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Investigation of dermal toxicity of ionic liquids in monolayer-cultured skin cells and 3D reconstructed human skin models

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ABSTRACT

Ionic liquids have gained increasing attention in the chemical industry as potential green substitutes for traditional solvents. However, little is known about toxicity of ionic liquids on the skin, a major exposure portal to toxic substances. Here, we evaluated dermal toxicity of ionic liquids using human keratinocyte and fibroblast cell line, 3D reconstructed human epidermis, and full-thickness model to investigate underlying mechanisms. Cytotoxicity of ionic liquids was evaluated for representative anions, [TFSI], [PF_6], [BF_4], and [DCA], as well as for cations, [EMIM], [BMPY], [TBA] and [Zn], in human keratinocyte cell line, HaCaT, and human dermal fibroblasts. In our results, significant cytotoxicity was induced by ionic liquids with [TFSI] in both cell lines. Notably, cytotoxicity of [TFSI] containing ionic liquids was comparable to xylene, a toxic conventional organic solvent. Fluorescent and flow cytometric analysis revealed that [TFSI]-exposed cells underwent necrotic cell death. Reactive oxygen species (ROS) was increased while the amount of glutathione was decreased by [TFSI] in dose-dependent manner, which was reversed by antioxidant, *N*-acetylcysteine. In 3D reconstructed human epidermis and full-thickness model, a single application of [TFSI] induced toxicity although it was minimal and largely limited to epidermal layer. Collectively, these results demonstrated potential dermal toxicity of ionic liquids.

1. Introduction

Although toxic and hazardous solvents are commonly used, their potential risk against people's health and environment should not be neglected. “Green chemistry,” which is defined as the design of chemical products and their processes to either eliminate or reduce the use and generation of hazardous substances (Anastas and Kirchhoff, 2002), has become a new norm in the chemical industry. In the aspect of “green chemistry,” “green solvents” are drawing attention as solvents are among the most heavily consumed materials in terms of “quantity.” There are four approaches for the employment of green solvents (Capello et al., 2007): (i) substitution of toxic and hazardous solvents with the ones that show better environmental, health, and safety (EHS) properties (Curzons et al., 1999); (ii) use of “bio-solvents”; (iii) substitution of organic solvents either with environmentally harmless supercritical fluids (Nalawade et al., 2006); or (iv) with ionic liquids showing low vapor pressure, and thus leading to less emission to the atmosphere (Lévêque and Cravotto, 2006).

Among these options, ionic liquids have received attention as green substitutes for traditional organic solvents. Market size of ionic liquids is estimated to reach 39.6 million USD by 2021, with a compound annual growth rate (CAGR) of 9.2% between 2016 and 2021 (Markets, 2016). Ionic liquids are mixtures of cations, anions, and molten salts with melting points around 100 °C (Moosavi, 2013). As ionic liquids are composed only of ions, their vapor pressures are negligible compared to traditional solvents that belong to volatile organic compounds (VOCs). The broad range of anion-cation combinations allow easy control of various solvent properties (Stepnowski et al., 2004). These desirable properties and enormous structural diversities fuel the demand for ionic liquids as they can be applied in various fields, such as catalytic synthesis, coordination chemistry, analytical chemistry, polymer materials, and nanotechnology, *etc.* (Zhao et al., 2007), as reviewed in detail (Welton, 1999).

While environmental friendliness of ionic liquids can be assured,

Abbreviation: TFSI, bis(trifluoromethanesulfonyl)imide; EMIM, 1-ethyl-3-methylimidazolium; PF6, hexafluorophosphate; BF4, tetrafluoroborate; DCA, dicyanamide; BMPY, 1-butyl-1-methylpyrrolidinium; TBA, tributylmethylammonium

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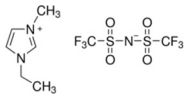
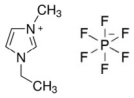
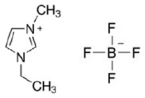
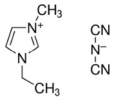
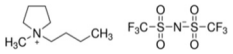
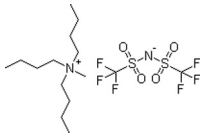
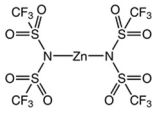
Table 1
Representative ionic liquids tested and their UN GHS category for skin irritation.

