Evaluation of ionic liquid "greenness"- cytotoxicity of ionic liquids

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Abstract. Ionic liquids have become a hot spot in research and application due to their own superiority. At the same time, the "risk" of ionic liquids has attracted more and more attention. The toxicity of ionic liquids varies according to the target. We have to obtain the toxicity data of ionic liquids on various organisms and cell lines, fill in the blanks of ionic liquid toxicity data and clarify the toxicity mechanism. Only in this way, can we lay the foundation for the design and synthesis of new ionic liquids that are truly non-toxic, environmentally friendly and meet functional requirements. In this experiment, 1-ethyl-3-imidazole diethyl phosphate ([Emim]DEP) was prepared by using 1-methylimidazole and triethyl phosphate as raw materials. The rat cranial anterior osteoblast cell line (MC3T3-E1) was used as the research object, using MTT method, fluorescent death-sense staining observation and flow cytometry to study the cytotoxicity of 1-allyl-3-methylimidazolium chloride ([Emim]DEP) type ionic liquid. This article aims to determine the median lethal concentration by the inhibition rate-concentration curve of [Emim]DEP type ionic liquid and measure cell survival index at this concentration.

1. Introduction

The ionic liquid is a salt, which is composed of an anion and a cation and is in a liquid state at 100 °C or lower [1, 2]. The development of ionic liquids is very rapidly. In 1888, Gabriel reported the world's first ionic liquid. In 1914, Walden et al. prepare the first true room temperature ionic liquid, ethylamine nitrate ([CH₃CH₂NH₂][NO₃]). In 1992, Wilkes et al. synthesized the first ionic liquid that is stable to both water and air, 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF₄]). At this point, the research and application of ionic liquids have attracted the attention of researchers, and a large number of ionic liquids have been synthesized one after another. Nowadays, the research on ionic liquids is deeper. Ionic liquids are not only used in organic reactions, but also in many other fields, such as chemical, pharmaceutical, food, textile, energy, and other fields [3-6].

Ionic liquids are composed of organic cations and inorganic or organic anions, so the physicochemical properties can be adjusted by combining anions and cations to design optimal ionic liquids to meet our needs [7]. Due to its extremely strong plasticity, ionic liquids have strong thermal stability (thermal decomposition temperature is usually between 250 °C and 450 °C), high electrical conductivity (about 10⁻³ S/cm), high solubility, low vapour pressure (10⁻¹¹-10⁻¹⁰ mbar) and
electrochemical window width (4.5-7 V) [8]. The research and development of functional ionic liquids has become the new direction and long-term research of ionic liquids.

Although ionic liquids claim to have "green" properties, in recent years, as scientists have more carefully assessed the life cycle of ionic liquids, different sounds have emerged. Ionic liquids have inevitable defects in preparation, purification, regeneration and application. In the past decade, a large number of articles on the environmental pollution of ionic liquids have been published, and the evaluation of ionic liquid toxicity is currently a hot issue. Ionic liquids are evaluated for toxicity in a number of different biological systems, including enzymes, cells, bacteria, fungi, plants and animals [9-12]. A large number of experimental studies have found that ionic liquids have certain toxic effects on various life-level research objects, such as animal acute toxicity, plant seed germination rate, cell death rate, cell viability, effects on cell morphology, cell membrane permeability and apoptosis.

A large number of literature publications on the environmental safety of ionic liquids have involved the preparation, application and environmental impact of ionic liquids. An important part of ionic liquid environmental risk assessment is its biological toxicity measurement, but the toxicity of ionic liquids varies according to the target. The same ionic liquid will have a strong inhibitory effect on some subjects, while others will show very weak, even no obvious effect. Only obtain the toxic liquid toxicity data of various organisms and cell lines, fill in the ionic liquid toxicity data blank, and clarify its toxicity mechanism, can we better determine its overall ecological environment and human health. The impact of this will lay the foundation for the future design of a new type of ionic liquid that is truly non-toxic, environmentally friendly and meets functional requirements.

2. Experimental

2.1. [Emim]DEP ionic liquid preparation
1-Methylimidazole (25.0000 g, 0.30 mol) and triethyl phosphate (50.0000 g, 0.30 mol) were uniformly mixed, stirred at 80 °C for 8 h under reflux, and then the reaction mixture was stirred at 150 °C for 10 h. The ionic liquid was cooled to room temperature and washed with diethyl ether (4×30 mL). And then the ionic liquid was evaporated under reduced pressure at 75 °C for 4 h to remove all volatile residues and vacuum dried at 80 °C for 48 h to obtain a light yellow liquid 1-Ethyl-3-methylimidazolium diethylphosphate ([Emim]DEP).

