Ecotoxicology and Environmental Safety 137 (2017) 103–112

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Two zinc-aminoclays' *in-vitro* cytotoxicity assessment in HeLa cells and *in-vivo* embryotoxicity assay in zebrafish

Hang-Suk Chun[a], Duckshin Park[b], Song Eun Lim[c], Kwang-Hun Jeong[c], Ji-Seon Park[a,d], Han-Jin Park[a], Shinyoung Kang[e], Kyoung Suk Kang[e], Hyun Gyu Park[e], Ha-Rim An[f], Yun Suk Huh[g], Young-Chul Lee[c,*]

[a] Department of Predictive Toxicology, Korea Institute of Toxicology (KIT), Daejeon 34114, Republic of Korea
[b] Korea Railroad Research Institute (KRRI), 176 Cheoldobakmulkwan-ro, Uiwang-si, Gyeonggi-do 16105, Republic of Korea
[c] Department of BioNano Technology, Gachon University, 1342 Seongnamdaero, Sujeong-gu, Seongnam-si, Gyeonggi-do 13120, Republic of Korea
[d] Human and Environmental Toxicology, School of Engineering, University of Science and Technology, Daejeon 34113, Republic of Korea
[e] Department of Chemical and Biomolecular Engineering (BK21 + program), KAIST, 291 Daehakno, Yuseong-gu, Daejeon 34141, Republic of Korea
[f] Advanced Nano-surface Research Group, Korea Basic Science Institute (KBSI), Daejeon 34133, Republic of Korea
[g] Department of Biological Engineering, College of Engineering, Inha University, Incheon 402-751, Republic of Korea

ARTICLE INFO

Keywords:
Zn-aminoclays
Cytotoxicity
HeLa cells
Embryotoxicity
Zebrafish

ABSTRACT

Two zinc-aminoclays [ZnACs] with functionalized primary amines [$(-CH_2)_3NH_2$] were prepared by a simple sol-gel reaction using cationic metal precursors of $ZnCl_2$ and $Zn(NO_3)_2$ with 3-aminopropyl triethoxysilane [APTES] under ambient conditions. Due to the facile interaction of heavy metals with primary amine sites and Zn-related intrinsic antimicrobial activity, toxicity assays of ZnACs nanoparticles (NPs) prior to their environmental and human-health applications are essential. However, such reports remain rare. Thus, in the present study, a cell viability assay of *in-vitro* HeLa cells comparing $ZnCl_2$, $Zn(NO_3)_2$ salts, and ZnO (~50 nm average diameter) NPs was performed. Interestingly, compared with the $ZnCl_2$, and $Zn(NO_3)_2$ salts, and ZnO NPs (18.73/18.12/51.49 μg/mL and 18.12/15.19/46.10 μg/mL of IC_{50} values for 24 and 48 h), the two ZnACs NPs exhibited the highest toxicity (IC_{50} values of 21.18/18.36 μg/mL and 18.37/17.09 μg/mL for 24 and 48 h, respectively), whose concentrations were calculated on Zn elemental composition. This might be due to the enhanced bioavailability and uptake into cells of ZnAC NPs themselves and their positively charged hydrophilicity by reactive oxygen species (ROS) generation, particularly as ZnACs exist in cationic NP's form, not in released Zn^{2+} ionic form (*i.e.*, dissolved nanometal). However, in an *in-vivo* embryotoxicity assay in zebrafish, ZnACs and ZnO NPs showed toxic effects at 50–100 μg/mL (corresponding to 37.88–75.76 of Zn wt% μg/mL). The hatching rate (%) of zebrafish was lowest for the ZnO NPs, particularly where ZnAC-[$(NO_3)_2$] is slightly more toxic than ZnAC-[Cl_2]. These results are all very pertinent to the issue of ZnACs' potential applications in the environmental and biomedical fields.

1. Introduction

Hybrid nanomaterials (Saleh et al., 2015) or single metal oxide nanoparticles (NPs) (Wehmas et al., 2015) such as TiO_2, CeO_2, SnO_2, and ZnO, among others, have caused environmental, health and safety concerns. Among engineered nanoscale semiconductor metal oxides, ZnO NPs, owing to their large surface-area-to-volume ratio, UV–visible absorption spectra, and long lifespan (Yu et al., 2004), are increasingly popular for employment in cosmetic/biomedical applications such as sunscreens, foot care, ointments and over-the-counter topical products, pigments and antimicrobial coatings for ultraviolet (UV) protection and fungicidal action in paints (Hu et al., 2003; Jones et al., 2008; Roduner, 2006), gas sensors (Lin et al., 1998), electronic devices (Kim et al., 2008; Ozgur et al., 2010; Sharma et al., 2015), and photocatalysts (Bai et al., 2013; Chakrabarti and Dutta, 2004; Curri et al., 2003; Lee et al., 2014b). Despite the common exposure to ZnO NPs in diverse routes of skin, airways and environments (Osmond and Mccall, 2010), the researching on the safety of ZnO NPs for animal cells are still demanding and its accurate mechanism is debating in academic fields (Vandebriel and De Jong, 2012).

