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Research paper

Cytotoxicity of natural allophane nanoparticles on human lung cancer A549 cells

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ABSTRACT

Clay minerals are mainly used around traditional cosmetics and industrial products, but currently their target application is continuously expanding into pharmaceutical industry and tissue engineering. To broaden the knowledge of in vitro cytotoxicity of allophane nanoparticles against human cancer cells, the cytotoxicity of both natural and synthetic allophane nanoparticles for cultured human alveolar basal epithelial (A549) cells was examined. For both natural and synthetic allophones, the A549 cell viability was maintained at >70% for concentration up to 3160 μg/mL, implying higher biocompatibility of allophane nanoparticles as compared with that of hectorite nanoparticles. The cell adhesion kinetics coupled with cytotoxic characteristics against A549 cells was analyzed using quartz crystal microbalance (QCM) technique to distinguish the dynamic cell adhesion signatures.

1. Introduction

Clays are commonly used in excipients and/or active substances in the pharmaceutics. Several studies are related with the reduction in the oral absorption of numerous medicines by way of the coadministration with clays. Nevertheless, such interactions may also be used to reach technological and biopharmaceutical advantages. In this regard, naturally abundant clay minerals (e.g., montmorillonite (Mt)) possessing multilayered structure and encapsulated organic compounds could be applied as a drug carrier for pharmaceutical materials (Aguzzi et al., 2007). This was the origin in the use of clays in systems of modern drug release.

During the past five years, the cell-based regenerative researches using clay nanoparticles provided new insights for the tissue engineering/regenerative medicine (TERM) (Kommireddy et al., 2005; Lewkowitz-Shpuntoffa et al., 2009; Dawson et al., 2011; Gaharwar et al., 2011; Mieszawska et al., 2011; Dawson et al., 2012). To enhance cell growth clay particles have been employed as encapsulating components within a polymer matrix. One of these studies demonstrated that the effectiveness of Laponite as an encapsulation medium to store human bone marrow stromal cells (HBMSCs) for 4 weeks. This approach has been applied for stabilization of the tubule formation of cultured human umbilical vein endothelial cells (HUVECs) (Dawson et al., 2011, 2012). In another study, multilayer halloysite shows the better adhesion of human fibroblasts, with fibroblasts maintaining their cellular phenotype (Kommireddy et al., 2005). All those properties of clays have suggested new opportunities to enhance differentiation/proliferation of fibroblast cells for bone regeneration, as well as functions for promoting osteogenesis.

On the other hand, there are only a few reports on toxicological effects upon exposure. High doses of organically-modified Mt with a polymer matrix (ethylene vinyl acetate copolymer) significantly reduced MC3T3-E1 mouse calvaria pre-osteoblasts proliferation (Lewkowitz-Shpuntoffa et al., 2009). Based on past research regarding potential toxicity of Mt particles (Li et al., 2010; Gaharwar et al., 2011; Lordan et al., 2011; Baek et al., 2012; Verma et al., 2012), Mt could cause no acute oral cytotoxic effects at high concentration in mice (up to 1000 mg/kg body weight) after long-time exposure. The Mt particles could be absorbed into the body within 2 h, it was not observed significantly accumulation in any specific organ (Baek et al., 2012). Laponite particles added directly to cell culture media did not exhibit loss of viability of MC3T3-E1 cells at high doses of 35 mg/mL (Gaharwar et al., 2011). Li et al. reported an interesting approach to evaluate the cytotoxicity of exfoliated Mt particles in Chinese hamster ovary (CHO) cells in vitro after 24 h incubation at concentrations up to 1000 μg/mL by using Mt (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) and LDH (lactate dehydrogenase activity) assays (Li et al., 2010). A low cytotoxicity <1 mg/mL (half maximal inhibitory concentration (IC_{50}) > 1 mg/mL) on CHO cells and dose-dependent effect were found after 24 h incubation. At the same time, the safety of the Mt particles for potential uses in biomedical areas was demonstrated. The researchers observed that

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the Mt particles could accumulate and adhered on the surface of cells. Nevertheless, the cell morphology does not show any obvious changes. Ironically, the presence of clay alters cells in permeability as revealed by releasing LDH. The possible reason behind the mechanism of interacting with living cells is not well explored in the literature. Furthermore, many studies have observed cytotoxicity only at high dose condition. Further information on the potential toxicity of clays and their mechanisms of toxicity are needed to fully understand their hazards.

