 664-666.

de Morais, P., Gonçalves, F., Coutinho, J. A. P., & Ventura, S. P. M. (2015). Ecotoxicity of Cholinium-Based Deep Eutectic Solvents. ACS Sustainable Chemistry & Engineering, 3(12), 3398-3404.

Deetlefs, M., & Seddon, K. R. (2010). Assessing the greenness of some typical laboratory ionic liquid preparations. Green Chemistry, 12(1), 17-30.

Deliconstantinos, G., Villiotou, V., & Stavrides, J. C. (1995). Modulation of particulate nitric oxide synthase activity and peroxynitrite synthesis in cholesterol enriched endothelial cell membranes. Biochemical Pharmacology, 49(11), 1589-1600.

Diedenhofen, M., & Klamt, A. (2010). COSMO-RS as a tool for property prediction of IL mixtures—A review. Fluid Phase Equilibria, 294(1–2), 31-38.

Dobrzynska, I., Skrzydlewska, E., & Figaszewski, Z. (2006). Parameters characterizing acid–base equilibria between cell membrane and solution and their application to monitoring the effect of various factors on the membrane. Bioelectrochemistry, 69(2), 142-147.

Domínguez de María, P., Yang, Z., Kohlmann, C., Greiner, L., Lozano, P., García-Verdugo, E.,Noto, R. (2012). Ionic Liquids In Biotransformations and Organocatalysis: Solvents and Beyond (P. D. d. María Ed. 1 ed.). New Jersey-U.S.A, Canada: John Wiley & Sons, Inc., Hoboken

Dong, K., Zhang, S., Wang, D., & Yao, X. (2006). Hydrogen Bonds in Imidazolium Ionic Liquids. The Journal of Physical Chemistry A, 110(31), 9775-9782.

Durand, E., Lecomte, J., Baréa, B., Piombo, G., Dubreucq, E., & Villeneuve, P. (2012). Evaluation of deep eutectic solvents as new media for Candida Antarctica B lipase catalyzed reactions. Process Biochemistry, 47(12), 2081-2089.

e Silva, F. A., Siopa, F., Figueiredo, B. F. H. T., Gonçalves, A. M. M., Pereira, J. L., Gonçalves, F., Ventura, S. P. M. (2014). Sustainable design for environment-friendly mono and dicationic cholinium-based ionic liquids. Ecotoxicology and Environmental Safety, 108(0), 302-310.

Egorova, K. S., Seitkalieva, M. M., Posvyatenko, A. V., & Ananikov, V. P. (2015). An unexpected increase of toxicity of amino acid-containing ionic liquids. Toxicology Research, 4(1), 152-159.

Escribá, P. V., González-Ros, J. M., Goñi, F. M., Kinnunen, P. K. J., Vigh, L., Sánchez-Magraner, L., Barceló-Coblijn, G. (2008). Membranes: a meeting point for lipids, proteins and therapies. Journal of Cellular and Molecular Medicine, 12(3), 829-875.

Fernandez-Gomez, F. J., Galindo, M. F., Gómez-Lázaro, M., Yuste, V. J., Comella, J. X., Aguirre, N., & Jordán, J. (2005). Malonate induces cell death via mitochondrial potential collapse and delayed swelling through an ROS-dependent pathway. British Journal of Pharmacology, 144(4), 528-537.

Florindo, C., Oliveira, F. S., Rebelo, L. P. N., Fernandes, A. M., & Marrucho, I. M. (2014). Insights into the Synthesis and Properties of Deep Eutectic Solvents Based on Cholinium Chloride and Carboxylic Acids. ACS Sustainable Chemistry & Engineering, 2, 2416−2425.

Ferraz, R., Branco, L. C., Prudêncio, C., Noronha, J. P., & Petrovski, Ž. (2011). Ionic Liquids as Active Pharmaceutical Ingredients. ChemMedChem, 6(6), 975-985.

Frade, R. F. M., & Afonso, C. A. M. (2010). Impact of ionic liquids in environment and humans: An overview. Human and Experimental Toxicology, 29(12), 1038-1054.

Francisco, M., van den Bruinhorst, A., & Kroon, M. C. (2013). Low-Transition-Temperature Mixtures (LTTMs): A New Generation of Designer Solvents. Angewandte Chemie International Edition, 52(11), 3074-3085.

Gal, N., Malferarri, D., Kolusheva, S., Galletti, P., Tagliavini, E., & Jelinek, R. (2012). Membrane interactions of ionic liquids: Possible determinants for biological activity and toxicity. Biochimica et Biophysica Acta, 1818, 2967-2974.

