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# Toxic effects of 1-methyl-3-octylimidazolium bromide on the early embryonic development of the frog *Rana nigromaculata*

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**Abstract**

Toxic effects of 1-methyl-3-octylimidazolium bromide ([$C_8$mim]Br) on the early embryonic development of the frog *Rana nigromaculata* were evaluated. Frog embryos in different developmental stages (early cleavage, early gastrula, or neural plate) were exposed to 0, 45, 63, or 88.2 mg/L of the ionic liquid [$C_8$mim]Br for 96 h. The 96-h median lethal concentration values at the early cleavage, early gastrula, and neural plate stages of development were 85.1, 43.4, and 42.4 mg/L, respectively. In embryos exposed to [$C_8$mim]Br, the duration of embryo dechorionation was prolonged in the early cleavage and neural plate, but not the early gastrula, stages of development compared with control embryos. Embryos in the neural plate developmental stage were found to have the highest mortality rate following [$C_8$mim]Br exposure. These results suggest that [$C_8$mim]Br has toxic effects on the early embryonic development of the frog.


*Keywords:* Ionic liquid; *Rana nigromaculata*; Developmental toxicity; Early embryonic development

## 1. Introduction

Ionic liquids (ILs) are compounds consisting entirely of ions with melting points below 100 °C (Sheldon, 2005) that have gained considerable attention in several fields, particularly chemistry, chemical technology, and environmental protection (Jastorff et al., 2003; Ranke et al., 2004, 2007; Docherty and Kulpa, 2005; Pretti et al., 2006). Compared to conventional organic solvents, ILs possess a number of unique properties, such as excellent solubility for a wide range of compounds, high thermal stability, immeasurably low vapor pressure (Sheldon, 2005; Ranke et al., 2007), and a broad temperature range over which they can remain in the fluid state. They have potential for application in chemical separation processes and chemical reactions (Dupont et al., 2002; Rantwijk et al., 2003; Pandey, 2006).

Because of their negligible vapor pressure, ILs have been considered to be 'green' solvents, meaning that they do not evaporate and cause air pollution. Nevertheless, most ILs are water soluble and have poor biodegradability (Garcia et al., 2005; Gathergood et al., 2004). If released into the environment, ILs could enter the aquatic ecosystem and potentially impact aquatic organisms. Therefore, studies on the toxicological properties of ILs are essential for further assessing the risks associated with the use of ILs. To date, few such studies have been performed.

Pernak et al. (2004) have reported that antimicrobial activity increased with an increase in the alkyl chain length of pyridinium, imidazolium, and quaternary ammonium salts. In a preliminary assessment of the toxicity of selected imidazolium ILs toward marine algae, Latala et al. (2005) have found that the growth of *Cyclotella meneghiniana* was effectively inhibited regardless of the ionic liquid concentration applied. Docherty and Kulpa (2005) have reported that hexyl- and octyl-imidazolium and pyridinium bromides had significant antimicrobial action on pure cultures of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Saccharomyces cerevisiae*.

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They also have found that lengthening the alkyl chain led to a concomitant increase in the toxicity of ILs. A similar trend of increased toxicity with increasing alkyl chain length was observed for other organisms, including the marine bacterium *Vibrio fischeri* (Ranke et al., 2004), the soil nematode *Caenorhabditis elegans* (Swatloski et al., 2004), and the freshwater snail *Physa acuta* (Bernot et al., 2005b). In addition, the effects of ILs have been reported on zebrafish (*Danio rerio*) (Pretti et al., 2006), the HeLa human tumor cell line (Stepnowski et al., 2004), the freshwater crustacean *Daphnia magna* (Bernot et al., 2005a), and acetylcholinesterase activity (Stock et al., 2004). Although toxicity testing of ILs was recently reviewed by Ranke et al. (2007), to the best of our knowledge there are no data available on the developmental toxicity of ILs in the frog, *Rana nigromaculata*.

The present study focused on the acute toxicity of 1-methyl-3-octylimidazolium bromide ([$C_8$mim]Br) on the early embryonic development of the frog *R. nigromaculata*. Amphibians are often the main vertebrate group at risk for exposure to contaminants in aquatic systems largely because their larvae live in water (Lahr, 1997; Mann and Bidwell, 2000). As an amphibian model for toxicity testing, the frog has several advantages. Large numbers of fertilized frog eggs are relatively easy to obtain and culture under laboratory conditions. In addition, the embryonic development of the frog is quite rapid and the developmental process can be observed clearly with the naked eye. Finally, the frog is a prevailing species in local areas of China. [$C_8$mim]Br, one common IL with an imidazolium cation, was selected for study because it is easily synthesized in the laboratory, is relatively toxic to organisms, and has more potential industrial applications than other ILs. Our aim was to evaluate the embryonic developmental toxicity of [$C_8$mim]Br on the frog, particularly with respect to its median lethal concentration, as well as its effects on the dechorionation duration, mortality, and malformation of frog embryos in different development stages.