Allophane (1-2 $SiO_2Al_2O_3$ 5-6H_2O) is a short-range-order clay mineral and occurs in some soils derived from volcanic ejecta and is able to protect the extracellular DNA and RNA molecules from ultraviolet light. The primary particle of the allophane is a hollow spherule with an outer diameter of 3.5–5.0 nm and a wall of about 0.6–1.0 nm thick, which has perforations (Brigatti et al., 2006; Iyoda et al., 2012). The surface area of allophane is as high as ~1000 m^2/g, which is often larger than activated carbon. In addition to this large surface area, the (OH)Al(OH_2) groups exposed on the wall perforations are the source of the pH-dependent charge characteristics of allophane nanoparticles.

Previous study recorded the morphological investigation to provide insight into the adsorption structure and characteristics of single-stranded DNA (ss-DNA) adsorbed by the allophane particles, which was the first time of the real images obtained from microscopic experiment (Matsuura et al., 2013). The adsorption of DNA to mineral surfaces is of great interest because of gene transfer, drug release, bio-adhesion (cell capture) and origins of life studies (Ferris et al., 1996; Joyce, 2002; Trevors and Pollack, 2005).

For some of aforementioned above, advances of clay utilization in bio-related fields prompt us to conduct a toxicology study for clays and clay minerals, at the same time more research seems necessary to establish the sources of potential cytotoxicity of allophane nanoparticles.

In this study, the cytotoxic activity of a natural and synthetic allophane nanoparticles was examined for cultured human alveolar basal epithelial (A549) cells. To better understand the potential toxicity of allophane, quartz crystal microbalance (QCM) is a very powerful tool on the basis of the change in cell morphology during cell adhesion (Marx et al., 2003; Zhu et al., 2012; Kandel et al., 2014; Nowacki et al., 2015). It motivated us to investigate the time development of the dynamic cell adhesion signatures and the effect of clays on the cell adhesion as revealed by the cell viscoelastic index (*CVI*), which reflects the formation of cytoskeleton structure and dynamic viscoelastic features of living cells. The effects of allophane nanoparticles were also compared to those of other clay nanoparticles (hectorite) frequently studied for biological and medical application. Knowledge of such a comparison should also be useful in assessing how does the clay nanoparticles affect the dynamic cell adhesion signatures.

2. Materials and methods

2.1. Allophanes and hectorite

The natural allophane sample was provided by Shinagawa Chemicals Ltd. and designated as AK70. AK70 was treated by H_2O_2 to remove of the humic substance and not further purified for use (Iyoda et al., 2012). The overall size of a single allophane particle is ~5 nm with a specific surface area of 250 m^2/g, which was estimated by the *t*-method (Fig. 1) (Lippens and de Boer, 1965; Iyoda et al., 2012). The functional groups (HO)Al(OH_2) exposed on the wall perforations (defects) play a significant role as active sites in the adsorption process. The Si/Al ratio (=0.58) of the sample was determined by dissolution in acidic ammonium oxalate solution (Theng et al., 1982; Iyoda et al., 2012).