Gill, I., & Vulfson, E. (1994). Enzymatic catalysis in heterogeneous eutectic mixtures of substrates. Trends in Biotechnology, 12(4), 118-122.

Gomes, R., Liteplo, R., & Meek, M. E. (2002). Ethylene Glycol: Human Health Aspects. In United Nations Environment Programme, International Labour Organization & W. H. Organization (Eds.), Concise International Chemical Assessment Documents (pp. 44). Stuttgart, Germany: World Health Organization.

Gorke, J., Srienc, F., & Kazlauskas, R. (2010). Toward Advanced Ionic Liquids. Polar, Enzyme-friendly Solvents for Biocatalysis. Biotechnology and Bioprocess Engineering, 15, 40-53.

Greene, J. G., Porter, R. H. P., Eller, R. V., & Greenamyre, J. T. (1993). Inhibition of Succinate Dehydrogenase by Malonic Acid Produces an "Excitotoxic" Lesion in Rat Striatum. Journal of Neurochemistry, *61*(3), 1151-1154.

Gutiérrez, M. C., Ferrer, M. L., Mateo, C. R., & del Monte, F. (2009). Freeze-Drying of Aqueous Solutions of Deep Eutectic Solvents: A Suitable Approach to Deep Eutectic Suspensions of Self-Assembled Structures. Langmuir, 25(10), 5509-5515.

Hallett, J. P., & Welton, T. (2011). Room-Temperature Ionic Liquids: Solvents for Synthesis and Catalysis 2. Chemical Reviews, 111, 1368-1375.

Hartmann, D. O., Shimizu, K., Siopa, F., Leitao, M. C., Afonso, C. A. M., Canongia Lopes, J. N., & Silva Pereira, C. (2015). Plasma membrane permeabilisation by ionic liquids: a matter of charge. Green Chemistry, *17*(9), 4587-4598.

Hayyan, A., Ali Hashim, M., Mjalli, F. S., Hayyan, M., & AlNashef, I. M. (2013a). A novel phosphonium-based deep eutectic catalyst for biodiesel production from industrial low grade crude palm oil. Chemical Engineering Science, 92(0), 81-88.

Hayyan, A., Hashim, M. A., Hayyan, M., Mjalli, F. S., & AlNashef, I. M. (2014). A new processing route for cleaner production of biodiesel fuel using a choline chloride based deep eutectic solvent. Journal of Cleaner Production, 65, 246-251

Hayyan, A., Mjalli, F. S., AlNashef, I. M., Al-Wahaibi, T., Al-Wahaibi, Y. M., & Hashim, M. A. (2012). Fruit sugar-based deep eutectic solvents and their physical properties. Thermochimica Acta, 541(0), 70-75.

Hayyan, M., Hashim, M. A., Al-Saadi, M. A., Hayyan, A., AlNashef, I. M., & Mirghani, M. E. S. (2013b). Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. Chemosphere, 93, 455–459.

Hayyan, M., Hashim, M. A., Hayyan, A., Al-Saadi, M. A., AlNashef, I. M., Mirghani, M. E. S., & Saheed, O. K. (2013c). Are deep eutectic solvents benign or toxic? Chemosphere, 90, 2193–2195.

Hayyan, A., Mjalli, F. S., AlNashef, I. M., Al-Wahaibi, Y. M., Al-Wahaibi, T., & Hashim, M. A. (2013d). Glucose-based deep eutectic solvents: Physical properties. Journal of Molecular Liquids, 178(0), 137-141.

Hayyan, M., Looi, C. Y., Hayyan, A., Wong, W. F., & Hashim, M. A. (2015). In Vitro and In Vivo Toxicity Profiling of Ammonium-Based Deep Eutectic Solvents. PloS One, 10(2), e0117934.

Hayyan, M., Mjalli, F. S., Hashim, M. A., & AlNashef, I. M. (2010). A novel technique for separating glycerine from palm oil-based biodiesel using ionic liquids. Fuel Processing Technology, 91(1), 116-120.

Helou, C., Marier, D., Jacolot, P., Abdennebi-Najar, L., Niquet-Léridon, C., Tessier, F. J., & Gadonna-Widehem, P. (2013). Microorganisms and Maillard reaction products: a review of the literature and recent findings. Amino Acids, 46(2), 267-277.

Herold, D. A., Keil, K., & Bruns, D. E. (1989). Oxidation of polyethylene glycols by alcohol dehydrogenase. Biochemical Pharmacology, 38(1), 73-76.

