CYTOTOXICITY OF COPPER DOPED SiO_2-HYDROXYPROPYL CELLULOSE COMPOSITES ON CANCER AND NON-CANCEROUS CELLS

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ABSTRACT

In the present paper the cytotoxicity of series of sol-gel based copper doped SiO_2/HPC composites on lung cancer cell line (A549) and primary human umbilical cord endothelial cells (HUVEC) was investigated. Hydroxypropyl cellulose (HPC), tetraethyl ortosilicate (TEOS) were used for preparation of amorphous matrix in which the copper was added as $Cu(NO_3)_2*3H_2O$ or $CuSO_4*5H_2O$ in concentration range of 0 - 5 wt. %. We observed that the composite materials at low concentration of Cu (0.5 wt. %) showed no toxic effects on both cell types. The composite material with $CuSO_4*5H_2O$ was found relatively more toxic, and furthermore, cancer cells are more sensitive to the copper concentration increase to non-cancer cells. The concentration of Cu ions of 2.5 and 5 wt. % were toxic to both cancer and non-cancer cells at 24th hour. At 72th hour of incubation some restoration of cell viability was observed.

Keywords: hydroxypropyl cellulose, tetraethyl ortosilicate, copper composites, cell viability.

INTRODUCTION

For many years the anti-microbial properties of Cu-based materials have been used for the creation of more than 300 antimicrobial products registered by the US Environmental Protection Agency (EPA) [1, 2]. In the last decade, interest in copper-containing materials with potential application in anti-tumor therapy and diagnostics has developed very rapidly due to their unique physical and chemical properties [3, 4]. In addition to being anti-microbial and anti-viral agents, copper composites show marked anti-tumor activity on various cancer cell lines, and the cytotoxicity towards cancer cell lines is specific [5, 6]. Cytotoxicity induced by copper ions overload is currently being studied in connection with the development of therapeutic methods. In recent years, many new cancer therapies based on Cu- based materials, such as chemodynamic therapy (CDT), radiodynamic therapy (RDT), immunotherapy, and sonodynamic therapy (SDT), have begun to emerge. In addition, Cu-based materials have also been reported to regulate the tumor microenvironment (TME) and thus to enhance cancer treatment [7]. There are many different types of Cu-based nanomaterials (NMs), mainly including copper oxides (e.g., Cu_2O, CuO), copper sulfides (e.g., Cu_2S, CuS, Cu_9S_5), etc. [8]. The synthesis strategies of Cu-based NMs are diversified and mainly include solvothermal/hydrothermal methods, high-temperature thermal decomposition methods, colloidal synthesis methods, microwave-assisted synthetic methods, cationic exchange methods, and template-oriented synthesis methods [9, 10]. The cytotoxicity of Cu-based materials is caused by their ability to generate a large number of hydroxyl radicals (OH), which can be used for CDT of tumors or to produce a large amount of reactive oxygen species (ROS) under

light irradiation, which can be used for photodynamic therapy (PDT) of tumors. Evidence is accumulating that copper homeostasis plays a regulatory role in various signaling pathways involved in the formation, proliferation and migration of cancer cells [11, 12]. Dysregulation of copper ion metabolism leads to the development of various diseases, including different types of cancer. Tumor cells usually have an increased amount of intracellular copper ions compared to normal cells, which in turn leads to upregulation of cellular processes and tumor progression [13].

Copper-based materials have been proven to be effective against various cancer cell lines. Copper nanoparticles (Cu NPs) was toxic to human lung carcinoma cell line (A549)137, human liver hepatoma (HepG2), Chinese hamster ovary (CHO), human osteosarcoma (Saos), and mouse embryonic fibroblast (3T3L1) cells in a dose-dependent manner [14, 15]. The study revealed that the capped Cu NPs by nontoxic aqueous extract of latex could be directly used for administration/in vivo delivery for cancer therapy [16]. Anticancer studies demonstrated the in vitro cytotoxicity values of Cu NPs against the tested human colon cancer Caco-2 cells, human hepatic cancer HepG2 cells and human breast cancer Mcf-7 cells, which can be used as a photothermal treatment to kill cancer cells [17].