IUPAC name/Trade name	Acronym	M.W.	CAS number	Chemical formula	ECHA (2016) Hazard category for skin irritation/corrosion
1-ethyl-3-methylimidazolium bis (trifluoromethanesulfonyl)imide	[EMIM] [TFSI]	391.31	174899-82-2		Category 1B Category 2
1-ethyl-3-methylimidazolium hexafluorophosphate	[EMIM] [PF_6]	256.13	155371-19-0		Category 1B Category 2
1-ethyl-3-methylimidazolium tetrafluoroborate	[EMIM] [BF_4]	197.97	143314-16-3		Category 1C Category 2
1-ethyl-3-methylimidazolium dicyanamide	[EMIM] [DCA]	177.21	370865-89-7		Category 2
1-butyl-1-methylpyrrolidinium bis (trifuloromethanesulfonyl)imide	[BMPY] [TFSI]	422.40	223437-11-4		Category 2
tributylmethylammonium bis(trifuloromethanesulfonyl) imide	[TBA] [TFSI]	480.53	405514-94-5		–
Zinc di[bis(trifuloromethanesulfonyl)imide]	[Zn] [$TFSI]_2$	625.65	168106-25-0		–

their effects on human health are not well established. Several research on toxicity of ionic liquids have been performed mainly concerning environmental toxicology (Jastorff et al., 2003), while cytotoxicity of ionic liquids has been reported to employ common cell-lines, such as HeLa (Stepnowski et al., 2004), CaCo-2 (Frade et al., 2009), MCF7 (Kumar et al., 2009), and PC12 (Samorì et al., 2010), without a sound toxicological rationale. Skin is one of the tissues that are directly exposed to toxic substances in the working environment and everyday life (Baudouin et al., 2002; Zanoni et al., 2014); therefore, outcome of chemical exposure to the skin should be fully studied for industry chemicals. EU REACh (Registration and Evaluation of Chemicals) also mandates testing of skin irritation or corrosion for chemicals that are used over 1 ton *per annum* before they are released into the market (Ha et al., 2016). In fact, ECHA (European Chemical Agency) database search (https://www.echa.europa.eu/search-for-chemicals, access date April 1st 2017) (ECHA, 2016) categorized some ionic liquids as irritants or corrosive (Table 1). However, to our best knowledge, toxicity of ionic liquids on the skin has not been fully established.

Here, we evaluated dermal toxicity of seven representative ionic liquids, [EMIM][TFSI], [EMIM][PF_6], [EMIM][BF_4], [EMIM][DCA], [BMPY][TFSI], [TBA][TFSI], and [Zn][$TFSI]_2$, using human keratinocyte cell line, HaCaT, and human fibroblast cell line, Hs68, and investigated their toxic mechanisms to assess the impact of ionic liquids on human health. We also used a 3D human epidermis and full-thickness model, which has *in vivo*-like morphological and growth characteristics, to further evaluate dermal toxicity of ionic liquids.

2. Materials and methods

2.1. Ionic liquids

The following ionic liquids used in our experiments were purchased from Tokyo Chemical Industry (Tokyo, Japan): [EMIM][TFSI], [EMIM][PF_6], [EMIM][BF_4], [EMIM][DCA], [BMPY][TFSI], [TBA][TFSI], and [Zn][$TFSI]_2$. Full names and chemical formula of these compounds, along with information on UN GHS skin irritation/corrosion categories, are listed in Table 1. FITC-Annexin V, PI, and 10 × binding buffer were purchased from BD Pharmingen (San Diego, CA, United States).

2.2. Materials

Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), and antibiotic solution (penicillin 10,000 units/mL and streptomycin 10,000 μg/mL) were purchased from Hyclone, GE healthcare (Little Chalfont, United Kingdom). Trypan blue and 0.05% trypsin-EDTA were purchased from Gibco, Thermo Fisher (Waltham, MA, United States). Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), *N*-acetyl-L-cysteine (NAC), hydrogen peroxide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Water-soluble tetrazolium salt (WST-1) was purchased from Roche (Basel, Switzerland). 2′,7′-dichlorofluorescin diacetate (DCFDA) was purchased from Life Technologies (Carlsbad, CA, United States). FITC-Annexin V, propidium iodide, and 10 × binding buffer were purchased from BD Pharmingen (San Diego, CA, United States). FITC-Annexin V, Ethidium Homodimer III, Hoechst 33342, and 5 × binding buffer were purchased from Promokine (Heidelberg, Germany). 3D reconstituted human full skin, Keraskin-FT™, and epidermis model, Keraskin™ (Jung et al., 2014), were purchased from Biosolution Co. (Seoul, Korea).

HaCaT (gift from AmorePacific Co. Yongin, Korea) and Hs68 (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C and 5% CO_2. Cells were harvested at 80% confluence using 0.05% trypsin-EDTA, and washed once with DMEM before each experiment.

2.3. Treatment of chemicals

Cells were then seeded in 48 well plates at density of 5×10^4 cells/well, or in 6 well plates at density of 3×10^5 cells/well. Stock solutions of the ionic liquids (2 M) were prepared in culture medium or DMSO.