2.2. MTT test detection [Emim]DEP type ionic liquid IC_{50}
After 7 days of MC3T3-E1 cell culture, the bottom of the culture flask was substantially covered, and the cell suspension was prepared by trypsin digestion, pipetting, centrifugation, etc. The cell density was measured by a hemocytometer, and the cell density was adjusted to 3×10^5 cells/mL. Inoculate 100 μL of the cell suspension in a 96-well plate, and after the cells are attached, add the ionic liquid (0 μg/mL, 1500 μg/mL, 2000 μg/mL, 2500 μg/mL, 3500 μg/mL, 5000 μg/mL) to be tested. The wells inoculated with the cells were cultured in an incubator, and the proliferation of MC3T3-E1 cells at 24 h, 48 h and 72 h of the ionic liquid action was measured by MTT method.

2.3. Annexin V-FITC/PI apoptosis detection
Semi-quantitative analysis was performed using flow cytometer to detect the degree of apoptosis in MC3T3-E1 cells. The cells were seeded on a six-well plate at the density of 1×10^6 cells/well, and the cells were attached to the ionic liquid to be tested for 12 h after the cells were attached. At the end of the ionic liquid treatment, cells were harvested and flow cytometry was performed immediately after the addition of Annexin V-FTIC and PI fluorescent dyes by standard methods. The number of cells per sample should be at least 1×10^4 cells/well. Because the experiment uses AnnexinV-FTIC and PI two fluorescent markers for cell labeling, it is necessary to set up negative control, FTIC single positive and PI single positive. In the control group, it is generally necessary to add 0.05% DMSO to promote dissolution, and the cells floating during the collection of cells should also be collected.
2.4. Fluorescence staining to observe the effect of [Emim]DEP type ionic liquid on cell proliferation

The cell suspension was prepared by trypsin digestion, pipetting, centrifugation, etc. The cell density was measured by a hemocytometer and the cell density was adjusted to $1 \times 10^5$ cells/mL. The cell suspension was inoculated into a 96-well plate. After the cells were attached, different concentrations of ionic liquid were added. The wells inoculated with the cells were cultured for 24 h in an incubator. The cells were observed by complex staining with three fluorescent stains.

3. Results and discussion

3.1. Characterization of Ionic liquid

The nuclear magnetic properties of 1-ethyl-3-methylimidazolium diethylphosphate was: $^1$HNMR (d6-DMSO with 0.03% v/v TMS, 600MHz): δ 1.07[t, 6H, J=7.1 Hz], 1.42[t, 3H, J=7.3 Hz], 3.62[p, 4H, J=7.0 Hz], 3.87[s, 3H], 4.22[q, 2H, J=7.3 Hz], 7.76[t, 1H, J=1.6 Hz], 7.85[t, 1H, J=1.7 Hz], 9.56[s, 1H]; $^{13}$CNMR (d6-DMSO with 0.03% v/v TMS, 151MHz): δ 15.1, 16.7, 35.6, 44.0, 59.0, 121.9, 123.5, 136.9. The mass spectrometry results are shown in figure 1. The test value is 111.0883, and the calculated value is 111.0901, which proves that the synthesized ionic liquid structure is correct.

3.2. IC$_{50}$ value of cells treated with [Emim]DEP ionic liquid

The results of the MTT experiment are shown in figure 2 (a). Compared with the blank group, the OD value of the experimental group was significantly decreased, indicating that the [Emim]DEP type ionic liquid inhibited the proliferation of MC3T3-E1 cells. As the concentration of ionic liquid increased, the OD value of the experimental group decreased and the inhibition of proliferation became stronger. From the time dimension, the OD value increased with the action time of ionic liquid in the concentration range of 0-1500 μg/mL, indicating that the inhibition of proliferation of ionic liquid in this range was weaker than that of cells. The proliferation inhibition effect of ionic liquid of 2000 μg/mL is consistent with the cell proliferation, and the inhibition of proliferation of ionic liquid is stronger than cell proliferation above 2500 μg/mL.

Figure (b-d) shows the cell inhibition rate-concentration curves measured by MTT assay. The goodness of fit of the fitting curve of 24 h is $R^2=0.99996$, the fitting curve of 48 h is $R^2=0.96117$ and fitting curve of 72 h is $R^2=0.96117$. The fitting effect is good and the fitting standard is reached. According to the fitting curve, the IC$_{50}$ value of [Emim]DEP type ionic liquid was 3959 μg/mL after 24 h, and the IC$_{50}$ value was 2226 μg/mL after 48 h, and its IC$_{50}$ value was 1884 μg/mL after 72 h.

