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Uptake of halloysite clay nanotubes by human cells: Colourimetric viability tests and microscopy study

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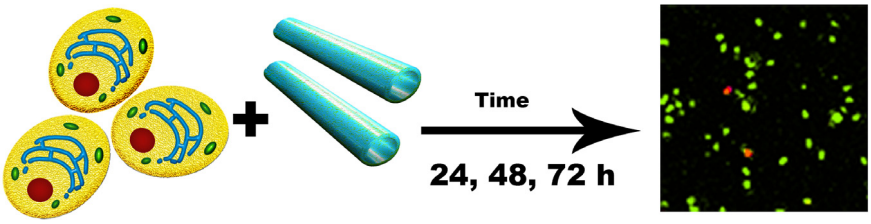
GRAPHICAL ABSTRACT



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ABSTRACT

This study is a systemic investigation of the uptake and toxicity of halloysite nanotubes using human adenocarcinoma epithelial cells (A549). A549 cells were chosen as a popular model of cancer cells extensively studied in nanotoxicity and drug delivery research. The adverse effects of a range of halloysite concentrations were evaluated. The viability of A549 cells was determined using several colourimetric assays. Dark-field microscopy was used to visualize the uptake and distribution of halloysite nanotubes in cells. The morphology of the cells was evaluated using dark-field, transmission electron and atomic force microscopies. The results showed that halloysite had a dose-dependent effect on human cells at concentrations of 5–900 μg per 10^5 cells in the MTT assay. The reduced toxicity of halloysite nanotubes at lower concentrations (5–75 μg per 10^5 cells) was additionally supported by the results of other colorimetric assays. Microscopy assays have demonstrated that the nanotubes, though affecting the biochemical processes, do not alter the morphology of the cells and do not penetrate into the nuclei.

1. Introduction

Nanomaterials are regarded to be a highly promising class of materials due to the global tendency for miniaturization in all fields of production. Nanomaterials application in various aspects of human life will certainly extend over time and become ubiquitous, and inevitably, human exposure to nanomaterials will grow. Physicochemical properties of materials at the nanoscale are different from those of the bulk materials, so new studies on toxicity and interaction of nanoscale materials with living cells are required [1]. The assessment of cytotoxicity of various nanomaterials in the *in vitro* models has become important [2–11]. For example, cancer cell lines have widely been applied for studying toxicity of nickel nanoparticles [12] or gold nanorods [13]. In biomedicine, nanomaterials are considered as perspective drug-delivery systems with nanotubes standing out among all the forms of nanomaterials because of their hollow structure and stability. However, a comprehensive toxicity testing is required prior to any medical application. For example, multiwalled carbon nanotubes are most unlikely to by applied in medicine due to their toxicity [14,15]. Note that nanomaterials have been prominently applied in different spheres of science and industry [16–29].

Among numerous nanomaterials, gaining popularity as drug delivery vehicles, halloysite nanotubes have attracted the enormous attention of researchers worldwide due to their remarkable structural and functional properties [30]. In particular, halloysite

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nanotubes are regarded as potent nanoobjects to fabricate nano-sized containers for drug delivery [31] and antimicrobial coatings [32]. Drugs can be loaded into clay nanotubes with 10–40 wt% efficiency, allowing for targeted and extended release [31]. Apart from biological applications halloysite has been successfully used for fabrication of flame-retardant polymers [33], composite coatings for wood cultural heritage objects preservation [34], food packaging films [35] and supports to synthesize composite nanoparticles [36,37]. Halloysite nanotubes have external diameters of ∼50 nm, internal diameters of 10–15 nm, and length of ∼1000 nm. Interestingly, these nanotubes exhibit different surface and lumen chemistry, having negatively-charged outer walls and positively-charged hollow cavity, thus allowing for the differential loading of drugs into or onto the nanotubes. The release time of substances loaded into the nanotubes can vary within hours, days and even weeks and can be additionally controlled by application of different surface treatments or modifications of the lumen [36]. Halloysite clay nanotubes are available in tons from natural deposits.

The biological applications of halloysite require the thorough and unbiased toxicity investigation before any clinical application. Among many models, human cells in culture provide a suitable platform to investigate the uptake and toxicity of halloysite. Here A549 cell line was used to analyze the halloysite nanotubes toxicity and influence on the metabolic activity of eukaryotic cells. This cell culture was chosen because of the high sensitivity of cells of pulmonary origin to airborne particles, which makes them a popular model for determination of toxicity [38]. Lung alveolar region is the prime site of deposition for inhaled fibrous nanomaterials. However, the description of the toxicity of halloysite nanotubes on well-characterized epithelial cells (A549) has not been reported yet, in addition there is no data about semi-lethal concentrations of halloysite nanotubes.

