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Antimicrobial Ionic Liquids

Brendan F. Gilmore
School of Pharmacy, Queen's University Belfast, UK

1. Introduction

First described almost a century ago (Walden, 1914), ionic liquids are a novel class of low temperature (typically <100°C) molten salts, comprised of discrete anions and cations (Seddon, 1997; Scammells et al., 2005; Stark & Seddon, 2007;). Many are liquid at room temperature. The majority of room temperature molten ionic liquids are salts with large nitrogen or phosphorous-bearing cations with alkyl chain substituents and anions such as halides, fluorophosphates, fluoroborates and so on. Over one million simple ionic liquids are theoretically possible, with mixtures of two or more ionic liquids making the possibilities for new reaction media almost limitless. Ionic liquids research has experienced a massive upsurge of interest in the past decade, primarily driven by their application in 'Green' chemistry, for example, as replacements for conventional organic solvents and volatile organic compounds (VOCs) in the chemical industry. Furthermore, ionic liquids have been utilized in multitude of diverse applications from synthetic chemistry (separation/extraction/catalysis) to novel biological applications. The most commonly used and extensively described cations and anions employed in ionic liquids are detailed in **Figure 1 (**adapted from Seddon et al, 2000).

The ability to 'tune' the physical, chemical and biological property sets of ionic liquids, by independent modification of the properties of the constituent anions and cations, has been the major driving force behind the huge interest in this rapidly expanding field of chemistry. 'Tuneability' of ionic liquids introduces an unparalleled flexibility in the design of reagents for a particular functional niche, these 'designer solvents' (Earle et al., 2006) are capable of providing a range of new reaction media potentially having greater diversity of character and application than that of the traditional solvents they are designed to replace (Scammells et al., 2005; Earle et al., 2006; Stark & Seddon, 2007). A summary of the physicochemical properties of common ionic liquids is given in **Table 1**. Whilst the majority of industry in this field has, to date, been directed towards 'green' applications, biological issues such as stability, biodegradability, recyclability and toxicity (Scammells et al., 2005) have received relatively little attention. However, these issues have attracted increased scrutiny recently, and the biological properties of ionic liquids, which in themselves are 'tuneable' have become one of the most debated topics in the ionic liquids arena.

Ionic liquids generally have properties such as near-zero vapour pressure (Earle et al., 2006) and thermal stability (Kosmulski et al., 2004). However, by altering the cation and anion, ionic liquids can be specifically created for a purpose or to possess particular properties suited to a given functional niche, and can therefore be described as tunable or designer

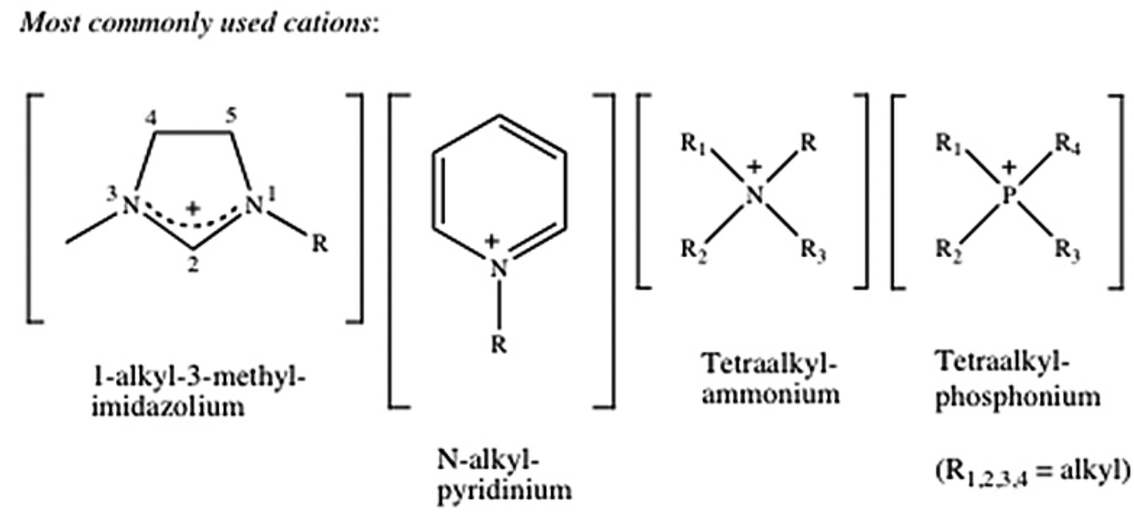


Fig. 1. Examples of the most commonly described ionic liquid cations and anions (Adapted from Seddon *et al.*, 2000).

chemicals (Freemantle, 1998; Davis, 2004). Ionic liquids have been validated as ideal replacements for organic solvents in a plethora of chemical processes (Villiagran et al., 2006; Huddleston et al., 2001; Mizuuchi et al., 2008). Perhaps one of the most attractive characteristics of employing ionic liquids in chemical processes is their potential for improving reaction yields, facilitating product recovery and their recyclability without loss of functionality. As a result ionic liquids have found applicability in an impressively diverse range of uses.

Physiochemical property	Ionic liquid
Conductivity	Good ionic conductivity compared to organic solvents/electrolyte systems. This is inversely linked to viscosity (Endres & Abedin, 2006)
Viscosity	Generally more viscous than common molecular solvents. Viscosity is determined by van der Waals forces and hydrogen bonding and alkyl chain length in the cation (Endres & Abedin, 2006)
Density	Generally more dense than water (Endres and Abedin, 2006)
Melting point	<100°C
Solubility	Ionic liquids can act as both hydrogen bond acceptors (anion) and donors (cation) and therefore interact with substances with both accepting and donating sites (Dupont & Suarez, 2006). Ionic liquids can be divided into two groups (water-miscible and water-immiscible) according to their solubility in water (Wei & Ivaska, 2008). Examples of water-immiscible ionic liquids include 1-butyl-3-methylimidazolium hexafluorophosphate and 1-decyl-3-methylimidazolium bis(trifluoro-methylsulfonyl)imide. Examples of water-miscible ionic liquids include [1-Butyl-3-methylimidazolium tetrafluoroborate. (Wei and Ivaska, 2008). Miscibility of ionic liquids in water is primarily dependent on the anion present it is also dependent on the structure of the cation (Seddon et al., 2000; Wei & Ivaska, 2008),
Thermal stability	Highly thermally stable (some up to temperatures of 450°C) (Endres & Abedin, 2006)
Chemical stability	Most are stable towards organic and inorganic substances (Dupont & Suarez, 2006)
Electrochemical window (defined as the electrochemical potential range over which the electrolyte is neither reduced or oxidised at an electrode)	Wide electrochemical window (Endres & Abedin, 2006)

Table 1. Physiochemical properties of Ionic liquids

2. Ionic liquids in 'green chemistry'

Green chemistry is defined as the design of chemical products and processes which reduce or eliminate the use and generation of hazardous substances (Anastas & Warner, 1998; Seddon et al., 2005). The design of safe and environmentally benign solvents has become increasingly important in the development of clean manufacturing processes. Conventional organic solvents are often toxic, flammable and volatile which when released into the environment can have potentially devastating effects. Ionic liquids have offered promise as reagents, which have the potential to replace many hazardous volatile organic solvents (including those banned by the Montreal protocol of 1989) (Anastas & Warner, 1998), and have therefore been cited as an important element of green chemistry. Ionic liquids have also been shown to have similar (if not superior and more diverse) properties to the organic/aqueous solvents they could potentially replace (Visser et al., 2000), whilst having negligible vapour pressure thus reducing the likely risk of atmospheric pollution (Fredlake et al., 2004). Attractive physicochemical attributes, improved reaction rates and yields, recyclability and design of ionic liquids lacking inherent biological toxicity all represent approaches for the 'greening' of chemical processes by ionic liquids.

