Cytotoxicity Elicited by Molybdenum Disulphide in Different Size of Particles in Human Airway Cells

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1. Introduction

Molybdenum disulphide (MoS_2) belongs to a class of layered materials well-known for their excellent lubrication performance. In the "bulk" form is used mainly in tribology area, as a dry and solid lubricant in, e.g. greases, dispersions, friction materials and bonded coatings. MoS_2 is used as an additive (in the amount of 0.5-30% by mass) for mineral oils, solid or liquid lubricants as well as composites. It can also constitute more than 80% of the mass of lubricants that exist in the form of powders or aerosols and which are available on the consumer market (IMOA 2018).

In recent years, the use of the nanoform of MoS_2 (MoS_2-NPs) has been increasing. Especially, two-dimensional nanoplates (2D nanomaterials) and fullerene-like structures are widely used in many tribological applications (Rapoport et al. 2005; Zhmud & Pasalskiy 2013; Feng & Cao 2016). The nanoplates/nanosheets of molybdenum disulfide are also used for the synthesis of analogues of such nanomaterials as: carbon nanotubes, fullerenes or graphene, in order to replace them in many consumer products (Teo et al. 2014; Jana & Rao, 2016). The use of MoS_2-NPs in dry lubricants is of particular importance in aviation, automotive, paper and food industries, in precision mechanics, electronics, chemical, glass, plastics and many other industries (Alazemi et al. 2016; Österle & Dmitriev 2016; Vidal-Abarca Garrido et al. 2016). The usage of MoS_2-NPs in lubricants in automotive and aviation is very beneficial for the environment, due to the reduction of particulate matter emission, which affects the smaller contamination of air, water and soil (Koppula et al. 2016; Vidal-Abarca Garrido et al. 2016). As a result, the reduction of emissions translates into a reduction in human morbidity, as solid particles (especially the fraction of ultrafine dust, with a diameter

equivalent to those of nanoparticles) are responsible for the generation of respiratory, cardiovascular, allergic and cancer diseases. In the other hands, dry lubricants are used in the form of powders and aerosols, which can lead to their significant emission to the environment, including the working environment. For this reason, the determination of the toxicity of the compounds in the nano-form is extremely important. According to toxicological reports, the use of nanomaterials, beneficial for technological reasons, is associated with an uncertain health risk (Drew & Hagen 2015). Both *in vivo* and *in vitro* studies confirm that the particle size of the same chemicals can significantly affect their toxic potential (Drew & Hagen 2015; ECETOC 2014). There is a concern that even substances with low toxicity may adversely affect the body if used in the form of nanoparticles. Despite existing and the still-emerging applications of MoS_2-NPs, only a few investigations into their biocompatibility and toxicity have been performed. What is important, mainly their modified forms were evaluated (Wu et al. 2011; Hao et al. 2017; Liu et al. 2014; Teo et al. 2014; Appel et al. 2016; Pardo et al. 2014; Wang et al. 2015). The results of these studies are inconclusive and indicate cytotoxic effects of MoS_2-NPs at both low (Chng et al. 2014; Wang et al., 2015) and high concentrations (Corazzari et al. 2014; Teo et al. 2014; Pardo et al. 2015; Hao et al. 2017; Liu et al. 2014).

Taking into account the trend of adding nanoscale MoS_2 to many applications, there is a need for toxicity studies on their unfunctionalized forms, especially in terms of potential health effects after inhalation.

The basic step in assessing the safety of nanomaterials is to determine their cytotoxic effects in in vitro conditions (Zapór 2012). The respiratory tract is often represented by bronchial (BEAS-2B) and alveolar (A549) epithelial cells. The BEAS-2B cells retain metabolic competence of normal bronchial epithelial cells, therefore they are considered a sensitive model for toxicity testing of environmental pollutants as well as nanomaterials, including molybdenum compounds (Haniu et al. 2011; Ekstrand-Hammarstroem et al. 2012; Gilbert et al. 2012; Wang et al. 2015). A549 cells, despite their neoplastic origin, retain the properties of normal alveolar epithelial cells.

The purpose of this study was to assess the cytotoxic effect of unmodified molybdenum disulfide in nano- (MoS_2-NPs) and micro- (MoS_2-MPs) size of particle toward human bronchial (BEAS-2B) and alveolar (A549) cells.

2. Materials and methods

2.1. Chemicals and reagents

The molybdenum disulphides with particle size < 100 nm (MoS_2-NPs) and > 1 µm (MoS_2-MPs) originated from US Research Nanomaterials, Inc, Huston, Texas. The media for cell cultures were provided by Gibco BRL (Life Technologies Ltd. Paisley, UK). Foetal Bovine Serum (FBS), trypsin solution (0,25%) with EDTA, were purchased from Sigma Chemical Co. (St Louis, MO, USA). For cytotoxicity assays were used: 3-(4.5 dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide (MTT), Neutral Red Solution (NRU), Hank's Balanced Salt Solution, Dulbeco's Phosphate Buffered Saline, dimethylsulphoxide (DMSO), Giemza solution, from Sigma.