## 2. Materials and methods

### 2.1. [$C_8$mim]Br preparation and chemicals

1-methyl-3-octylimidazolium bromide was prepared as previously described (Bonhôte et al., 1996). Briefly, 273 g (1.205 mol) of freshly distilled 1-bromooctane was added dropwise over 1 h into a vigorously stirred solution of 100 g (1.205 mol) 1-methylimidazolium dissolved in 300 mL of 1,1,1-trichloroethane at 70 °C. The mixture was refluxed for 12 h at 80 °C. The 1-methyl-3-octylimidazolium bromide was decanted from the hot solution in a separatory funnel, washed with ethyl acetate, and dried in an oven at 70 °C under reduced pressure. $^1$H NMR spectra determined for this [$C_8$mim]Br preparation were in good agreement with those reported in the literature (Chun et al., 2001).

All other reagents were obtained from commercial sources, and were of analytical grade.

### 2.2. Embryo collection and incubation

Fertilized eggs of *R. nigromaculata* were collected from the suburbs of Xinxiang, China, during the natural reproduction period of the frog in April 2006 and sent to the laboratory immediately. The eggs were incubated in standard frog embryo teratogenicity assay (FETAX) solution, which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.4 mM KCl, 0.14 mM $CaCl_2$, 0.44 mM $CaSO_4$, and 0.58 mM $MgSO_4$ (ASTM, 1993; Fort et al., 1997; Mann and Bidwell, 2000; Dvořáková et al., 2002). Throughout all experiments, the incubation temperature was 23 ± 1 °C, the oxygen level was 7.7 ± 0.1 mg/L, and the pH was 7.2 ± 0.1. The animals were handled according to the guidelines in the China Law for Animal Health Protection and Instructions for Granting Permit for Animal Experimentation for Scientific Purposes (ethics approval no. SCXK (YU) 2005-0001).

### 2.3. [$C_8$mim]Br exposure and assessment

Test solutions of the IL [$C_8$mim]Br were prepared by pipetting the required amount of 1 g/L stock solution ([$C_8$mim]Br dissolved in distilled water) into known volumes of FETAX solution. The final concentrations of [$C_8$mim]Br used in these studies were 45, 63, and 88.2 mg/L, based on results of previous acute toxicity testing, with [$C_8$mim]Br-free FETAX solution serving as the control. Embryos were transferred with a wide-mouth finely polished pipette into culture dishes (12 cm in diameter) containing 50 mL of test solution. Exposures were performed at three development stages (early cleavage, early gastrula, and neural plate) as described by Wang (1958). Each treatment was replicated two times and included 40 individuals per replicate. The exposure duration was 96 h. Following exposure, the [$C_8$mim]Br-treated embryos were transferred to [$C_8$mim]Br-free FETAX solution. Every 24 h, the number of dead and viable embryos in each dish were recorded, then the exposure solution changed. Dead embryos were removed with the solution change.

Embryonic development was monitored daily for 96 h. The time required for the natural dechorionation of the embryos, the number of embryonic deaths within 96 h, and the morphological malformation of embryos were also evaluated.

The acute toxicity of [$C_8$mim]Br on frog embryos was determined according to Eaton and Klaassen (2001) and Zhang and Liu (1997). Briefly, embryos were placed in culture dishes (12 cm in diameter; 40 individuals per dish) containing 50 mL of test solution. Groups of embryos were exposed to one of five concentrations of [$C_8$mim]Br, with each treatment conducted in triplication. Embryos were exposed at different development stages (early cleavage, early gastrula, or neural plate) for 96 h. The acute toxicity of [$C_8$mim]Br on frog embryos was assessed by calculating the percentage of dead embryos in each of the [$C_8$mim]Br-treated groups, and the $LC_{50}$ was expressed as the median concentration that would kill 50% of embryos after a 96-h exposure at each development stage. The $LC_{50}$ values and their 95% confidence limits were calculated using SPSS 11.5 for Windows.

### 2.4. Statistical analyses

Data were analyzed using one-way analysis of variance followed by least significant difference determination using SPSS 11.5 for Windows. $p < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. 96-h $LC_{50}$

The 96-h $LC_{50}$ value of [$C_8$mim]Br for frog embryos exposed at the early cleavage stage was 85.1 mg/L, with a 95% confidence interval of 79.9–90.6 mg/L, while the values at the early gastrula and neural plate stages were 43.4 mg/L (95% confidence interval, 40.4–46.6 mg/L) and 42.4 mg/L (95% confidence interval, 40.2–44.7 mg/L), respectively.