Ionic liquids have already been reported as alternative 'green' solvents for a wide range of reactions (Wasserscheid et al., 2002; Prasad et al., 2005; Tao et al., 2006), however, in addition to possible concerns about the recyclability of ionic liquids there have also been concerns raised over the biodegradability or environmental persistence of ionic liquids (Garcia et al., 2004; Garcia et al., 2005; Stolte et al., 2008). A series of imidazolium compounds were shown to be poorly biodegradable and it was found that bacteria did not use them as a source of carbon under the conditions of the investigation (Romero et al., 2008), making them potentially persistent polluters. In this study, it was also demonstrated that imidazolium based ionic liquids have a wide range of toxicities in this relevant bioassay. Generally, toxicity (EC_{50} value) was found to correlate directly with the length of the *n*-alkyl substituent in the methylimidazolium cation, while the anion has no apparent effect on the EC_{50} value. The authors conclude that the ionic liquids tested are more toxic than conventional organic solvents. In tests against *Vibrio fischeri* and mammalian cell lines, a series of imidazolium ionic liquids of varying alkyl chain length were shown to exhibit significant toxicity (Ranke et al., 2004), once again dependent on the length of the cationic *n*-alkyl substituent.

Many ionic liquids are water-soluble and as such could contribute to pollution of aquatic environments. For example, it has been demonstrated that imidazolium, pyridinium and pyrrolidinium ionic liquids had LC_{50} > 100 mg/L against *Danio rero* (zebra fish), and as such can be regarded as non-lethal (Pretti et al., 2006). On the other hand, however, the ammonium based ionic liquids had LC_{50} values remarkably lower than that reported for organic solvents and yet proved fatal when zebrafish were exposed to them. Ecotoxicological tests on several ionic liquids have revealed that imidazolium and pyridinium ionic liquids exhibit significant toxicity towards the freshwater algae *Pseudokirchneriella subcapitata* (Pham et al., 2008), while imidazolium ionic liquids are toxic to the freshwater crustacean *Daphnia magna* (Wells & Coombe et al., 2004) and *Caenorhabditis elegans* (Swatloski et al., 2004). A number of recent studies have also demonstrated the potential of certain ionic liquids to exhibit excellent antimicrobial activity, discussed below, thus presenting the exciting possibility that ionic liquids could have application as biocidal agents in the control of microorganisms in the environment for contamination and infection control.

3. Methods for evaluating the antimicrobial activity of ionic liquids

A number of methods exist for the accurate determination of microbial susceptibility to antimicrobial/antibiotic compounds. Such methods yield vital data regarding fundamental sensitivity or tolerance to a given antimicrobial biocide or antibiotic and are therefore vital to the successful treatment and management of microbial infections. Furthermore, such tests are useful for determining relative potency of an antimicrobial agent across a range of species and for identifying antimicrobial synergies. The basic testing procedures, which have been used in the assessment of the antimicrobial activity of ionic liquids are considered briefly below. Whilst the majority of these tests have relied on basic planktonic susceptibility assays (minimum inhibitory concentrations (MIC) or minimum bactericidal/fungicidal concentration (MBC/MFC)) or agar diffusion techniques, the importance of evaluation of antimicrobial activity against microbial biofilms is also discussed. In our group, we have pioneered the use of high throughput screening of ionic liquids against clinically relevant microorganisms grown as biofilms, by determination of minimum biofilm eradication concentration (MBEC) (Carson et al., 2009; Busetti et al., 2010).

3.1 Agar diffusion tests

The agar diffusion technique (also known as the Kirby-Bauer test (Bauer et al., 1966) but described somewhat earlier by Abraham am co-workers in 1941 (Abraham et al., 1941)) is a simple and commonly employed technique for determination of MIC on solid media. The basic method requires antibiotic/biocide impregnated discs to be placed on the surface of agar plates seeded or spread with the appropriate test strain of bacteria or fungi. Antimicrobial agent(s) may also be added (as a solution) to wells punched in the agar. The diffusion of antimicrobial agent into the surrounding agar results in inhibition of growth around the reservoir/source and gives rise to zones or clearance where (for sensitive organisms) microbial growth is inhibited. Generally, the diameter of these zones of inhibition or clearance increases with increasing concentration of antimicrobial agent, and this may be measured to determine qualitatively the relative degree of toxicity. The MIC may also be determined from the zero intercept of a linear regression of the squared size of these zones of inhibition, x, versus the natural logarithm of the antibiotic concentration (Bonov et al, 2008). This is described in the equation below, where D is the diffusion coefficient (assumed to be independent of concentration) and t the time over which antibiotic diffusion occurs (incubation time):

$$\ln(MIC) = \ln(c) - \frac{x^2}{4Dt}$$

The technique is also useful for empirical determination of antimicrobial activity of a given compound or assessing relative antimicrobial potency by measuring zones of inhibition of bacterial or fungal growth around the antimicrobial site of application. Recently, this method has been championed by Stephens and co-workers (Rebros et al., 2009; Wood & Stephens, 2010) as a simple method for rapid determination of relative toxicity of ionic liquids. This simple, inexpensive method has been suggested as a basic requirement in the toxicological assessment of ionic liquids and, since it requires neither specialist equipment nor advanced microbiological techniques, may be performed routinely in laboratories conducting research into ionic liquids with minimum microbiological expertise. However,

the method is not without inherent limitations and consequently, care must be taken in interpretation of the data obtained. For example, it is well established that some antibiotics deviate from the behaviour described above by interacting with components of the growth media; similar effects might be expected with some ionic liquids especially those having hydrophobic or amphipathic character. Interaction of ionic liquids and other ionic components of the growth media (dissolved salts, nutrients etc.), chemical reactivity of the reagent and interaction with the agar itself may all result in erroneous data. Furthermore, the method is unlikely to be of any practical use for ionic liquids which are immiscible with water, since agar is >98% water, and thus water miscibility will have a significant effect on the extent of diffusion through the medium. Despite this, the use of agar diffusion assays will provide basic toxicity information for a large number of ionic liquids and provides a rapid, high-throughput 'first look' in the hierarchical screening of antimicrobial activity of ionic liquids.