Hill, E. H., Stratton, K., Whitten, D. G., & Evans, D. G. (2012). Molecular Dynamics Simulation Study of the Interaction of Cationic Biocides with Lipid Bilayers: Aggregation Effects and Bilayer Damage. Langmuir, *28*(42),14849-14854.

Hosoya, N., & Kawada, N. (1958). Malonate Metabolism In Human Placenta The Journal of Biochemistry, 45(5), 363-365.

Hosoya, N., Kawada, N., & Matsumura, Y. (1960). Regulatory Effect Of Malonate On Glucose Metabolism In Human Earlier Placenta. The Journal of Biochemistry, 48(2), 262-266.

Huang, Z.-L., Wu, B.-P., Wen, Q., Yang, T.-X., & Yang, Z. (2014). Deep eutectic solvents can be viable enzyme activators and stabilizers. Journal of Chemical Technology and Biotechnology, 89, 1975–1981.

Hunt, P. A., Ashworth, C. R., & Matthews, R. P. (2015). Hydrogen bonding in ionic liquids. Chemical Society Reviews, 44(5), 1257-1288.

Instruments, D. (2006). Surface tension values of some common test liquids for surface energy analysis. Retrieved 12/1/2015, 2015, from www.surface-tension.de

Iolitec. (2011). Ionic Liquids Today. In T. J. S. Schubert (Ed.), (1 ed., Vol. 11, pp. 25). Germany.

Jenkins, H. D. B., & Marcus, Y. (1995). Viscosity B-Coefficients of Ions in Solution. Chemical Reviews, 95(8), 2695-2724.

Jeong, S., Ha, S. H., Han, S.-H., Lim, M.-C., Kim, S. M., Kim, Y.-R., Jeon, T.-J. (2012). Elucidation of molecular interactions between lipid membranes and ionic liquids using model cell membranes. Soft Matter, *8*(20), 5501-5506.

Jeong, K. M., Lee, M. S., Nam, M. W., Zhao, J., Jin, Y., Lee, D.-K., Lee, J. (2015). Tailoring and recycling of deep eutectic solvents as sustainable and efficient extraction media. Journal of Chromatography A, 1424, 10-17.

Jessop, P. G. (2011). Searching for green solvents. Green Chemistry, 13(6), 1391-1398.

Jibril, B., Mjalli, F., Naser, J., & Gano, Z. (2014). New tetrapropylammonium bromide-based deep eutectic solvents: Synthesis and characterizations. Journal of Molecular Liquids, 199(0), 462-469.

Jing, B., Lan, N., Qiu, J., & Zhu, Y. (2016). Interaction of Ionic Liquids with a Lipid Bilayer: A Biophysical Study of Ionic Liquid Cytotoxicity. The Journal of Physical Chemistry B, 120(10), 2781-2789.

Juneidi, I., Hayyan, M., & Hashim, M. A. (2015). Evaluation of toxicity and biodegradability for cholinium-based deep eutectic solvents. RSC Advances., 5(102), 83636-83647.

Juneidi, I., Hayyan, M., & Mohd Ali, O. (2016). Toxicity profile of choline chloride-based deep eutectic solvents for fungi and Cyprinus carpio fish. Environmental Science and Pollution Research, 23(8), 7648-7659.

Kasai, H., Iwamoto-Tanaka, N., & Fukada, S. (1998). DNA modifications by the mutagen glyoxal: adduction to G and C, deamination of C and GC and GA cross-linking. Carcinogenesis, 19(8), 1459-1465.

Kerton, F. M. (2009). Alternative Solvents for Green Chemistry. Royal Society of Chemistry, Thomas Graham House,Science Park, Milton Road, Cambridge, CB4 0WF, UK: RSC Publishing.

Kim, Y.-S. (2002). Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. BMB Reports, 35(5), 443-451.

Klamt, A. (2005). COSMO-RS: From Quantum Chemistry to Fluid Phase Thermodynamics and Drug Design: Elsevier.

Kuimova, M. K., Botchway, S. W., Parker, A. W., Balaz, M., Collins, H. A., Anderson, H. L., Ogilby, P. R. (2009). Imaging intracellular viscosity of a single cell during photoinduced cell death. Nature Chemistry, 1(1), 69-73.

Kumar, A. K., Parikh, B. S., & Pravakar, M. (2016). Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. Environmental Science and Pollution Research, 23(10), 9265-9275.

Kumar, V., & Malhotra, S. V. (2009). Study on the potential anti-cancer activity of phosphonium and ammonium-based ionic liquids. Bioorganic & Medicinal Chemistry Letters, 19(16), 4643-4646.

