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Using *Caenorhabditis elegans* to probe toxicity of 1-alkyl-3-methylimidazolium chloride based ionic liquids

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Ionic liquids are gaining attention as new solvents within the green chemistry community; however this attention has quickly outstripped current environmental and toxicological data available. In the present communication, we establish the use of *Caenorhabditis elegans* as a model organism for inexpensively and quickly exploring toxicological effects of 1-alkyl-3-methylimidazolium chloride ionic liquids.

Ionic liquids are quickly gaining attention as new solvents within the green chemistry community.[1] This attention is due in part to the negligible vapor pressures of ionic liquids when compared to traditional volatile organic solvents. To date there has been very little work presented which examines the potential toxicity of ionic liquids,[2] and investigations of their utility in synthesis and separation systems have outstripped what little current environmental and toxicological data is available. Due to the immense range of possible ionic liquids, there is a real need for an inexpensive, quick toxicological screening process for new and existing ionic liquids.

To examine the putative toxicological effects of ionic liquids, we have employed the nematode model organism, *Caenorhabditis elegans*.† *C. elegans* is a well-studied free-living soil roundworm with a transparent anatomy. It is rapidly cultured, with only a 3-day life cycle, making it ideal for studies of longevity and toxicity. This worm is genetically tractable with over 30 years of mutational data available.[3] Notably, *C. elegans* is the first multicellular organism to have its total genomic DNA sequence determined, and is the only animal for which a defined cell lineage and complete neuronal connectivity are described.[4] Moreover, approximately 50% of all genes linked to human disease have a counterpart in *C. elegans*.[5]

C. elegans has been used as a model organism in order to probe toxicity and accumulation of pesticides[6] as well as various metal ions.[7] In this study, we establish *C. elegans* as a system by which to gauge the toxicological effects of water soluble ionic liquids, and report here an initial analysis of *C. elegans* survival in response to a range of concentrations of 1-butyl-3-methylimidazolium chloride (C_4mimCl), 1-methyl-3-octylimidazolium chloride (C_8mimCl), and 1-methyl-3-tetradecylimidazolium chloride (C_{14}mimCl).

The ionic liquids were synthesized using previously described methods.[8] Before toxicity studies were carried out, the ionic liquids were rigorously dried by heating to 70 °C under reduced pressure. The water contents were determined using a volumetric Aquastar Karl Fischer titrator (EM Science, Gibbstown, NJ). Duplicate measurements were performed on each sample, and agreed to within 100 ppm. To ensure purity (*i.e.*, complete reaction) the ionic liquids were analyzed using NMR (^{1}H at 360.13 MHz), and the analysis indicated no residual reactants. The C_4mimCl and C_{14}mimCl were off-white crystalline solids at room temperature, and the C_8mimCl was a colorless viscous liquid at room temperature.

Stock solutions were prepared by dissolving the ionic liquids in sterile water at the following final concentrations: C_4mimCl 840 mg mL^{-1}, C_8mimCl 350 mg mL^{-1}, and C_{14}mimCl 312 mg mL^{-1}. After complete dissolution the solutions were sterilized by passing them through 0.22 μm pore-size filters.

Wild-type Bristol N2 worms were cultured using standard conditions.[3] The various ionic liquids were added to Nematode Growth Media (NGM) plates at a final concentration of 1.0, 2.5, or 5.0 mg mL^{-1} by pipeting the appropriate amount of stock solution and allowing it to soak into the medium for 24 h. Concentrated OP50 bacterium was then introduced onto the plates as a food source for the worms. Next, 50 L4-stage worms were added to each plate, incubated at 25 °C, and then assayed for viability approximately 20 hours later, a significant percentage of the nematode's life span, by gently prodding the worms with platinum wire. Unresponsive worms were scored as unviable. The authors are aware of possible anti-microbial activities of some ionic liquids,[9] and it should be noted that in the studies reported here the bacteria concentrations were adequate for the short incubation times.

The set of imidazolium chloride ionic liquids was chosen for a variety of reasons. First, their relatively high water solubility makes them easy to work with, they represent the starting blocks to numerous commonly used ionic liquids, and finally, it is thought that lipophilicity (K_{ow}), or increasing alkyl chain length, is a major factor in determining bioaccumulation/toxicity.[10]

In the present study, as the alkyl chain length increased, the lethality of the ionic liquids increased. Perhaps this is due to the more lipophilic nature of the larger compounds or alternatively the smaller ones may be cleared more rapidly from the excretory system. When animals were exposed to 1.0 mg mL^{-1} ionic liquid, the lethality went from 0.0% with C_4mim, to 11% with C_8mim, to 97% with C_{14}mim. Likewise the trend continued with 5.0 mg mL^{-1} ionic liquid exposure: 1.0% lethality for C_4mim, 66% lethality for C_8mim, and 100% lethality with C_{14}mim (see Fig. 1). *C. elegans* exposed to C_4mimCl at any of the concentrations tested (1.0–5.0