3.2 Dilution tests

Dilution tests are routinely used for the determination of the two most fundamental parameters in antimicrobial susceptibility testing; the minimum biofilm eradication concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC), sometimes referred to as the minimum lethal concentration (MLC). Dilution tests usually involve the use of liquid media but agar may also be used (as discussed above). Doubling dilutions of the antimicrobial agent are prepared and added to a defined inoculum of test microorganism taken from the logarithmic phase of growth, such that a final inoculum of 5 x 10^5 colony forming units (CFU or viable cells)/ml is achieved. Following incubation at 35^{o}C ± 2.5^{o}C overnight (18 hours), the MIC is determined as the concentration of antimicrobial contained in the first clear tube/well. Therefore the MIC is defined as the minimum concentration of antimicrobial agent that inhibits the growth of an overnight culture of microorganism. The conditions used for the test and appropriate control tests (which must be included) are most commonly obtained either from the Clinical and Laboratory Standards Institue (CLSI) formerly the National Committee for Clinical Laboratory Standard (NCCLS) (NCCLS document M27-A, 1997; NCCLS document M7-A5) or the British Society for Antimicrobial Chemotherapy (Andrews, 2001).

The MBC is the lowest concentration (in mg/L) of antimicrobial that results in ≥99.9% killing of the bacterium under test. The 99.9% cut-off is an arbitrary *in vitro* value with 95% confidence limits that has uncertain clinical relevance. MBCs are determined by spreading 0.1-ml (100-ml) volumes of all clear (no growth) tubes from a dilution MIC test onto separate agar plates (residual antimicrobial in the 0.1-ml sample is 'diluted' out over the plate). After incubation at 35°C overnight (or longer for slow-growing bacteria), the numbers of colonies growing on each plate are recorded. The first concentration of drug that produces <50 colonies after subculture is considered the MBC. Minimum fungicidal concentrations are determined in the same manner, however, different growth media is necessary (e.g. use of RPMI 1640 plus 2% dextrose) and the inoculum density (yeast cells or spores) is reduced (c. 10^4 CFU/ml).

3.3 Evaluating biofilm susceptibility to antimicrobial agents

Both the MIC and MBC/MFC evaluations are suspension tests, which test the susceptibility of planktonic (free floating) microorganisms grown under optimum conditions to a given

antimicrobial challenge. However, evaluation of the antimicrobial susceptibility of microbial biofilms is now recognized as a more physiologically relevant assay. A biofilm may be defined as *'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit altered phenotype with respect to growth rate and gene transcription'* (Donlan & Costerton, 2002). Biofilms represent the predominant mode of growth of microorganisms and also the most persistent, phentypically resistant mode of growth, with increased tolerance to antimicrobial challenge. Irrespective of site, biofilm formation follows a series of discreet events, summarized below in Figure 2.

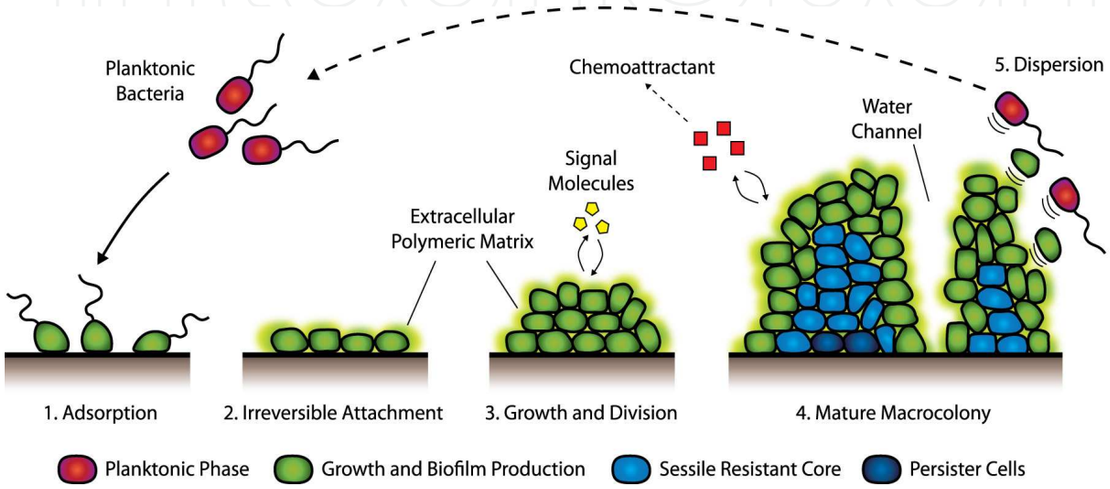


Fig. 2. Microbial Surface Colonisation; Main Stages in Surface attachment and biofilm formation (Adapted from Harrison et al., 2005)

As the importance of microbial biofilms in medicine, industry and agriculture has become clear, a huge amount of industry has been invested into studying their growth and control. As a result of this a number of *in vitro* models have been developed to facilitate elucidation of the mechanisms central to this important microbiological process. Each model has relative advantages and disadvantages, depending on the aspect of biofilm physiology the models were designed study. These are expertly reviewed in (McBain, 2009; Coeyne & Nelis, 2010). However, to date the only model used for the study of biofilm susceptibility of ionic liquids is that employed in our group, namely the Calgary Biofilm Device (commercially available from Innovotech Inc., Edmonton, AB, Canada as the MBEC Assay). The MBEC assay, developed in 1999 by Ceri and co-workers (Ceri et al., 1999) was developed specifically to evaluate biofilm susceptibility to antimicrobials. Essentially, the device consists of a 96-well plate and a lid bearing 96 polycarbonate pegs, each peg protrudes into the 96 wells and provides a surface onto which the bacteria/fungi may attach and form a biofilm, as shown below in Figure 3.

Shear forces (provided by gyration of the plate) stimulates microbial attachment and biofilm formation, the density of which may be determined by sonication of the biofilm back into fresh growth media followed by enumeration *via* standard plate counting. Biofilms grown on pegs may then be transferred to a 96-well challenge plate, set up in the same manner as an MIC assay with varying concentrations of antimicrobial agent(s) alone or in combination.