In previous investigations, we examined that silver doped SiO_2/hydroxypropyl cellulose materials do not influence the vitality of 3T3 cells after 24 h of incubation in presence of materials [18]. In another study concerning the cytotoxicity effect of silver doped SiO_2/pectin composites we found out that cytoskeleton organization and apoptosis induction revealed satisfied rate at Ag content up to 1 wt. % [19].

The main goal of this work is to compare the cytotoxicity and morphological alterations of two types of copper doped SiO_2/HPC composites with different copper source.

EXPERIMENTAL

Materials

The reagents used for fluorescent preparations were Phalloidin - TRITC (high affinity F-actin probe conjugated to the red-orange dye tetramethylrhodamine), DAPI (4',6-diamidino-2-phenylindole) - both purchased from Sigma, and MTT (3-(4,5-dimethithiasol-2-il)-2,3-dipheniltetrsol bromide) - purchased from Alfa Aesar, Germany. The latter is dissolved in phosphate buffer PBS (pH = 7.3), and filtered through a filter with a 0.45 μm pore diameter. The solution was stored in the dark at 2°C - 8°C temperature.

Cells

Lung cancer cell line (A549) and primary umbilical cord endothelial cells (HUVEC) were used in the experiments. The cells were purchased from the American Cell Culture Bank (ATCC, Manassas, VA, USA). The cells were incubated in phenol red-free Dulbecco's modified eagle medium (DMEM) with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C and 5 % CO_2. Cells were passaged every 3 - 5 days, no more than 15 times. All solutions, buffers, and media used for cell culture were purchased from PAN-Biotech (Aidenbach, Germany).

Methods

Synthesis of copper doped SiO_2/HPC composites

In this study, the cytotoxicity of two types of composite materials in the silica-hydroxypolypyl cellulose-copper system was tested. The materials were obtained by the sol-gel method using TEOS (as SiO_2 source, Alfa Aesar) and HPC (Sigma-Aldrich) as precursors and $Cu(NO_3)_2*3H_2O$ or $CuSO_4*5H_2O$ (Valerus Ltd) as a source of copper ions. The amount of copper as varied from 0 to 5 weight percentage (0; 0.5; 2.5 and 5 wt. %) to the amount of SiO_2 in the samples. The procedure for the synthesis of composites was described in details in our previous study [20]. The abbreviation used to designate the composites is as follows: for samples obtained by $CuSO_4$ the abbreviation will be SiO_2HPC*x*wt. % Cu-1; while for the specimens obtained by $Cu(NO_3)_2$ - SiO_2HPC*x*wt. % Cu-2 abbreviation will be used.

Incubation of composites in culture medium

1 mg mL^{-1} Cu composites in different cupper concentration (0.5, 2.5 and 5 wt. %) were incubated in DMEM. The materials were transferred to sterile 6-well polystyrene plates, placed under UV in a laminar box for one hour for sterilization, then the required amount of culture medium was added. The materials were incubated for 72 hours.

Colorimetric test for cell viability

The test is based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan crystals. A549 and HUVEC were grown, trypsinized and after that centrifuged at 1500 rpm for 5 minutes. The supernatant is then aspirated with a vacuum pump without disturbing the cell pellet. Cells with density of $1x10^4$ were added to the well plate and were incubated for 24 hours at 37°C and 5 % CO_2. Then, the medium was aspirated and new medium in which materials were incubated was added. The well plate was additionally incubated for 24, 48 and 72 hours at 37°C and 5 % CO_2. After that, 20 μL MTT solution at a concentration of 5 mg mL^{-1} in PBS was added to each well and was incubated for 3 hours at 37°C and 5 % CO_2. At the end of the incubation period, the medium from each well was carefully aspirated and the formazan crystals were dissolved in a 100 μL per well solution of 5 % formic acid in isopropanol. The absorption was determined spectrophotometrically at 570 nm on a microplate reader Tecan Infinite F200 Pro (Tecan, Austria). Cell survival is calculated as a percentage of the untreated cell control.

Phase contrast microscopy

Phase contrast microscopy was performed to reveal changes in cell morphology after treatment with composites. For that purpose, MEIJI microscope (Japan) and an Opticam B1 camera (484501, Optica, Italy) were used.