The precursor gels (Si/Al ratio = 0.50, 0.75 and 1.0) for the allophane synthesis were prepared by mixing and stirring (for 1 h) of 100 mM of sodium silicate, ortho ($NaSiO_4$, Nacalai-Tesque) and aluminum chloride hexahydrate ($AlCl_3.6H_2O$, Sigma-Aldrich). The sodium chloride formed was removed by centrifugation at a speed of 5000 rpm for 5 min. The precursors were then autoclaved at 100 °C for 48 h. The synthesized

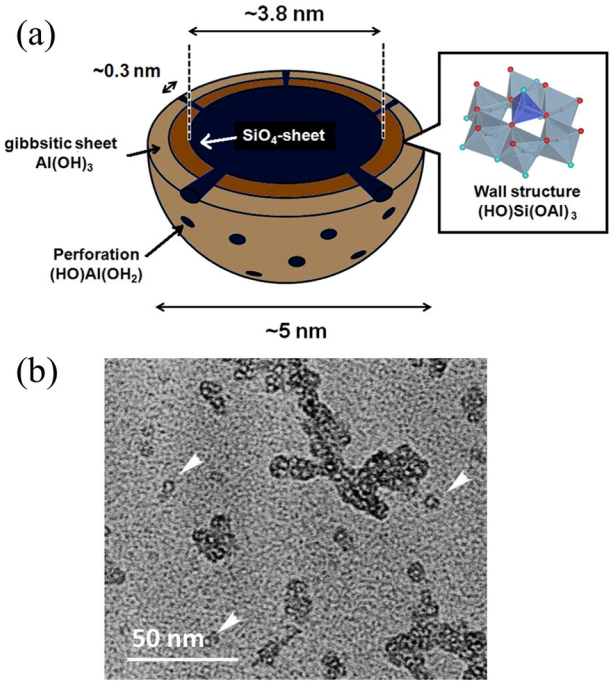

Fig. 1. Allophane structure and TEM bright field image. (a) Schematic representation of allophane structure. The overall size of a single allophane particle is ~5 nm. (b) High resolution TEM image of clustered particles of AK70, rather than singular particles. Arrows indicate single unit particles of allophane [12]. The surface area of allophane is as high as ~1000 m^2/g, which is often larger than activated carbon. (Adapted with permission from [12] Copyright 2012: Elsevier).

samples were designated as allophane(Si/Al)-x (x: Si/Al ratio) and The details of the synthesis experiments were described in previous paper (Iyoda et al., 2012).

Synthetic hydrophilic hectorite clay (SWN) ($Na_{0.33}(Mg_{2.67}Li_{0.33})SiO_4O_{10}(OH)_2$) (thickness ~ 1 nm, diameter ~ 100 nm, and cation exchange capacity = 0.87 meq/g, density = 2.13 g/cm^3) was purchased from CO-OP Chemical Co. Ltd., Japan.

Prior to culture initiation, AK70 nanoparticles were autoclaved and/or sterilized with ethanol at room temperature to assess appropriate sterilization methods.

2.2. Characterization

The surface charge characteristics of both allophane nanoparticles and SWN in water (0.05 wt.%) were determined by electrophoresis (Zetasizer Nano ZS, Malvern Instruments, UK) by the technique of laser Doppler anemometry. The method involved washing AK70 several times with water and adjusting the pH of the dispersion in the range of 2–11 using dilute HCl and NaOH (Nacalai-Tesque). All measurements were performed for four replicates and averaged to get the final value (Kawachi et al., 2013).

The average diameter of the nanoparticles was measured by dynamic light scattering (DLS) using Zetasizer Nano ZS (wavelength = 532 nm). The dynamic information can be retrieved by examining the autocorrelation function $g(t)$ of the time-dependent intensity (Nishida et al., 2015).

2.3. In vitro cell culture and cell viability

Human lung carcinoma A549 cells (ATCC) were used as a cancer cell and were cultured in RPMI-1640 (Wako Pure Chemical Industries) supplemented with 10% FBS including 1% antibiotic-antimycotic mixture. Upon reaching confluence, cells were cultured on 10 cm dishes in an atmosphere of 5% CO_2 and 95% relative humidity at 37 °C.

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