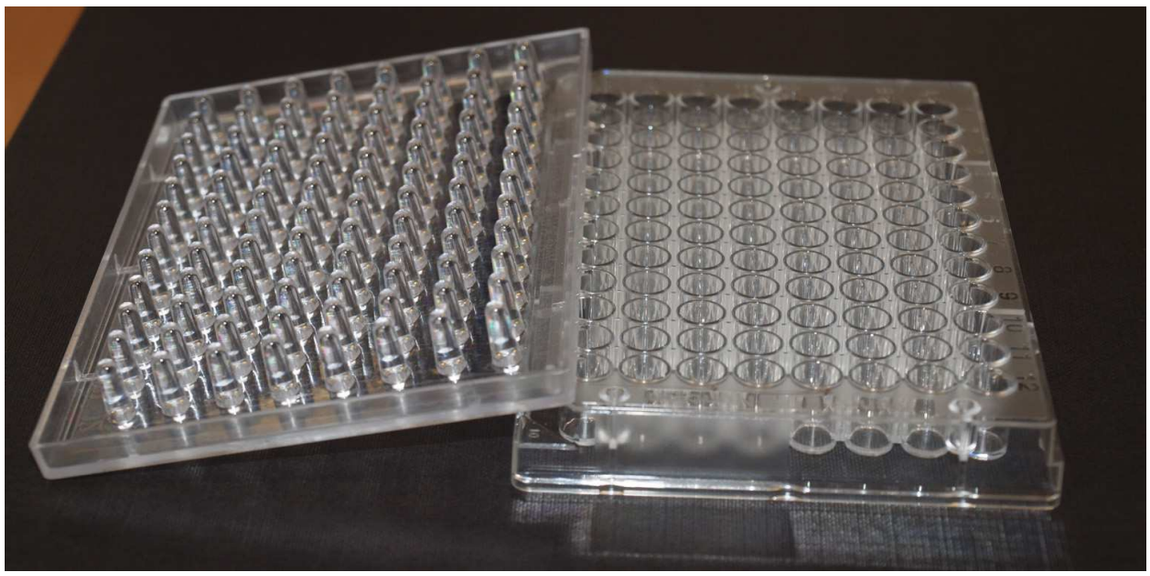


Fig. 3. The commercially available Calgary Biofilm Device/MBEC Assay Plate

Following antimicrobial exposure bacteria would again be sonicated from the pegs and counted to determine the biofilm MIC (BMIC), biofilm bactericidal concentration (BMBC) and biofilm eradication concentration (MBEC) in a highly standardized and reproducible assay based on existing MIC technology.

4. Antimicrobial and antibiofilm activities of ionic liquids

The toxicity shown in the studies highlighted previously raises issues over the validity of the classification of ionic liquids as 'green' compounds. However, toxicity itself a tuneable property which may be of utility in a number of other applications, for example, in the development of antiseptics, disinfectants and anti-fouling reagents (Pernak et al., 2004a; Pernak et al., 2004b; Pernak et al., 2007a; Fischmeister et al., 2007). The antimicrobial activities of five new groups of choline-like quaternary ammonium chloride ionic liquids were evaluated against a range of Gram positive and Gram negative bacteria (Pernak & Chwala, 2003). The ionic liquids tested all showed good antimicrobial activity, and confirmed that lipophlicity was the main factor in determining antimicrobial activity. Compounds with an alkyl chain substituent of 12 carbon atoms on the cation all exhibited the highest antimicrobial activity across all groups of ionic liquids tested, for a range of test microorganisms.

In a similar study, a series of 3-alkoxymethyl-1-methylimidazolium ionic liquids bearing [Cl], [BF_4] and [PF_6] anions were tested against a range of bacterial species, as well as fungi (Pernak et al., 2003). This study demonstrated that shorter cationic alkyl chain substituents resulted in reduced antimicrobial activity compared to the imidazolium compounds containing 10, 11, 12 and 14 carbon atoms in their alkoxy group, confirming earlier findings (Pernak et al., 2004a). Again, the imidazolium ionic liquids with alkoxy substituents of twelve carbon atoms were the most active against the bacteria and fungi tested. Another study showed that 1, 3 - (dialkloxymethyl)-substituted imidazolium ionic liquids (Pernak et al., 2004b) also exhibited broad-spectrum antimicrobial activity against various bacterial rods, cocci and fungi.

Pyrrolidinium ionic liquids with varying alkyl chain substituents were shown to possess good antimicrobial activity against rods, cocci and fungi (Demberelnyamba et al., 2004). Compounds exhibiting the greatest antimicrobial activity were those having 14 carbon atoms in the alkyl chain. In a recent study, Pernak and co-workers tested a range of trigeminal tricationic ionic liquids for antimicrobial activity (Pernak et al., 2007b), it was found that, in addition to their broad spectrum antimicrobial activity, their potency was much better than the commercially available benzalkonium chloride. A further study on chiral ammonium-based ionic liquids (Pernak & Feder-Kubis, 2005) revealed that compounds with 11 carbons in the alkyl substituent showed the most activity against a range of bacteria and fungi. In a study carried out on a number of ionic liquids with varying anions (Docherty & Kulpa, 2005), it was found that improved antimicrobial activity resulted from increasing alkyl group chain length as well as increasing the number of alkyl groups substituted on the cation ring. Varying the anion present in the compound did not significantly alter toxicity. Recently, the antimicrobial activity of multifunctional long-alkyl-chain quaternary ammonium azolate based ionic liquids has been described (Walkiewicz et al., 2010). These ionic liquids, based on didecylmethylammonium, benzalkonium, domiphen and hexadecyltrimethlammonium cations combined with benzotriazole, 1,2,4-triazolate, 4-nitroimidazolate or 2-methyl-4-nitroimidazolate anions all exhibited excellent, broad spectrum anti-bacterial and antifungal activity, which was comparable or superior to that of the original benzalkonium chloride (Walkiewicz et al., 2010).

According to the studies discussed above, a general feature common to the ionic liquids is a dependency on substituent alkyl chain length for antimicrobial potency, indicating a general mechanism for antimicrobial activity. Other studies have indicated that the mechanism of antimicrobial activity of ionic liquids is *via* membrane disruption. This seems likely given the structural similarity between ionic liquids and antimicrobial agents whose mechanism is more fully elucidated (Li et al., 1998; Pernak et al., 2001). Many ionic liquids have a similar structure to cationic surfactants whose primary mode of action membrane-bound protein disruption (Bernot et al., 2005). Another suggested mechanism of toxicity and antimicrobial activity is the inhibition of the enzyme acetylcholinesterase, as illustrated in studies of the inhibitory effects of imidazolium and pyridinium ionic liquids which were shown to inhibit purified enzyme with EC_{50} levels as low as 13 μM (Stock et al., 2004).

4.1 Antibiofilm activity of 1-alkyl-3-methylimidazolium chloride and 1-alkylquinolinium bromide ionic liquids

All microbiological toxicity studies conducted to date have described antimicrobial activity against planktonic, or free swimming, microbial phenotypes. However, the predominant mode of growth of both pathogenic and environmental microorganisms, is as highly-ordered surface-adhered communities encased within a self-produced protective extracellular polymeric matrix (glycocalyx), collectively known as a biofilm (Donlan & Costerton, 2002; Hall-Stoodley et al., 2004). A general characteristic of biofilm communities is that they tend to exhibit significant tolerance/resistance to antibiotics and antimicrobial/biocidal challenge compared with planktonic bacteria of the same species (Ceri et al., 1999; Stewart & Costerton, 2001; Gilbert et al., 2002; Stewart, 2002). Therefore, significant limitations exist when attempting to extrapolate planktonic culture susceptibility data to environmental or clinical scenarios where the majority of microbial growth is as biofilms. This is illustrated by the NIH estimation that up to 80% of all chronic human

infections are biofilm-mediated and that 99.9% of bacteria in aquatic ecosystems live as biofilm communities (Lewis, 2001; Costerton & Wilson, 2004). Indeed, it has been demonstrated that there is often no correlation between planktonic susceptibility to antimicrobials (MIC values) and biofilm susceptibility of the same species and strain to those same antimicrobial agents (Smith et al., 2003).