RESULTS AND DISCUSSION

Cytotoxicity of copper doped SiO_2/HPC composites

HUVEC

The composites, without copper ions, does not showed cytotoxicity to control HUVEC cells 24 hours after incubation. Cell proliferation remained high (80 % - 100 %) until the end of the experiment (Fig. 1 (A, B) 0 wt. %)). At treated cells with both materials containing 0.5 wt. % copper, also no cytotoxic effect throughout the observed period up to the 72nd hour was observed. (Fig. 1 - 0.5 wt. %).

At the 48th and 72nd hours of incubation, significant differences in the action of the two materials with 2.5 and 5 wt. % Cu content, respectively, were reported. As can be seen from Fig. 1, composites Cu-2 containing 2.5 wt. % did not reduce cell viability (Fig. 1, 80 %), but Cu-1 composite lowered the cell viability to 50 % (Fig. 1). The both types of composites with the highest concentration of the copper content were highly toxic, with cell viability falling below 50 % 24 hours after treatment. Interestingly, at the 72th hour, some recovery of cell metabolism was observed after treatment with the highest concentration of copper ions compared to 24 hours at both types of composites (Fig. 1 A, B – 5 wt. %).

A549 cell

A549 cells did not change their cell viability when they were treated with composites with lower copper concentration (0.5 wt. %), (Fig. 2) till the 48th hour after incubation.

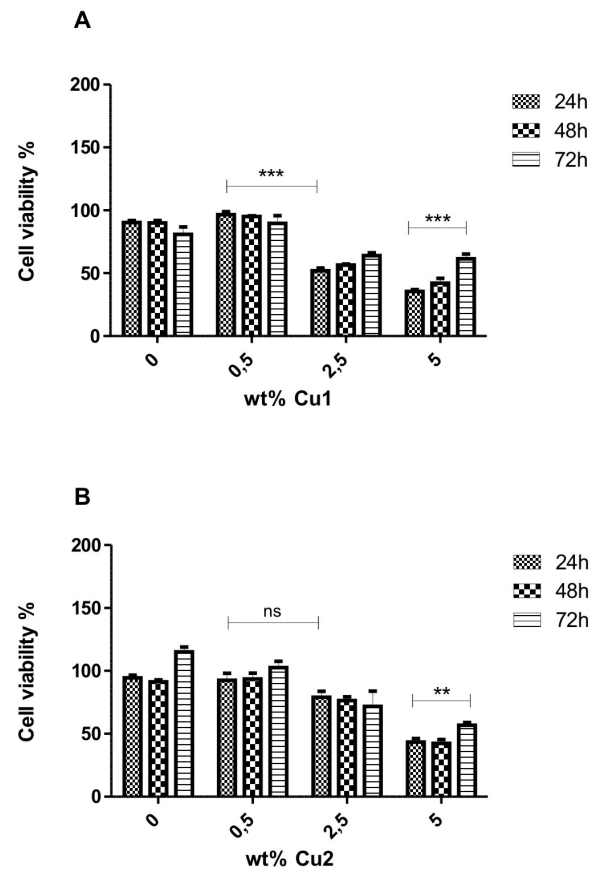

Fig. 1. Cell viability of HUVEC incubated for 24, 48, 72 hours in presence of copper doped SiO_2/HPC composites (A) Cu-1 and (B) Cu-2. Statistics were made with ANOVA one-way test and Tukey-Kramer posttest (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The data were normalized to control - cells that have not been in contact with composites.