Kunz, W., & Häckl, K. (2016). The hype with ionic liquids as solvents. Chemical Physics Letters, 661, 6-12.

Kurtoglu, M., & Lampidis, T. J. (2009). From delocalized lipophilic cations to hypoxia: Blocking tumor cell mitochondrial function leads to therapeutic gain with glycolytic inhibitors. Molecular Nutrition & Food Research, 53(1), 68-75.

Laforenza, U., Bottino, C., & Gastaldi, G. (2016). Mammalian aquaglyceroporin function in metabolism. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1858(1), 1-11.

Latała, A., Nedzi, M., & Piotr, S. (2009). Toxicity of imidazolium and pyridinium based ionic liquids towards algae. Chlorella vulgaris, Oocystis submarina (green algae) and Cyclotella meneghiniana, Skeletonema marinoi (diatoms). Green Chemistry, 11, 580-588.

Levine, A. J. p53, the Cellular Gatekeeper for Growth and Division. Cell, 88(3), 323-331.

Li, Z., & Lee, P. I. (2016). Investigation on drug solubility enhancement using deep eutectic solvents and their derivatives. International Journal of Pharmaceutics, 505(1–2), 283-288.

Liew, S. Y., Looi, C. Y., Paydar, M., Cheah, F. K., Leong, K. H., Wong, W. F., Awang, K. (2014). Subditine, a New Monoterpenoid Indole Alkaloid from Bark of Nauclea subdita (Korth.) Steud. Induces Apoptosis in Human Prostate Cancer Cells. PLoS ONE, 9(2), e87286.

Lin, E. C. C. (1977). Glycerol Utilization and its Regulation in Mammals. Annual Review of Biochemistry, *46*(1), 765-795.

Liu, H., & Heaney, A. P. (2011). Refined fructose and cancer. Expert Opinion on Therapeutic Targets, 15(9), 1049-1059.

Liu, H., Huang, D., McArthur, D. L., Boros, L. G., Nissen, N., & Heaney, A. P. (2010). Fructose Induces Transketolase Flux to Promote Pancreatic Cancer Growth. Cancer Research, 70(15), 6368-6376.

Liu, K. J.-M., Drucker, Y., & Jarad, J. (1995). Hepatic Glycerol Metabolism in Tumorous Rats: A 131C Nuclear Magnetic Resonance Study. Cancer Research, 55(4), 761-766.

Liu, Q.-P., Hou, X.-D., Li, N., & Zong, M.-H. (2012). Ionic liquids from renewable biomaterials: synthesis, characterization and application in the pretreatment of biomass. Green Chemistry, 14(2), 304-307.

Liu, T., Liu, X., Spring, D. R., Qian, X., Cui, J., & Xu, Z. (2014). Quantitatively Mapping Cellular Viscosity with Detailed Organelle Information via a Designed PET Fluorescent Probe. Scientific Reports,4, 5418.

Liu, T., Zhu, L., Wang, J., Wang, J., & Tan, M. (2016). Phytotoxicity of imidazolium-based ILs with different anions in soil on Vicia faba seedlings and the influence of anions on toxicity. Chemosphere, 145, 269-276.

Liu, Y., Garzon, J., Friesen, J. B., Zhang, Y., McAlpine, J. B., Lankin, D. C., Pauli, G. F. (2016). Countercurrent assisted quantitative recovery of metabolites from plant-associated natural deep eutectic solvents. Fitoterapia, 112, 30-37.

Lobo, H. R., Singh, B. S., & Shankarling, G. S. (2012). Deep eutectic solvents and glycerol: a simple, environmentally benign and efficient catalyst/reaction media for synthesis of N-aryl phthalimide derivatives. Green Chemistry Letters and Reviews, 5(4), 487-533.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). Molecular Cell Biology. New York, NY: Freeman, W. H.

Looi, C., Moharram, B., Paydar, M., Wong, Y., Leong, K., Mohamad, K., Mustafa, M. (2013). Induction of apoptosis in melanoma A375 cells by a chloroform fraction of Centratherum anthelminticum (L.) seeds involves NF-kappaB, p53 and Bcl-2-controlled mitochondrial signaling pathways. BMC Complementary and Alternative Medicine, 13(1), 1-14.

Ludwig, D. S. (2013). Examining the health effects of fructose. JAMA, 310(1), 33-34.

Lundholm, K., Edström, S., Karlberg, I., Ekman, L., & Schersten, T. (1982). Glucose turnover, gluconeogenesis from glycerol, and estimation of net glucose cycling in cancer patients. Cancer, 50(6), 1142-1150.