Biofilms are a major survival strategy for microbial populations in the face of environmental stresses and have been linked to a host of industrially and clinically relevant complications; from chronic plant, animal and human infections, to failure of implanted medical devices and microbially-influenced biocorrosion. Therefore, knowledge of the antibiofilm activity of ionic liquids is both environmentally and clinically relevant.

In a recent study, Carson and colleagues reported for the first time the *in vitro* antibiofilm activity of a library of 1-alkyl-3-methylimidazolium chloride ionic liquids (the general structure is given below in Figure 4) against a panel of clinically relevant pathogenic bacteria (including MRSA) and fungi using the Calgary Biofilm Device (CBD), a high-throughput micro-titre plate-based technology for screening antimicrobial susceptibility of microbial biofilms, which permits the determination of minimum biofilm eradication concentration (MBEC), or the concentration of an antimicrobial agent required to kill a microbial biofilm. This study illustrated that antibiofilm activity of these ionic liquids was also dependent on alkyl chain length, with the MBEC value decreasing (increased antibiofilm potency) with increasing alkyl chain length. Ionic liquids [$C_n$mim]Cl where $n \geq 10$ exhibited potent, broad spectrum antimicrobial activity. In general, of the compounds tested in this series, [$C_n$mim]Cl where $n = 14$, exhibited greatest antibiofilm activity against all microbial biofilms. The data from this study indicate that Gram positive microbial biofilms (in keeping with planktonic cultures) are generally more susceptible to 1-alkylmethylimidazolium ionic liquids than Gram negative bacterial biofilms, whilst *Candida tropicalis* biofilms exhibited a similar susceptibility profile to these reagents as the representative Gram positive organisms tested in this study (Carson et al., 2009).

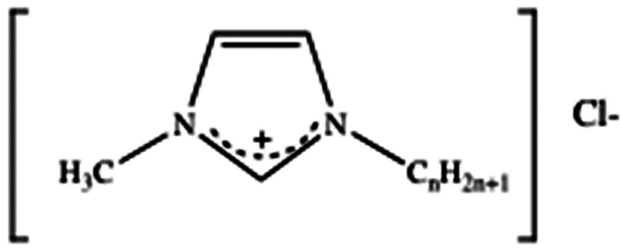


Fig. 4. General structure of 1-alkyl-3-methylimidazolium chlorides [$C_n$mim]Cl which exhibited antibiofilm activity across a range of clinically relevant pathogens.

In a further study from the same group, Busetti and co-workers, described the antimicrobial and antibiofilm activities of a range of 1-alkylquinolinium bromide ionic liquids (Busetti et al., 2010). In general, these ionic liquids are the most potent antibiofilm ionic liquids tested so far, having a superior microbiological toxicity to the 1-alkyl-3-methylimidazolium ionic liquids against both planktonic and biofilm cultures of a range of bacteria and fungi commonly implicated in nosocomial and device associated infections, including *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella aerogenes* and *Bacillus cereus*. In keeping with the observations from our previous studies, the antimicrobial activity is

dependent on the length of alkyl chain substituent, with compounds having alkyl chain lengths of 12-14 carbon atoms exhibiting greatest antimicrobial potency. The general structure of the 1-alkylquinolinium bromides is given below in Figure 5.

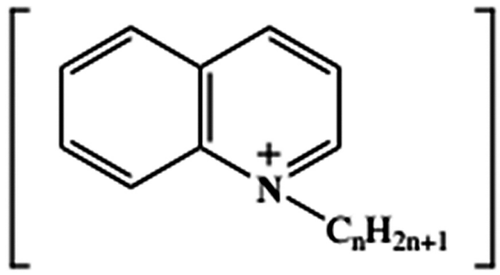


Fig. 5. General structure of 1-alkylquinolinium bromides with demonstrated antimicrobial and antibiofilm activities

These important studies not only highlight the potential environmental effects of ionic liquids to the microbial ecosystem (which are susceptible to their antimicrobial activities even in their predominant environmental mode of growth as biofilms) but also opens up the real possibility of employing ionic liquids for a plethora of beneficial uses as antimicrobials in disinfectants, preservatives, antiseptics and development of anti-infective medical device surfaces for use in healthcare and as antibiofouling reagents for a host of industrial applications.

The challenges that remain in bringing the first ionic liquid based biocides to the clinical setting as either disinfectants, antiseptics, sterilants for medical devices/instruments or preservatives include demonstrating suitably rapid rates of kill, for example high level disinfectants would typically be required to be sporocidal in <7 minutes, and (as an industry 'rule of thumb') reduce the original (vegetative) bioburden of 5 microbial species by 5 log reductions (99.999% kill) within 5 minutes. The factors that attenuate the activity of ionic liquids as disinfectants have not, as yet, received sufficient attention. Furthermore, the toxicological profile of these compounds is, for the present, not elucidated (although ongoing work in our laboratory is aimed at addressing this lacuna in our knowledge). Despite this, ionic liquids appear to hold great promise in the future development of biocides for use in clinical and industrial infection and contamination control.

5.0 Potential regulatory challenges to antimicrobial applications of ionic liquids

As with all novel compounds with potential application as biocidal products, legislative and commercial barriers to their entry to the market exist. In the following sections, the current European legislation regarding biocidal products and new chemical entities coming onto the EU market are discussed. These legislative instruments are necessary in safeguarding public safety and will no doubt prove burdensome in bringing ionic liquids from the bench to various in-use settings where we predict they will be usefully employed as biocides.

5.1 The Biocidal Products Directive (98/8/EC)

Directive 98/8/EC of the European Parliament and of the Council on the placing on the market of biocidal products was adopted in 1998. The Biocidal Products Directive aims to

harmonise both the issues of biocide manufacture and use, and the European market for biocidal products. Furthermore, the directive aims to provide a high level of protection for humans, animals and the environment. The scope of the directive is wide, covering some 23 different product types including disinfectants (classified by use in given areas), chemicals for preservation of products and material (such as timber), non-agricultural pesticides and anti-fouling agents used to prevent hull-fouling on vessels. However, medicines and cosmetics fall outside the remit of the directive, potentially allowing ionic liquids to be used as preservatives in the first instance.