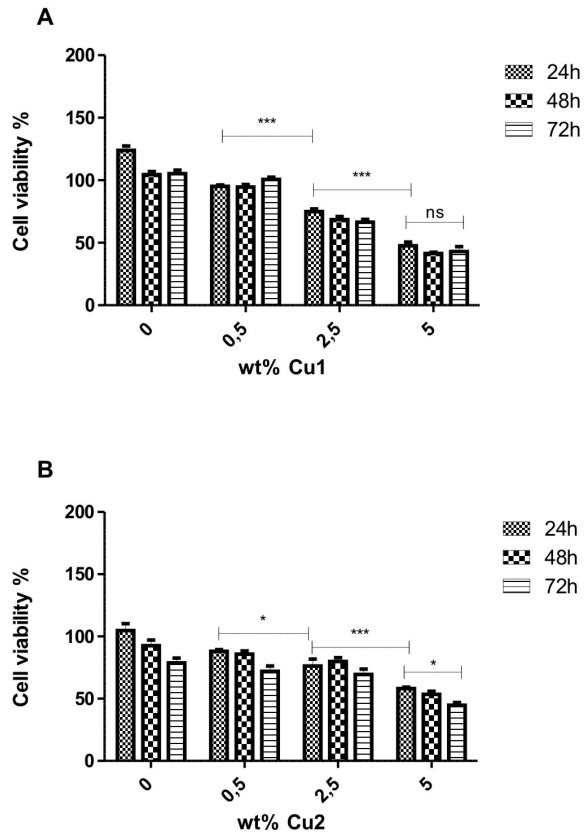

Fig. 2. Cell viability of A549 cells incubated for 24, 48, 72 hours in presence of copper doped SiO_2/HPC composites from (A) Cu-1 and (B) Cu-2. Statistics were made with ANOVA one-way test and Tukey-Kramer posttest (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The data were normalized to control - cells that has not been in contact with composites.

As it is shown in Fig. 2 (A and B), the composites containing 2.5 wt. % and 5 wt. % Cu-1 was more cytotoxic than Cu-2 at the same Cu concentrations as at 72th hour of incubation the cell survival falls below 50 %.

Influence of copper ions on HUVEC morphology

After incubation of cells with composites without Cu content (0 wt. %), no morphological changes were observed like the control group, even at the 72th hour an increasing number of cells and formation of a monolayer can be clearly seen (Fig. 3 A-C). The samples containing 0.5 and 2.5 wt. % copper from both materials ($CuSO_4*5H_2O$ or $Cu(NO_3)_2*3H_2O$) did not affect also cell morphology and therefore were not toxic to HUVEC. Only the material with 5 wt. % copper sulfate (Cu-1) induced minimal morphological changes, as the cells were more elongated, but remain well spread to the substrate (Fig. 3).

Influence of copper ions on A549 cancer cell morphology.

Similar to endothelial cells, no morphological changes were observed for A549 cells after treatment with composites without Cu content (0 wt. %).

Materials with 0.5 wt. % copper were not toxic for A549 cells at the whole times of incubation and no changes in cell morphology were observed (Fig. 4).

The composite materials containing 2.5 wt. % copper caused alteration in cell morphology of A549 cells after 48 hours of incubation (Fig. 4, left and right