Mai, N. L., Ahn, K., & Koo, Y.-M. (2014). Methods for recovery of ionic liquids—A review. Process Biochemistry, 49(5), 872-881.

Marrucho, I. M., Branco, L. C., & Rebelo, L. P. N. (2014). Ionic Liquids in Pharmaceutical Applications. Annual review of chemical and biomolecular engineering, *5*(1), 527-546.

McKennis, H., Turner, R. A., Turnbull, L. B., Bowman, E. R., Muelder, W. W., Neidhardt, M. P., Spencer, S. (1962). The excretion and metabolism of triethylene glycol. Toxicology and Applied Pharmacology, 4(4), 411-431.

Mehrkesh, A., & Karunanithi, A. T. (2015). Life-Cycle Perspectives on Aquatic Ecotoxicity of Common Ionic Liquids. Environmental Science & Technology, 50(13), 6814-6821.

Michel, V., Yuan, Z., Ramsubir, S., & Bakovic, M. (2006). Choline transport for phospholipid synthesis. Experimental Biology and Medicine, 231(5), 490-504.

Mirjafari, A., Pham, L. N., McCabe, J. R., Mobarrez, N., Salter, E. A., Wierzbicki, A., Davis, J. H. (2013). Building a bridge between aprotic and protic ionic liquids. RSC Advances, 3(2), 337-340.

Modica-Napolitano, J. S., & Aprille, J. R. (2001). Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. Advanced Drug Delivery Reviews, 49(1–2), 63-70.

Muhammad, N., Elsheikh, Y. A., Mutalib, M. I. A., Bazmi, A. A., Khan, R. A., Khan, H., . khan, I. (2015). An overview of the role of ionic liquids in biodiesel reactions. Journal of Industrial and Engineering Chemistry, 21, 1-10.

Muller, P. A. J., Vousden, K. H., & Norman, J. C. (2011). p53 and its mutants in tumor cell migration and invasion. The Journal of Cell Biology, 192(2), 209-218.

Mulyono, S., Hizaddin, H. F., Alnashef, I. M., Hashim, M. A., Fakeeha, A. H., & Hadj-Kali, M. K. (2014). Separation of BTEX aromatics from n-octane using a (tetrabutylammonium bromide + sulfolane) deep eutectic solvent - experiments and COSMO-RS prediction. RSC Advances, 4(34), 17597-17606.

Nadiv, O., Shinitzky, M., Manu, H., Hecht, D., Roberts, C. T., LeRoith, D., & Zick, Y. (1994). Elevated protein tyrosine phosphatase activity and increased membrane

viscosity are associated with impaired activation of the insulin receptor kinase in old rats. Biochemical Journal, 298(Pt 2), 443-450.

Oak, J.-H., Nakagawa, K., & Miyazawa, T. (2000). Synthetically prepared Amadori-glycated phosphatidylethanolamine can trigger lipid peroxidation via free radical reactions. FEBS Letters, 481(1), 26-30.

Oliveira, M. V. S., Vidal, B. T., Melo, C. M., de Miranda, R. d. C. M., Soares, C. M. F., Coutinho, J. A. P., Lima, Á. S. (2016). (Eco)toxicity and biodegradability of protic ionic liquids. Chemosphere, 147, 460-466.

Olivier-Bourbigou, H., Magna, L., & Morvan, D. (2002). Ionic liquids and catalysis: Recent progress from knowledge to applications. Applied Catalysis A: General, 373, 1-56.

Owusu-Ansah, E., Yavari, A., & Banerjee, U. (2008). A protocol for in vivo detection of reactive oxygen species. Protocol Exchange (2008) doi:10.1038/nprot.2008.23

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R. L., & Duarte, A. R. C. (2014a). Natural Deep Eutectic Solvents – Solvents for the 21st Century. ACS Sustainable Chemistry & Engineering, 2(5), 1063-1071.

Paiva A., Craveiro R., Martins M., Rocha A., Aroso I. M., Barreiros S., Reis R. L., and Duarte A. R. C., "THEDES? Therapeutic Deep Eutectic Solvents: New solvents for the functionalization of natural-based polymers", 14th European Meeting on Supercritical Fluids, 2014b.

Pandey, A., Rai, R., Pal, M., & Pandey, S. (2014). How polar are choline chloride-based deep eutectic solvents? Physical Chemistry Chemical Physics, 16, 1559-1568.