The basic objective of the directive is to produce a list of active biocidal products that are licensed for use across all member states, known as 'Annex I'. Active substances must be assessed and any decision on their inclusion in Annex I will be taken at Community level. Only products containing active substances listed in Annex I will be authorised for use in the EC. The two-tier system mandated by the directive requires that firstly, active substances must be assessed and a decision reached as to their suitability for inclusion in Annex I and secondly, the producers and formulators responsible for the placing of the market of the biocidal products and their active substances must apply for authorisation of the biocidal product. In each member state, it is the responsibility of the national competent authority (in the GB this is the Health and Safety Executive) to authorise products containing active substances included in Annex I. The principle of mutual recognition outlined in Article 4 of the directive means that once a product containing an Annex I active substance has been authorised by one member state it can be recognised in as an authorised product in other member states.

The legislation related to the directive came into force in September 2000, with guidance on deadlines for identification and notification of active substances. In June 2009, based on experience of working under Directive 98/8/EC, the European Commission adopted a proposal for a Regulation concerning the placing on the market and use of biocidal products (COM(2009)267), which is intended as a full revision of the existing directive which it will repeal and replace. This revision is a response to a 2008 report on the implementation of the directive, which highlighted the inherent weaknesses of the original directive, primarily the complexity of the legal framework and the high costs associated with compliance (especially the cost of compiling a dossier in support of inclusion of an active substance in Annex I). The proposed new regulation is scheduled to enter into force on January 1st 2013.

Although there is unlikely to be any direct impact on the pharmaceutical industry *per se*, the biocidal products directive and proposed revision *is likely to present a significant restriction to bringing new disinfectants to market*. This is likely to have some impact on the use of new ionic liquids for biocidal applications. The experience under the original directive indicated that expense was a major issue in supporting the addition of an active substance to Annex I. It remains to be seen if the revised regulation will streamline the process and reduce costs associated.

5.2 Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

EC Regulation (EC1907/2006) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) entered into force on June 1st 2007, replacing a number of European Directives and Regulations within a single legislative framework. The former EC legislative framework for chemical substances was a collection of numerous different directives and regulations, which had developed historically. Part of the problem, which REACH sought to

address, was the different rules governing 'existing' and 'new' chemicals. This distinction between existing and new chemical substances was introduced under regulation (EC) 793/93 based on a cut-off date of 1981. Chemical substances which were reported as being on the EC market between 1st January 1971 and 18th September 1981 were called 'existing' chemicals (~100,000, listed in the European Inventory of Existing Commercial Chemical Substances (EINECS)); those introduced to the market after the cut-off date were classified as 'new' chemicals (~3800). Whilst new chemicals must undergo rigorous testing before being placed on the market, there were no such regulations for existing chemicals, as a result there is generally insufficient publically accessible information available to accurately assess and control these substances effectively.

Further issues which led to the drafting of this Regulation include the pre-REACH allocation of responsibilities whereby public authorities were responsible for undertaking risk assessments of substances, with no such responsibilities on downstream users (manufacturers, importers, end users). Manufacturers and importers were required to provide information on use of the substances, but downstream users (industrial users, formulators) were not. In addition, the threshold for notification and testing of new chemical substances could be as low as 10 kg per year. This has been regarded as a significant barrier to innovation within the EU chemical industry, since the resultant trend has been away from developing new chemicals and towards using exiting agents.

The aims of REACH are:

i. To provide a high level of protection of human health and the environment from the use of chemicals
ii. To give those who place chemical substances on the market (manufacturers and importers) responsibility for understancing and managing the risks associated with their use
iii. To allow free movement of substances on the EU market
iv. To enhance the innovation in and competitiveness of the EU chemicals industry
v. To promote the use of alternative methods (other than animal studies) for assessment of hazardous properties of substances, e.g. QSAR studies.

REACH is based on the concept that the chemical industry itself is best placed to ensure that chemicals placed on the market in the EU do not adversely affect human health or the environment. REACH creates a single system for both new and existing chemical substances. Substances are now described as 'non-phase-in' substances (i.e. those not produced or marketed prior to the entry into force of REACH) and 'phase-in' substances (i.e. those substances listed in EINECS, or those that have been manufactured in the Community, but not placed on the Community market, in the last 15 years or the so called "no longer polymers" of Directive 67/548). A major part of REACH is the requirement for manufacturers or importers of substances to register them with a central European Chemicals Agency (ECHA), with a standard set of data to be submitted for each substance. If the substance is not registered, data will not be available and the substance will no longer be able to be legally manufactured or supplied.

REACH proposes a number of benefits over the existing patchwork of legislation and regulations. Primarily, by creating parity for 'existing' and 'new' chemical substances with respect to risk management and the making available of data for all substances in relation to this. Furthermore, it simplifies the existing EU level regulation by replacing 40 existing pieces of legislation and creates a single system for all chemicals, removing the distinction of

'existing' and 'new' substances. REACH will result in better risk characterisation of chemicals and mandates improved information flows in the supply chain. REACH will close the knowledge gap for over 30,000 existing substances, providing information on both acute and long-term toxicity. REACH provisions are intended to be phased in over a period of 11 years. Manufacturers of biocides and biocidal products are examining closely the likely impact of REACH and it is generally regarded that REACH will impose a significant burden, but one which must be borne for commercial reasons. In this respect, the bringing onto the market of ionic liquid based biocidal products will require (i) demonstration that these reagents are more effective or have superior properties and in-use characteristics compared with existing biocides and (ii) significant financial investment to satisfy the compliance programmes required by REACH. That said, there exists a pressing need for new and effective biocides, in the face of increasing emergence of resistance to most conventional biocides. Ionic liquids have numerous attractive properties, which render them excellent candidates for biocidal applications, and, to date, no reports of resistance have been published.

6. Conclusion

Since ionic liquids are tunable and designer chemicals, they have been used in a wide variety of applications. Many have been developed for use as solvents in industrial chemistry, and generally their use confers a number of advantages over using other solvents; superior reaction rates, recyclability of reactants and catalysts and improved product recovery. Having negligible vapour pressure, it has been suggested that ionic liquids will not contribute to air pollution and are thus green alternative to conventional organic solvents which are generally volatile, flammable and toxic. As a result of this, many studies have been conducted on the use of ionic liquids as novel green solvents to replace established solvents for particular reactions.

However, toxicity of ionic liquids has been demonstrated by a number of groups, including our own, in a variety of environmental niches, and raises questions over their 'green' credentials. Nonetheless the toxicity of ionic liquids is in itself a property which can be tuned and exploited for other beneficial uses, for example in developing novel antimicrobials. We have demonstrated the utility of ionic liquids as antibiofilm agents; biofilms are complex, organised communities of bacteria which have been shown to have greater tolerance and resistance to antimicrobials, accounting for the majority of chronic and acute infections as well as the majority of bacterial communities in aquatic environments. In summary, the biological properties of ionic liquids may yet prove their most exciting and the benefits of rationally designed, bespoke ionic liquid-based antimicrobials to human health has yet to be harnessed.