Previously, cytotoxicity of halloysite nanotubes was explored in various cell lines, including cancer cell lines Caco2 and HT29-MTX [39], HeLa and MCF-7 [40]. So, only few *in vitro* studies have examined the effects of halloysite exposure, and those that did demonstrated variable effects, including significant declines in viability in Hela (cervical) and MCF-7 (breast) cancer cell lines [41] after 24 h, but no effect on 3T3 (fibroblast) cells over 48 h [42]. Cell viability (Hela and MCF-7) decreased to 80% of the control after exposure to 500 $\mu g\ ml^{-1}$ halloysite (a very high concentration of *c*. 4×10^8 nanotubes per ml) [40].

Here, the cytotoxicity of clay minerals for basic and cancer human cells was extensively characterized by using several analytical methods such as enhanced dark-field microscopy, transmission electron microscopy (TEM), and atomic force microscopy (AFM) and diversity of colorimetric tests. After that, the IC50 for A549 cell line was determined with MTT-test.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), trypsin, penicillin–streptomycin, fetal bovine serum (FBS), Dimethylsulfoxide (DM SO), 3-(4,5-dimethylthiazol-2-yl-9,2,5-diphenyltetrazolium bromide (MTT), 7-hydroxy-3H-Phenoxazin-3-one 10-oxide (resazurin sodium salt), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral red), N-methyl-D-glucamine, lactate, NAD^+ (LDH reagents), ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Sigma-Aldrich) were used as purchased without additional purification.

Halloysite nanotubes (Dragon mine) were obtained from Applied Minerals (USA).

2.2. Cell culture

Adenocarcinomic human alveolar basal epithelial cells (A549) were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Sigma, USA) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (PAA, Austria), 45 I.U/ml penicillin and 45 mg/ml streptomycin (PAA, Austria). The cells were cultured at 37 °C in 80% humidified atmosphere with 5% CO_2. Primary human skin fibroblasts (HSF) were isolated from biopsy sample of adult skin and cultivated in minimal Eagle's medium Alfa (αMEM) supplemented with 0.2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% of heat-inactivated bovine serum (PAA laboratories). The fibroblasts used in experiments were 5–12 passages. Cells were routinely examined using an inverted light microscope Carl Zeiss Observer equipped with a Carl Zeiss MRC CCD camera.

2.3. Administration of halloysite nanotubes

Cells were seeded in a flat-bottom 6-well plate at 10^5 cells per well. After 24 h, when the cells reached ∼80% confluency, halloysite nanotubes were added to the wells at various concentrations (5–900 μg per 10^5 cells), then the cells were cultured at 37 °C in 80% humidified atmosphere with 5% CO_2 for the next 24 h.

2.4. MTT assay

MTT assay was carried out as published elsewhere [43]. Briefly, A549 cells were seeded in a flat-bottom 96-well plate at 2×10^3 cells per well. After incubation for 24 h, halloysite nanotubes at 5–75 μg per 10^5 cells were added, then the cells were cultivated for 24 h. After that 20 μl of MTT solution was added into each well and incubated for 4 h. Next, 200 μl of media was aspirated from each well and 200 μl of DMSO was added. Absorbance was recorded at 540 nm using Tecan Infinite M200 microplate spectrophotometer.

2.5. Neutral red uptake assay

Neutral red is a slightly cationic dye, which can permeate through the cell membrane by the non-ionic passive diffusion and concentrate in lysosomes thereby staining them. The assay was carried out as described elsewhere [44]. Briefly, A549 cells were seeded in a flat-bottom 96-well plate at 2×10^3 cells per well. After incubation for 24 h, halloysite nanotubes at 5–75 μg per 10^5 cells were added, then the cells were cultivated for 24 h. Then, 200 μl of media was aspirated from each well containing cells and 200 μl of neutral red working solution was added and incubated for 4 h. The dye working solution (0.033%) in the culture media was prepared before the experiment. Then, the solution was replaced by the solvent (1% glacial acetic acid in 50% ethanol) for dye extraction. Finally, absorbance was recorded at 540 nm and 690 nm.

2.6. Resazurin reduction assay

The cytotoxicity of the halloysite nanotubes was determined using the water-soluble resazurin dye. The method is based on the ability of live cells to reduce blue weakly fluorescent resazurin into the pink resorufin. It should be noted that resazurin, unlike MTT, can be reduced with a wider range of enzymes. The assay was carried out as described elsewhere [45]. Briefly, A549 cells were seeded in a flat-bottom 96-well plate at 2×10^3 cells per well. After incubation for 24 h, halloysite nanotubes at 5–75 μg per 10^5 cells were added, then the cells were cultivated for 24 h. After that 1 μl of 50 μM resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) (Sigma-Aldrich) was added into each well and incubated for 24 h. Absorbance was recorded at 590 nm.