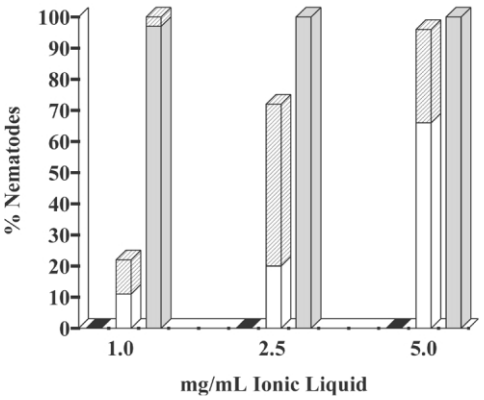

Fig. 1 Representation of lethality (solid bars) and aversion (hatched bars) *vs.* concentration of ionic liquid, C_4mimCl (dark gray), C_8mimCl (white), and C_{14}mimCl (light gray); concentrations from left to right are 1.0, 2.5, and 5.0 mg mL^{-1}. The increase in lethality with increasing concentration, and with increasing alkyl chain length on the ionic liquid cation, can be clearly observed.

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mg mL^{-1}) did not display adverse effects, while C_{14}mimCl was lethal to *C. elegans* at all concentrations tested (see Fig. 1).

Worms exposed to C_8mimCl displayed an obvious chemical aversion response. Normally, when worms are placed onto a Petri dish with a lawn of bacteria they remain there, eating. In contrast to this, when *C. elegans* were introduced onto the Petri dishes containing C_8mimCl, they did not begin eating, rather they crawled off the plates as depicted in Fig. 1 with the hatched bars. This does not appear to be a problem with bacteria viability as worms that stayed on the plates continued to eat, develop, and reproduce successfully.

Lethality in the nematodes is shown in Fig. 2. The visual distinction between viable (A) and unviable (B) is readily identifiable by simple differential interference contrast (DIC) microscopic analysis. Specifically, in addition to a complete loss of locomotion and pharyngeal pumping, unviable animals exhibit a generally stiff and visibly rigid appearance, whereas viable *C. elegans* exhibit an overtly more turgid and resilient morphology, while also maintaining motility and consistent pharyngeal activity.

The use of *C. elegans* as a model for assessing environmental toxicants represents an efficient, inexpensive, and rapid opportunity to discern the cytological and molecular effects of a variety of ionic liquids. In this regard, we observed that lengthening the alkyl chain led to a concomitant increase in toxicity/lethality presumably through surfactant/detergent effects. At 1 mg mL^{-1}, C_{14}mimCl resulted in essentially complete lethality over the studied period; the shortest chain length, and most common ionic liquid, C_4mimCl was effectively benign. Using guidelines established by Kamrin[12] based on LC_{50} for acute toxicity (Table 1), C_4mimCl and C_8mimCl appear to be *not acutely toxic*, while C_{14}mimCl is at least *slightly toxic*. Despite differences in the methodology employed here and by Ranke *et al.*[11] (the latter study assayed two mammalian cell lines, glial and hematopoeitic, and *Vibrio fischeri*), the trend of increasing toxicity with increasing alkyl chain length was observed by both groups.

For all compounds tested, worms that survived initial chemical exposure appeared healthy and reproduced successfully. In future studies, reproductive, neurological and behavioral phenotypes can be readily assessed. Whereas the human brain has > 100 billion neurons, this worm contains exactly 302 neurons. Yet, most common neurological pathways and molecules (ion channels, synaptic proteins, and neurotransmitters like serotonin, dopamine, GABA and acetylcholine) are conserved from humans to worms.

C. elegans is a simple, extensively studied organism which allows for rapid toxicological screening of a large number of ionic liquids. The ionic liquids examined in this study contained the same anion. However, to assess a broader range of ionic liquids, a comprehensive matrix (PF_6, BF_4, *etc*. salts) of ionic liquids will be assayed. Future studies will provide a more in depth analysis of bioaccumulation/lipophilicity *via* methods such as tagging techniques (radiochemical or fluorescence) and/or NMR. These studies should generate a comprehensive view of relative toxicological effects, and help establish the cationic and anionic factors that contribute to toxicity. Elucidation of lethality thresholds for various ionic liquids, as well as the ability to correlate worm toxicity with non-animal (K_{ow}) methods of determining toxicity should also be possible.

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Fig. 2 Differential interference contrast microscopic analysis of viable (A) and unviable (B) nematodes.

Table 1 Narrative descriptions of acute toxicity[12] based on LC_{50}

Toxicity category	LC_{50}/mg mL^{-1}
Very highly toxic	$< 10^{-4}$
Highly toxic	10^{-4}–10^{-3}
Moderately toxic	10^{-3}–10^{-2}
Slightly toxic	10^{-2}–10^{-1}
Not acutely toxic	$> 10^{-1}$

Notes and references

† Work with the invertebrate nematode model organism, *C. elegans*, does not require IACUC (Institutional Animal Care and Use Committee) approval, as stated by the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS).

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