7. References

Abraham, E.P., Gardner, A.D., Chain, E.B., Heatley, N.G., Fletcher, C.M., Jennings, M.A., Florey, H.W. (1941) Further observations on penicillin. Lancet, ii: 177-189.

Andrews, J. M. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*, 48 *Suppl. S1*. 5-16.

Anastas, P.T. and Warner, J.C. (1998) *Green Chemistry: Theory and Practice*; Oxford University Press: New York, 1998, p.30.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*. 45, 493-496.

Bonev, B., Hooper, J., Parisot, J. (2008) Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method, *J. Antimicrob. Chemother*., 61, 1295-1301.

Bernot R. J., Kennedy, E. E., Lamberti, G. A., (2005) Effects of ionic liquids on the survival, movement and feeding behaviour of the freshwater snail, *Physa acuta*. *Environ. Toxicol. Chem.,* 24, 1759-1765.

Busetti, A., Crawford, D.E., Earle, M.J., Gilea, M., Gilmore, B.F., Gorman, S.P., Laverty, G., Lowry, A.F., McLaughlin, M., Seddon, K.R. (2010) Antimicrobial and antibiofilm activities of 1-alkylquinolinium bromide ionic liquids. *Green Chem*., 12, 420-425.

Carson, L., Chau, P. K. W., Earle, M. J., Gilea, M. A., Gilmore, B. F., Gorman, S. P., McCann, M. T., Seddon K. R. (2009) Antibiofilm activities of 1-alkyl-3-methylimidazolium chloride ionic liquids. *Green Chem.,* 11(4) 492-497.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*., 37, 1771-1776.

Coeyne, T. & Nelis, H.J. (2010) *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Methods,* Sept 16 – ahead of print.

Costerton, J. W. & Wilson, M. (2004) Introducing *Biofilms. Biofilms*, 1, 1-4.

Davis, J.H. (2004) Task-specific ionic liquids. *Chem. Lett*., 33, 1072-1077.

Demberelnyamba, D., Kim, K., Choi, S., Park, S., Lee, H., Kim, C., Yoo, I. (2004) Synthesis and antimicrobial properties of imidazolium and pyrrolidinium salts. *Bioorg. Med. Chem*., 12, 853–857.

Docherty, K. & C.F. Kulpa Jr. (2005), Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids *Green Chem.,* 7, 185–189.

R. M. Donlan and Costerton, J. W. (2002) Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev*., 15, 167-193

Dupont J. & Suarez, P.A.Z. (2006) Physico-chemical processes in imidazolium ionic liquids *Phys. Chem. Chem. Phys*., 8, 2441-2452.

Earle, M.J., Esperancüa, J.M.S.S., Gilea, M.A., Canongia Lopes, J.N., Rebelo, L.P.N., Magee, J.W., Seddon, K.R., Widegren, J.A. (2006) The distillation and volatility of ionic liquids *Nature,* 439, 831.

Endres, F. & El Abedin, S.Z. (2006) Air and water stable ionic liquids in physical chemistry *Phys. Chem. Chem. Phys*., 8, 2101-2116.

Fischmeister, C., Griffin, S.T., Rogers, R.D. (2007) Synthesis and properties of chiral imidazolium ionic liquids with a (1*R*,2*S*,5*R*)-(–)-menthoxymethyl substituent *New J. Chem.,* 31, 879-892.

Fredlake, C. P., Crosthwaite, J. M., Hert, D. G., Aki, S. N. V. K., Brennecke, J. F. (2004) Thermophysical Properties of Imidazolium-Based Ionic Liquids, *J. Chem. Eng. Data.,* 49, 954-964.

Freemantle, M. (1998) Designer solvents – Ionic Liquids may boost clean technology development. *Chem. Eng. News,* 76, 32.

Gathergood, N., Garcia, M.T., Scammells P.J. (2004) Biodegradable ionic liquids: Part I. Concept, preliminary targets and evaluation. *Green. Chem*., 6, 166–175.

Garcia, M.T., Gathergood, N., Scammells, P.J. (2005) Biodegradable ionic liquids Part II. Effect of the anion and toxicology *Green. Chem*., 7, 9–14.

Gilbert, P. T., Maira-Litran, T., McBain, A. J., Rickard, A. H., Whyte, F. (2002) The physiology and collective recalcitrance of microbial biofilm communities *Adv. Microbial. Phys*., 46, 203-256.

Hall-Stoodley, L., Costerton, J. W., Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol*., 2, 95108.

Harrison, J.J., Turner, R.J., Marques, L.R. & Ceri, H. (2005) Biofilms: A new understanding of these microbial communities is driving a revolution that may transform the science of microbiology. *American Scientist*, 93, 508-515.

Huddleston J.G., Visser, A.E., Reichart, W.M., Willauer, H.D., Broker, G.A., Rogers, R.D. (2001) Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation. *Green Chem*., 3, 156-164.

Kosmulski, M., Gustafsson, J., Rosenholm, J.B. (2004) Thermal stability of low temperature ionic liquids revisited *Thermochim. Acta*., 412, 47-53.

Li, G., Shen, J., Zhu, Y. (1998) Study of pyridinium-type functional polymers. II. Antibacterial activity of soluble pyridinium-type polymers. *J. Appl. Polym. Sci*., 67, 1761-1768.

Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrob. Agents Chemother*., 45, 999-1007.

McBain A. (2009). In vitro biofilm models: an overview. *Advances in Applied Microbiology*, 69, 99-132.

Mizuuchi, H., Jaitely, V., Murdan, S., Florence, A.T. (2008) Room temperature ionic liquids and their aqueous mixtures as potential drug solvents. *Eur. Journ. Pharm. Sci.,* 33, 326-331

National Committee for Clinical Laboratory Standards (1997) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard. NCCLS document M27-A, Wayne, PA.

National Committee for Clinical Laboratory Standards (2000) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th edn. NCCLS document M7-A5, Wayne, PA.

Pernak, J., Rogoza, J., Mirska, I. (2001) Synthesis and antimicrobial activities of new pyridinium and benzimidazolium chlorides. *Eur. J. Med. Chem*., 36, 313-320.

Pernak, J. & Chwala, P. (2003) Synthesis and anti-microbial activities of choline-like quaternary ammonium chorides. *Chem. Eur. J*., 38, 1035-1042.

Pernak, J., Sobaszkiewicz, K., Mirska, I. (2003) Anti-microbial activities of ionic liquids *Green Chem.*, 5, 52–56.

Pernak, J., Goc, I., Mirska, I. (2004a) Anti-microbial activities of protic ionic liquids with lactate anion. *Green Chem*., 6, 323-329.

Pernak, J., Sobaszkiewicz, K. Foksowicz-Flaczyk, J. (2004b) Ionic Liquids with Symmetrical Dialkoxymethyl-Substituted Imidazolium Cations. *Chem. Eur*., 10, 3479-3485.