Pena-Pereira, F., Kloskowski, A., & Namiesnik, J. (2015). Perspectives on the replacement of harmful organic solvents in analytical methodologies: a framework toward the implementation of a generation of eco-friendly alternatives. Green Chemistry, 17(7), 3687-3705.

Pena-Perreira, F., & Namiesnik, J. (2014). Ionic Liquids and Deep Eutectic Mixtures: Sustainable Solvents for Extraction Processes. ChemSusChem Reviews, 1784-1800

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M. A., Arning, J., Stolte, S. (2013). Ecotoxicity and biodegradability of selected protic and aprotic ionic liquids. Journal of Hazardous Materials, 261, 99-105.

Perkins, S. L., Painter, P., & Colina, C. M. (2014). Experimental and Computational Studies of Choline Chloride-Based Deep Eutectic Solvents. Journal of Chemical and Engineering Data, 59(11), 3652-3662.

Petkovic, M., Seddon, K. R., Rebeloa, L. P. N., & Pereira, C. S. (2010). Ionic liquids: a pathway to environmental acceptability. Chemical Society Reviews, 40(20), 1383-1403.

Pham, T. P. T., Cho, C.-W., & Yun, Y.-S. (2009). Environmental fate and toxicity of ionic liquids: A review. Water Research, 44, 352-372.

Pischetsrieder, M., Seidel, W., Münch, G., & Schinzel, R. (1999). N 2-(1-Carboxyethyl) deoxyguanosine, a nonenzymatic glycation adduct of DNA, induces single-strand breaks and increases mutation frequencies. Biochemical and Biophysical Research Communications, 264(2), 544-549.

Plagemaen, P. G. W. (1971). Choline metabolism and membrane formation in rat hepatoma cells grown in suspension culture. III. Choline transport and uptake by simple diffusion and lack of direct exchange with phosphatidylcholine. Journal of Lipid Research, 12(6), 715-724.

Plum, L. M., Rink, L., & Haase, H. (2010). The Essential Toxin: Impact of Zinc on Human Health. International Journal of Environmental Research and Public Health, *7*(4), 1342-1365.

Port, A. M., Ruth, M. R., & Istfan, N. W. (2012). Fructose consumption and cancer: is there a connection? Current Opinion in Endocrinology, Diabetes and Obesity, 19(5), 367-374.

Pun, P. B. L., & Murphy, M. P. (2012). Pathological Significance of Mitochondrial Glycation. International Journal of Cell Biology, 2012, 1-13.

Qi, X.-L., Peng, X., Huang, Y.-Y., Li, L., Wei, Z.-F., Zu, Y.-G., & Fu, Y.-J. (2015). Green and efficient extraction of bioactive flavonoids from Equisetumpalustre L. by deep eutectic solvents-based negative pressurecavitation method combined with macroporous resin enrichment. Industrial Crops and Products, 70, 142–148.

Radošević, K., Ćurko, N., Gaurina Srček, V., Cvjetko Bubalo, M., Tomašević, M., Kovačević Ganić, K., & Radojčić Redovniković, I. (2016). Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. LWT - Food Science and Technology, 73, 45-51.

Radošević, K., Cvjetko Bubalo, M., Gaurina Srček, V., Grgas, D., Landeka Dragičević, T., & Radojčić Redovniković, I. (2015). Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. Ecotoxicology and Environmental Safety, 112(0),46-53.

Radošević, K., Železnjak, J., Cvjetko Bubalo, M., Radojčić Redovniković, I., Slivac, I., & Gaurina Srček, V. (2016). Comparative in vitro study of cholinium-based ionic liquids and deep eutectic solvents toward fish cell line. Ecotoxicology and Environmental Safety, 131, 30-36.

Ranke, J., Mölter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffmann, J., Jastorff, B. (2004). Biological effects of imidazolium ionic liquids with varying chain lengths in acute Vibrio fischeri and WST-1 cell viability assays. Ecotoxicology and Environmental Safety, 58(3), 396-404.

Ravandi, A., Kuksis, A., Marai, L., Myher, J., Steiner, G., Lewisa, G., & Kamido, H. (1996). Isolation and identification of glycated aminophospholipids from red cells and plasma of diabetic blood. FEBS Letters, 381(1-2), 77-81.

Requena, J. R., Ahmed, M. U., Fountain, C. W., Degenhardt, T. P., Reddy, S., Perez, C., Thorpe, S. R. (1997). Carboxymethylethanolamine, a biomarker of phospholipid

modification during the maillard reaction in vivo. Journal of Biological Chemistry, 272(28), 17473-17479.