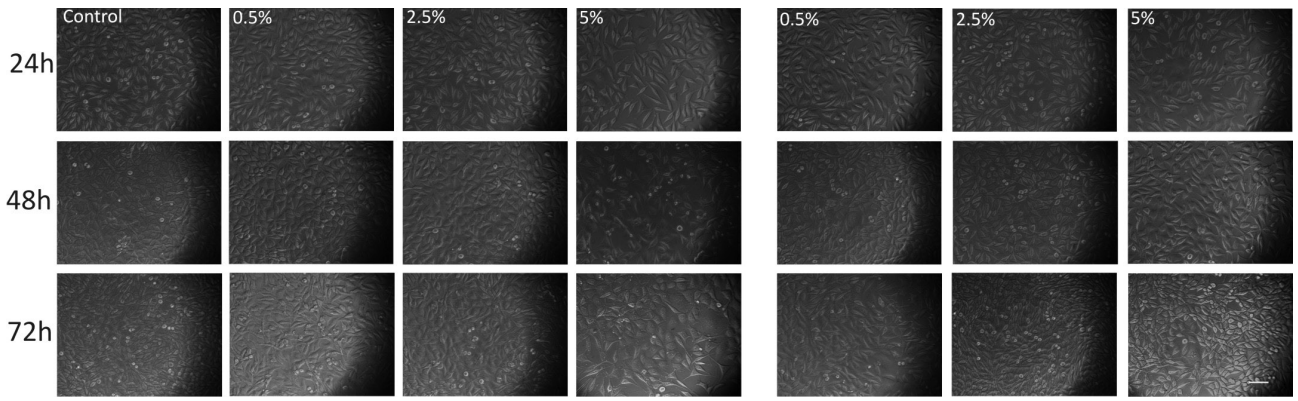

Fig. 3. Microscopic images of untreated HUVEC cells - control incubated for 24, 48 and 72 hours; cells treated with the Cu-1 incubated for 24, 48 and 72 hours - left panel; cells treated with the Cu-2 incubated for 24, 48 and 72 hours - right panel; Bar - 50μm.

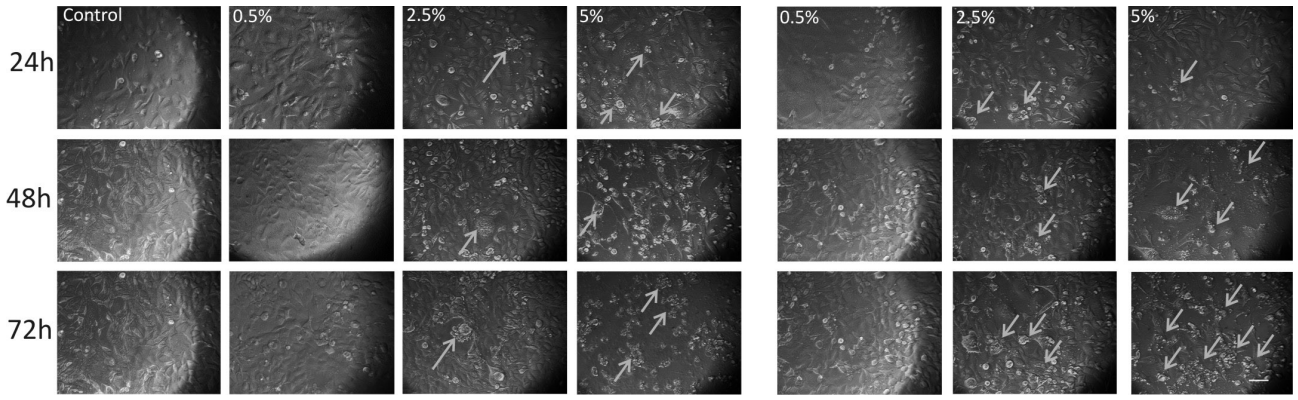

Fig. 4. Microscopic images of A549 cells: treated with composites Cu-1 - left panel, and with composites Cu-2 - right panel. Arrows show apoptotic cells. Bar - 50μm.

panel). Numerous round cells together with shrunken cells with fragmented membrane-bound apoptotic bodies (apoptotic cells) were observed, and after 72 hours the effect was even more pronounced (Fig. 4).

Composites with 5 wt. % copper content were the most toxic for the cells and significantly reduced their viability. The number of adhered cells was significantly reduced and many apoptotic cells were observed (Fig. 4). Phase contrast images confirmed the cytotoxic effect observed by MTT assay.

It can be summarized that the composites show cell-specific cytotoxicity, but the Cu-1 materials exhibited significantly higher cytotoxicity to both cell types. If we compare the cytotoxicity between the two types of cells, the cancer cells were more sensitive to the both types of copper ions. The results are in conformation to other studies which selectively targeting the cancer cells in vitro [6, 21, 22], due to the sensitivity of Cu to oxygen concentration and its inherent specific ability to inhibit the growth of cancer cells in relation to non-tumorigenic cells.

CONCLUSIONS

The cytotoxicity and morphological changes of two series of silica/HPC composites doped with copper, was studied. The results show that the cytotoxic effect of the materials used depends on the Cu source used (copper sulfate or nitrate), the amount of copper ions in the composite materials, the cell type and the incubation time. Cell viability was affected by both type composites, with the material obtained with $CuSO_4*5H_2O$ being more cytotoxic.

Of both cells, A549 cancer cells were more susceptible to toxic shock.

Thus, synthesized composite materials can serve for further research and development, with the aim of applying them as a new and/or adjuvant cancer therapy.

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