Pernak J. & Feder-Kubis, J. (2005) Synthesis and properties of chiral ammonium-based ionic liquids. *Chem. Eur. J.,* 11, 4441- 4449.

Pernak, J., Syguda, A., Mirska, I., Pernak, A., Nawrot, J., Pradzynska, A., Griffin, S.T., Rogers, R.D. (2007a) Choline-Derivative-Based Ionic Liquids *Chem. Eur. J.,* 2007a, 13, 6817-6827.

Pernak, J., Skrzypczak, A., Lota, G., Frackowiak, E. (2007b) Synthesis and Properties of Trigeminal Tricationic Ionic Liquids. *Chem. Eur. J.,* 13, 3106-3112.

Pham, T. P. T., Cho, C.W., Min, J., Yun, Y. S. (2008) Alkyl-chain length effects of imidazolium and pyridinium ionic liquids on photosynthetic response of *Pseudokirchneriella subcapitata. J. Biosci. Bioeng*., 105, 425-428.

Prasad, A.K., Kumar, V., Malhotra, S., Ravikumar, V.T., Sanghvi Y.S., Parmar, V.S. (2005) 'Green' methodology for efficient and selective benzoylation of nucleosides using benzoyl cyanide in an ionic liquid *Bioorg. Med. Chem*., 13, 4467-4472.

Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., Intorre, L. (2006) Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*) *Green Chem.,* 8, 238–240.

Ranke, J., Mölter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffman, J., Ondrussckka, B., 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, 396–404.

Rebros, M., Gunaratne, H.Q.N., Ferguson, J., Seddon, K.R., Stephens, G. (2009) A high throughput screen to test the biocompatibility of water-miscible ionic liquids, *Green Chem.*, 11, 402-408

Romero, A., Santos, A., Tojo, J., Rodriguez, A. (2008) Toxicity and biodegradability of imidazolium ionic liquids. *J. Hazard. Mater.,* 151, 268–273.

Scammells, P.J., Scott, J.L., Singer, R.D. (2005) Ionic Liquids: The Neglected Issues. *Aust. J. Chem*. 58, 155-169.

Seddon K. R. (1997) Ionic liquids for clean technology. *J. Chem. Technol. Biotechnol*., 68, 351-356.

Seddon, K.R., Stark, A., Torres, M.J. (2000) Influence of chloride, water, and organic solvents on the physical properties of ionic liquids. *Pure Appl. Chem*., 72, 2275-2287.

Smith, A. L., Fiel, S. B., Mayer-Hamblett, N., Ramsey, B., Burns, J. L. (2003) Susceptibility Testing of Pseudomonas aeruginosa Isolates and Clinical Response to Parenteral Antibiotic Administration : Lack of Association in Cystic Fibrosis. *CHEST*, 123, 1495-1502.

Stark, A. & Seddon, K. R. (2007) 'Ionic Liquids' in '*Kirk-Othmer Encyclopaedia of Chemical Technology*', ed. A. Seidel, John Wiley & Sons, Inc., Hoboken, New Jersey, 2007, vol. 26, pp. 836–920.

Stewart, P. S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol*., 292, 107-113.

Stewart, P. S. & Costerton, J. W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet*., 358, 135-138.

Stock, F., Hoffmann, J., Ranke, J., Stormann, R., Ondruachka B., Jastorff, B. (2004) Effects of ionic liquids on the acetylcholinesterase – a structure–activity relationship consideration. *Green Chem.,* 6, 286-290.

Stolte, S., Abdulkarim, S., Arnig, J., Blomeze-Nienstedt, A., Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thorning, J. (2008) Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds. *Green Chem*., 10, 214-224.

Swatloski, R. P., Holbrey, J. D., Memon, S. B., Caldwell, G. A., Caldwell K. A., Rogers, R. D. (2004) Using *Caenorhabditis elegans* to probe toxicity of 1-alkyl-3-methylimidazolium chloride based ionic liquids, *Chem. Commun*., 668-669.

Tao, G., He, L., Lui, W., Xu, L., Xiong, W., Wang, T., Kou, Y. (2006) Preparation, characterization and application of amino acid-based green ionic liquids. *Green Chem*., 8, 639-646.

Villiagran, C., Aldous, L., Lagunas, M.C., Compton, R.G., Hardacre, C. (2006) Electrochemistry of phenol in bis{(trifluoromethyl)sulfonyl}amide ($[NTf_2]^-$) based ionic liquids. *J. Electoanalytical Chem.,* 588, 27-31.

Visser, A. E., Swatloski, R. P., Rogers, R. D. (2000) pH-Dependent Partitioning in Room Temperature Ionic Liquids Provides a Link to Traditional Solvent Extraction Behavior. *Green Chem.,* 2000, 2, 1-4.

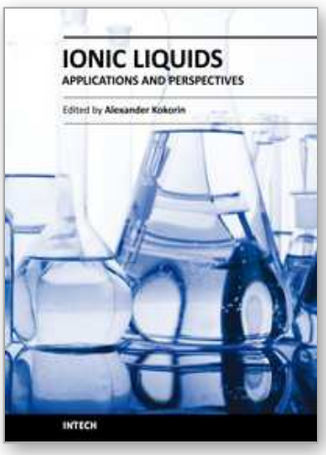
Walden, P. (1914) Molecular weights and electrical conductivity of several fused salts. *Bull Acad Sci St. Petersbourg*. 405-422.

Wasserscheid, P., van Hal R., Bösmann, A. (2002) 1-*n*-Butyl-3-methylimidazolium ([bmim]) octylsulfate—an even 'greener' ionic liquid, *Green Chem*., 2002, 4, 400-404.

Wei, D. & Ivaska, A. (2008) Applications of ionic liquids in electrochemical sensors *Anal. Chim. Acta*, 607, 126-135.

Wells A. & Coombe, V.T. (2006) On the Freshwater Ecotoxicity and Biodegradation Properties of Some Common Ionic Liquids, *Org. Process Res. Dev.,* 2006, 10, 794-798.

Wood, N. & Stephens, G. (2010) Accelerating the discovery of biocompatible ionic liquids. *Phys. Chem. Chem. Phys*., 12, 1670-1674.



Ionic Liquids: Applications and Perspectives

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This book is the second in the series of publications in this field by this publisher, and contains a number of latest research developments on ionic liquids (ILs). This promising new area has received a lot of attention during the last 20 years. Readers will find 30 chapters collected in 6 sections on recent applications of ILs in polymer sciences, material chemistry, catalysis, nanotechnology, biotechnology and electrochemical applications. The authors of each chapter are scientists and technologists from different countries with strong expertise in their respective fields. You will be able to perceive a trend analysis and examine recent developments in different areas of ILs chemistry and technologies. The book should help in systematization of knowledges in ILs science, creation of new approaches in this field and further promotion of ILs technologies for the future.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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