As a result, prior to ZnO NPs' applications, concentration-dependent cytotoxicity assessment of ZnO NPs has been actively pursued for

* Corresponding author.
E-mail address: dreamdbs@gachon.ac.kr (Y.-C. Lee).

http://dx.doi.org/10.1016/j.ecoenv.2016.11.022
Received 16 September 2016; Received in revised form 22 November 2016; Accepted 25 November 2016
Available online 19 December 2016
0147-6513/

various cell types (see Table S1, Supplementary Information) (Vandebriel and De Jong, 2012). *In-vitro* immunotoxicity in mammalian systems (Roy et al., 2015) and ecotoxicity results (Ma et al., 2013) of ZnO NPs were focused on cytotoxicity mechanism, then resulting in higher concentration causes cell death. It has long been known that ZnO NPs can indirectly produce reactive oxygen species (ROS) by fully occupying oxidative stress defense compounds such as metallothionein, thereby preventing them from binding Fe or Cu ions, which remain free to catalyze Fenton-type reactions (Chevion, 1988). Cells exposed to ZnO NPs thus can suffer marked decreases in mitochondrial membrane potential as well as increases in both hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) levels (Guo et al., 2013). Additionally, caspase-12 mediates an endoplasmic reticulum (ER)-specific apoptosis pathway, thus effecting cell death (Nakagawa et al., 2000). When, in a recent study, target cells were exposed to increasing levels of ZnO NPs, concentration-dependent increases in caspase-12 levels were observed (Guo et al., 2013). Therefore, other research groups have attempted to modify ZnO NPs' surfaces to reduce their toxicity and potentiate their "smart" role as a targeted delivery carrier in biomedical applications (Owens et al., 2016; Punnoose et al., 2014). For example, silica (SiO_2) and poly methyl acrylic acid (PMAA) (Ramasamy et al., 2014; Yin et al., 2010), chitosan (Li et al., 2010) and surfactant-coated ZnO NPs [27] were found to reduce ROS-generation levels without affecting UV absorbance (Ramasamy et al., 2014; Yin et al., 2010).

In 1997, Mann et al. developed 3-aminopropyl-functionalized magnesium phyllosilicate (magnesium aminoclay, MgAC). Beyond bio(nano)composites (Mann, 2009), research into MgAC has expanded from environmental to bio-medical applications; among the achievements are effective removal in oxyanions (Lee et al., 2011a) and heavy metals (Lee et al., 2011b) and the minimization of *in-vitro* cytotoxic effects in mammalian cells (Han et al., 2011b) and *in-vivo* mice (Yang et al., 2014). Recently, Lee et al. reported that carbon-nanodot-conjugated FeAC showed no toxic effects on HeLa cells *in-vitro* based on simultaneous bio-imaging (Kang et al., 2015). Interestingly, ZnACs with an injection of NaOH at 338 K or 373 K temperatures were synthesized by Airoldi et al. (da Fonseca et al., 2004) and applied to the removal of toxic Cu^{2+} ions by coordination with primary amines between layers bonded to inorganic frameworks. AC-family preparations have been actively developed for multifaceted applications (Datta et al., 2013). However, relevant cytotoxicity studies of ACs have as yet been rare, and the available dataset is limited, despite their importance to the potential environmental and human-health applications, compared to previously reported cytotoxicity evaluation of ZnO NPs (Vandebriel and De Jong, 2012).

In the present study, in order to investigate the feasibility of two ZnACs NPs for environmental and biomedical applications, a cytotoxicity assay in HeLa cells was performed in comparison with ZnO NPs (~50 nm), 3-aminopropyltriethoxysilane (APTES), $ZnCl_2$, and $Zn(NO_3)_2$ as precursors of ZnAC preparation. Additionally, the two ZnACs and ZnO NPs were tested for *in-vivo* embryotoxicity in zebrafish. In order to reveal the cytotoxicity mechanisms of the ZnACs and ZnO NPs, their cellular uptakes were investigated by intracellular ROS measurement and fluorescence-activated cell sorting (FACS) as well as observing by confocal microscopy.