Riemann, A., Schneider, B., Ihling, A., Nowak, M., Sauvant, C., Thews, O., & Gekle, M. (2011). Acidic Environment Leads to ROS-Induced MAPK Signaling in Cancer Cells. PloS One, 6(7), e22445.

Ru, J., Hua, Y., Xu, C., Li, J., Li, Y., Wang, D., Jie, Y. (2015). Morphology-controlled preparation of lead powders by electrodeposition from different PbO-containing choline chloride-urea deep eutectic solvent. Applied Surface Science, 335(0), 153-159.

Samorì, C., Malferrari, D., Valbonesi, P., Montecavalli, A., Moretti, F., Galletti, P., Pasteris, A. (2010). Introduction of oxygenated side chain into imidazolium ionic liquids: Evaluation of the effects at different biological organization levels. Ecotoxicology and Environmental Safety, 73(6), 1456-1464.

Sanchez-Leija, R. J. J., Pojman, A., Luna-Barcenas, G., & Mota-Morales, J. D. (2014). Controlled release of lidocaine hydrochloride from polymerized drug-based deep-eutectic solvents. Journal of Materials Chemistry B, 2, 7495–7501.

Santos, C. R., & Schulze, A. (2012). Lipid metabolism in cancer. FEBS Journal, 279(15), 2610-2623.

Scurto, A. M., Aki, S. N. V. K., & Brennecke, J. F. (2002). CO2 as a Separation Switch for Ionic Liquid/Organic Mixtures. Journal of the American Chemical Society, 124(35), 10276-10277.

Sedev, R. (2011). Surface tension, interfacial tension and contact angles of ionic liquids. Current Opinion in Colloid & Interface Science, 16(4), 310-316.

Shahbaz, K., Baroutian, S., Mjalli, F. S., Hashim, M. A., & AlNashef, I. M. (2012). Densities of ammonium and phosphonium based deep eutectic solvents: Prediction using artificial intelligence and group contribution techniques. Thermochimica Acta, 527(0), 59-66.

Shahriari, S., Tome, L. C., Araujo, J. M. M., Rebelo, L. P. N., Coutinho, J. A. P., Marrucho, I. M., & Freire, M. G. (2013). Aqueous biphasic systems: a benign route using cholinium-based ionic liquids. RSC Advances, 3(6), 1835-1843.

Shamsuri, A. A., & Abdullah, D. K. (2010). Ionic Liquids: Preparations and Limitations. Makara Sains, 14(2), 101-106.

Siedlecka, E. M., Czerwicka, M., Neumann, J., Stepnowski, P., Fernández, J. F., & Thöming, J. (2011). Ionic Liquids: Methods of Degradation and Recovery. In P. A. Kokorin (Ed.), Ionic Liquids: Theory, Properties, New Approaches: InTech.

Singh, A. S., Shendage, S. S., & Nagarkar, J. M. (2014). Choline chloride based deep eutectic solvent as an efficient solvent for the benzylation of phenols. Tetrahedron Letters, 55(52), 7243-7246

Singh, B., Lobo, H., & Shankarling, G. (2010). Selective N-Alkylation of Aromatic Primary Amines Catalyzed by Bio-catalyst or Deep Eutectic Solvent. Catalysis Letters, 141(1), 178-182.

Smith, E. L., Abbott, A. P., & Ryder, K. S. (2014). Deep Eutectic Solvents (DES) and Their Applications. Chemical Reviews, 114(21), 11060–11082.

Sowmiah, S., Srinivasadesikan, V., Tseng, M.-C., & Chu, Y.-H. (2009). On the Chemical Stabilities of Ionic Liquids Molecules, 14, 3780-3813.

Szachowicz-Petelska, B., Dobrzyńska, I., Sulkowski, S., & Figaszewski, Z. A. (2012). Characterization of the Cell Membrane during Cancer Transformation. In D. R. Ettarh (Ed.), Colorectal Cancer Biology-From Genes to Tumor: InTech.

Tang, B., & Row, K. H. (2013). Recent developments in deep eutectic solvents in chemical sciences. Monatshefte für Chemie - Chemical Monthly, 144(10), 1427-1454.

Tang, S., Baker, G. A., & Zhao, H. (2012). Ether- and Alcohol-Functionalized Task-Specific Ionic Liquids: Attractive Properties and Applications. Chemical Society Reviews, 41(10), 4030-4066.

Ventura, S. P. M., e Silva, F. A., Gonçalves, A. M. M., Pereira, J. L., Gonçalves, F., & Coutinho, J. A. P. (2014). Ecotoxicity analysis of cholinium-based ionic liquids to Vibrio fischeri marine bacteria. Ecotoxicology and Environmental Safety, 102(0), 48-54.