The cell count of Annexin V/PI staining was used to analyze the state of MC3T3-E1 cells after ionic liquid treatment by flow cytometry. The results are shown in figure (e). The apoptotic cells in the control group (0 μg/mL) accounted for only 2.6% of the total number of cells, and most of the cells grew normally. With the increase of the concentration of ionic liquid treatment, the percentage of cells entering the apoptotic state increased to 21% (1000 μg/mL), 49.3% (3959 μg/mL), and 84% (5000 μg/mL). As expected, the higher the concentration of ionic liquid, the greater the proportion of
apoptotic and necrotic cells, indicating that the ionic liquid controls the growth of MC3T3-E1 cells by activating the pathway that promotes apoptosis. When the concentration of ionic liquid is 3959 μg/mL, apoptosis and necrotic cells accounted for 49.3% of the total number of cells, which was consistent with the IC$_{50}$ value measured by the previous MTT assay.

![Figure 2](Color on line). (a) OD value of MC3T3-E1 cells treated with different concentrations of [Emim]DEP type ionic liquid for different time. (b-d) [Emim]DEP type ionic liquid for 24 h, 48 h, 72 h after MC3T3-E1 cell inhibition rate-concentration fitting Curves. (e) MC3T3-E1 cells were treated with different concentrations of [Emim]DEP ionic liquid and apoptosis was detected by Annexin V-FITC/PI staining flow cytometry.

3.3. Compound fluorescence staining to observe cell life and death

After the [Emim]DEP type ionic liquid was applied, the MC3T3-E1 cells were fluorescently stained. The results of fluorescent staining of cells after 24 h of ionic liquid treatment are shown in figure 3. In the blank group without ionic liquid, MC3T3-E1 cells adhered to the wall and covered 80% of the bottom of the well plate, and the cells were mutually connected to form a network of cells. As the concentration of ionic liquid increased, the green fluorescence decreased and the red fluorescence increased significantly. In Hoescht staining, the number of nuclear deep staining increased significantly, indicating that the number of MC3T3-E1 living cells decreased significantly with the increase of ionic liquid concentration. Experimental results show that ionic liquid has obvious inhibition of proliferation on MC3T3-E1 cells.
Fluorescence staining of MC3T3-E1 cells after [Emim]DEP type ionic liquid treated 24 h (40×) Scale: 500 μm.

Fluorescence staining at magnification is shown in figure 4. Figure 4 (a) shows the results of Calcein staining. When the concentration of ionic liquid is 0 and 1000 μg/mL, the number of cells in the microscope field is about the same, but the cells in figure 4 (a₁) spread well and the cell morphology is intact. Some cells showed normal apoptotic state and the morphology of cells in figure 4 (a₂) was almost complete, but most of the cells showed obvious organelle rupture. The number of figure 4 (a₁) and figure 4 (a₄) cells was significantly reduced. The reason was that a large number of cells were apoptotic under the action of ionic liquid, and the adhesion ability of dead cells is reduced and falls off from the bottom of the culture bottle, and the morphology of the cells changes, which has a tendency to shrink.

Figure 4 (b) shows Hoechst staining. As the concentration of ionic liquid increases, the number of blue fluorescence decreases significantly, indicating that the number of living cells is significantly reduced, and the number of dead cells is significantly increased. The cell adhesion is significantly reduced due to cell death, and it cannot continue adherent growth on the well plate resulted in a significant decrease in blue fluorescence. In addition, after apoptotic cells were stained, the nucleus was dark blue. From figure 4 (b), the proportion of nuclear deep staining was observed to increase, indicating that the proportion of apoptosis increased with the increase of ionic liquid concentration. When the concentration of the ionic liquid is 5000 μg/mL and the duration of action is 24 h, the cells are completely broken and there is no intact cell morphology in the visual field.

Figure 4 (Color on line). (a) Calcein staining of MC3T3-E1 cells after [Emim]DEP-type ionic liquid treated for 24 h. (b) Hoechst staining of MC3T3-E1 cells after [Emim]DEP-type ionic liquid treated for 24 h. The concentration of 1-4 is 0, 1000, 3959, 5000 μg/mL respectively. Scale: 200μm
Conclusion
In this experiment, 1-ethyl-3-methylimidazolium diethylphosphate ([Emim]DEP) was prepared by "two-step synthesis" with 1-methylimidazole and triethyl phosphate as raw materials. And testing including MTT test, flow cytometry and complex fluorescence staining for scientific exploration. It can be seen from the results of MTT test that [Emim]DEP-type ionic liquid has an inhibitory effect on the proliferation of MC3T3-E1 cells. This effect has time-related and concentration-related effects and the inhibitory effect increased with the action time and the concentration of action Enhanced.

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