2. Material and methods

2.1. Chemicals used in this study

Zinc oxide (ZnO, ~50 nm average diameter, 81.39 g/mol), zinc chloride ($ZnCl_2$, 136.30 g/mol), zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, 297.49 g/mol), (3-aminopropyl) triethoxysilane (APTES, 221.37 g/mol), fluorescein isothiocyanate isomer I (FITC, 389.38 g/mol), 2′,7′-dicholorofluorescin diacetate (DCFH-DA, 487.29 g/mol), dipyridamole (504.63 g/mol), dimethyl sulfoxide (DMSO, 78.13 g/mol), phosphate-buffered saline (PBS) tablets (1 tablet is dissolved in 200 mL of deionized [DI] water to yield 0.01 M phosphate buffer, pH 7.4 at 25 °C), cell-proliferation reagent water-soluble tetrazolium salt-1 (WST-1), and trypan blue (0.4%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bulk ethanol (18 L) was received from Samchun Pure Chemical Co. Ltd (Gyeonggi-do, Korea). Roswell Park Memorial Institute(RPMI) 1640, fetal bovine serum (FBS), and trypsin-EDTA (0.05%) were supplied by Gibco® *via* Life Technologies (Rockville, MD, USA), and and L-Glutamine 200 mM (100×) and Penicillin/Streptomycin (Pen/Strep, 100×) were obtained from Invitrogen (Carlsbad, CA, USA). Staining of HeLa cells was performed with the Annexin V apoptosis detection kit 1 (BD Pharmingen®, San Diego, CA, USA) for *fluorescence-activated cell sorting* (FACS) measurement. Milli-Q DI water was used throughout the experiments (conductivity < 18.2 MΩ cm^2;Milli-Q Millipore filter system; Millipore Co., Bedford, MA, USA).

2.2. Preparation of two ZnACs

Each ZnAC, ZnAC-[Cl_2] and ZnAC-[$(NO_3)_2$], was prepared using $ZnCl_2$ and $Zn(NO_3)_2 \cdot 6H_2O$ precursors, respectively. Briefly, ~8.4g of $ZnCl_2$ or $Zn(NO_3)_2 \cdot 6H_2O$ was dissolved in 200 mL of ethanol solution. After 10 min magnetic stirring, ~13 mL of APTES was drop-wise added to the Zn ethanolic solution (da Fonseca et al., 2004) and adjusted to ~1.34 Zn:Si molar ratio by a simple sol-gel reaction where hydrolysis and condensation of organosilanes results in production of network of siloxane [Si-O-Si] bonds (da Fonseca et al., 1999). The resultant solution was stirred overnight preparatory to its collection in white-slurry form. Then, each ZnAC was centrifuged at 3000 rpm for 15 min, after which the separated ZnAC was washed twice with 100 mL of ethanol solution, oven-dried at 60 °C for 24 h, and finally powdered by pestle and mortar and denoted ZnAC-[Cl_2] or ZnAC-[$(NO_3)_2$].

2.3. Characterizations of ZnAC-[Cl_2], ZnAC-[$(NO_3)_2$], ZnO, $ZnCl_2$, and $Zn(NO_3)_2$

Morphological micrographs of ZnAC-[Cl_2], ZnAC-[$(NO_3)_2$] and ZnO at 0.5 mg/mL dispersed in DI water were observed by field-emission transmission electron microscope (FE-TEM, 200 kV, Tecnai F20, Philips, Netherlands) at the KARA research center. A cold-type field-emission scanning electron microscopic (FE-SEM-4700) images by equipped with an energy-dispersive X-ray spectrometer (EDX) were captured. Powder X-ray diffraction (XRD) patterns of $ZnCl_2$, ZnAC-[Cl_2], $Zn(NO_3)_2$, ZnAC-[$(NO_3)_2$], and ZnO were examined by using normal powder X-ray diffractometer (D/MAX-2500, RIGAKU, USA) at 40 kV and 300 mA, scanning at $2\theta = 3–70^\circ$ in 0.02 increments. High-resolution X-ray photoelectron spectroscopy (HR-XPS) with monochromatic Al Kα X-ray radiation ($h\nu$ = 1486.6 eV) operated at 120 W (Kratos Analytical, AXIS Nova, Manchester, UK) was employed to investigate the surface chemical interaction in O1s, Zn_{2p}, and N_{1s} species for the ZnAC-[Cl_2], ZnAC-[$(NO_3)_2$], and ZnO. The shift of the binding energy as a result of relative surface charging was corrected using the C_{1s} level at 284.6 eV as an internal standard (Lee et al., 2014a). The covalent bondings of the organic functionalities in ZnACs were recorded by pellet-mode Fourier transform infrared (FTIR) spectrometry (FT-IR 4100, Jasco, Japan). The Zn (wt%) content in the ZnACs and ZnO was analyzed by energy-dispersive X-ray (EDX) analysis. The UV–Vis spectra of ZnAC-[Cl_2] and ZnAC-[$(NO_3)_2$] at 0.625 mg/mL, and of ZnO at 0.156 mg/mL, were taken by UV–vis spectrophotometry (VARIAN, 50 Bio CARY, USA). Both the zeta potential and the dynamic light scattering (DLS)-based hydrodynamic size of ZnAC-[Cl_2], ZnAC-[$(NO_3)_2$], and ZnO at 0.625 mg/mL and dispersed in serum-containing media were measured by particle-size analysis (Zetasizer Nano ZS, Malvern, UK). The released Zn^{2+} ion concentrations of ZnO-treated HeLa cells and zebrafish culture media were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES, OPTIMA 7300 DV, Perkin-Elmer, USA). The samples of