Ventura, S. P. M., Marques, C. S., Rosatella, A. A., Afonso, C. A. M., Gonçalves, F., & Coutinho, J. A. P. (2012). Toxicity assessment of various ionic liquid families towards Vibrio fischeri marine bacteria. Ecotoxicology and Environmental Safety, 76(0), 162-168.

Vlachy, N., Jagoda-Cwiklik, B., Vácha, R., Touraud, D., Jungwirth, P., & Kunz, W. (2009). Hofmeister series and specific interactions of charged headgroups with aqueous ions. Advances in Colloid and Interface Science, 146(1–2), 42-47.

Wagle, D. V., Zhao, H., & Baker, G. A. (2014). Deep Eutectic Solvents: Sustainable Media for Nanoscale and Functional Materials. Accounts of Chemical Research, 47(8), 2299-2308.

Wei, Z.-F., Wang, X.-Q., Peng, X., Wang, W., Zhao, C.-J., Zu, Y.-G., & Fu, Y.-J. (2015). Fast and green extraction and separation of main bioactive flavonoids from Radix Scutellariae. Industrial Crops and Products, 63, 175–181.

Weiner, I. D., Mitch, W. E., & Sands, J. M. (2015). Urea and Ammonia Metabolism and the Control of Renal Nitrogen Excretion. Clinical Journal of the American Society of Nephrology, *10*(8), 1444-1458.

Welton, T. (1999). Room-Temperature Ionic Liquids. Solvents for Synthesis and Catalysis. Chemical Reviews, 99(8), 2071-2084.

Wen, Q., Chen, J.-X., Tang, Y.-L., Wang, J., & Yang, Z. (2015). Assessing the toxicity and biodegradability of deep eutectic solvents. Chemosphere, 132, 63-69.

Wike-Hooley, J. L., Haveman, J., & Reinhold, H. S. (1984). The relevance of tumour pH to the treatment of malignant disease. Radiotherapy and Oncology, 2(4), 343-366.

Wilkes, J. S., & Zaworotko, M. J. (1992). Air and water stable 1-ethyl-3-methylimidazolium based ionic liquids. Journal of the Chemical Society, Chemical Communications (13), 965-967.

Wu, B.-P., Wen, Q., Xu, H., & Yang, Z. (2014). Insights into the impact of deep eutectic solvents on horseradish peroxidase: Activity, stability and structure. Journal of Molecular Catalysis B: Enzymatic, 101, 101– 107.

Wu, W., & Zhao, S. (2013). Metabolic changes in cancer: beyond the Warburg effect. Acta Biochimica et Biophysica Sinica, 45(1), 18-26.

Zeisel, S. H., & da Costa, K.-A. (2009). Choline: an essential nutrient for public health. Nutrition Reviews, 67(11), 615-623.

Zhang, Q., Vigier, K. D. O., Royer, S., & Jerome, F. (2012). Deep eutectic solvents: syntheses, properties and applications. Chemical Society Reviews, 41, 7108-7146.

Zhang, Y., Bakshi, B. R., & Demessie, E. S. (2008). Life Cycle Assessment of an Ionic Liquid versus Molecular Solvents and Their Applications. Environmental Science & Technology, 42(5), 1724-1730.

Zhang, H., Tang, B., & Row, K. H. (2014). A Green Deep Eutectic Solvent-Based Ultrasound-Assisted Method to Extract Astaxanthin from Shrimp Byproducts. Analytical Letters, *47*(5), 742-749.

Zhao, B.-Y., Xu, P., Yang, F.-X., Wu, H., Zong, M.-H., & Lou, W.-Y. (2015). Biocompatible Deep Eutectic Solvents Based on Choline Chloride: Characterization and Application to the Extraction of Rutin from Sophora japonica. ACS Sustainable Chemistry & Engineering, 3(11), 2746-2755.

Zhao, H., & Baker, G. A. (2012). Ionic liquids and deep eutectic solvents for biodiesel synthesis: a review. Journal of Chemical Technology and Biotechnology, 88, 3-12.

Zhao, H., Baker, G. A., & Holmes, S. (2011). New eutectic ionic liquids for lipase activation and enzymatic preparation of biodiesel. Organic & Biomolecular Chemistry, 9(6), 1908-1916.

Yoo, B., Shah, J. K., Zhu, Y., & Maginn, E. J. (2014). Amphiphilic interactions of ionic liquids with lipid biomembranes: a molecular simulation study. Soft Matter, 10(43), 8641-8651.