2.2. Characterization of MoS_2-NPs and MoS_2-MPs

The particles of MoS_2-NPs and MoS_2-MPs were broken down and dispersed into colloidal form by means of ultrasonic gun (1 cycle, 15 min., 100% amplitude, water bath with ice). Then, an aqueous suspension of particles was stabilized with poly(vinylpyrrolidone) – PVP of molecular weight M = 360 000, in the ratio MoS2:PVP = 1:1. The choice of PVP as a stabilizer was dictated primarily by its physiological inertness, besides the compound being a non-ionic polymer does not change the pH of the culture media, and as non-toxic it is also used in medicine, pharmacology and cosmetics (Kariduraganavar et al. 2014). Not without significance is the fact that PVP is often used in tribology as a lubricant improving agent (Sułek et al. 2011).

The particle size and morphology was characterized using scanning electron microscopy (SEM, Nova NanoSEM 450 (FEI). Crystalline structure of particles was confirmed by X-ray diffraction (XRD), with Empyrean (PANalytical) diffractometer with CoKa line (1.78901 A). The measurement data was processed using ICDD PDF 4 database and HighScore Plus software. The particle size and size distribution was carried out in aqueous suspension of MoS_2 particles with stabilizer (PVP) using Dynamic Light Scattering (DLS) (Litesizer TM 500, Anton Paar). Particle suspensions with an initial concentration of 12.5 mg MoS_2/mL were prepared successively during the experiment. The preparation of nanoparticles, as well as the preparation of their suspensions and the characteristics was carried out at the Department of Materials Technology and Chemistry of the University of Lodz.

2.3. Cell culture and treatment

The normal bronchial epithelium cells BEAS-2B (CRL-9609) were purchased from American Type Culture Collection (LGC Standards Sp. z o.o.). The cells were grown in the LHC-9 serum free medium in the culture flasks coated with collagen type 1 (Greiner). The A549 cells were cultured in a DMEM medium supplemented with 7% FBS and with 1% antibiotic-antimycotic in sterile tissue culture flasks (Nunc, USA). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Before starting the experiment, the cells were removed from the flask by trypsinisation. Cell number and viability were determined in a Bürker chamber by the trypan blue exclusion method. Cells whose viability was over 90% were used in experiments. The cells were screened for *Mycoplasma sp.* infection using MycoAlert[TM] PLUS Mycoplasma Detection Kit (Lonza, Walkersville, Inc.).

2.4. Cytotoxicity studies

To assess cytotoxicity of MoS_2-NPs and MoS_2-MPs two assays were used. The MTT assay is based on the uptake and the reduction by mitochondrial succinate dehydrogenase of the soluble yellow MTT tetrazolium salt to an insoluble blue MTT formazan product (Denizot & Lang 1986; Mosmann 1983). The NRU assay is based on the uptake and lysosomal accumulation of the supravital dye, neutral red (Borenfreund & Puerner 1985). Cells were plated at a density of 1 x 10^4/well in a 96-well culture plate and cultured overnight to allow adherence and recovery. After this period, non-attached cells were aspirated and suspension of MoS_2-NPs or MoS_2-MPs in different concentrations (1÷200 μg/mL) were added, and incubated for 24. 48. or 72 hrs. Then, the medium was removed and reagents for cytotoxicity tests were added. Cytotoxicity tests were performed in at least three independent replications. Based on the absorbance values obtained in MTT and NRU tests, the viability ratio of cells exposed to the tested compounds, i.e. the percentage of viable cells compared to control was calculated. The possibility of interference nanoparticles with the test reagents was examined. To this end, nanoparticles were incubated in the absence of the cells and then MTT and NRU tests were performed (Kroll et al. 2012).

2.5. Clonogenic assay (Colony Forming Efficiency Assay, CFEA)

The clonogenic assay was conducted according the procedure described by Franken et al. (2006) and adapted from Kruszewski et al. (2013). Briefly: Exponentially growing cells were harvested and seeded in Petri dish 60 x 15 mm (21 cm^2) (Iwaki Cell Biology, Japan) at a density 500 cells/dish together with tested compound. Each dish finally contained 5 mL of cell culture medium with MoS_2-NPs or MoS_2-MPs in appropriate concentrations at least in three replicates

for each treatment. Cells were exposed to particles over the time period they needed to form colonies, that is 10 days. After this period, particle solutions were removed, cells were washed with PBS, fixed (ethanol), stained (0.4% Giemza), and colonies were counted. Then plating efficiency (PE) and surviving fraction (SF) was calculated, as below:

PE = (number of colonies formed / number of cells seeded)

SF = no. of colonies formed after treatment / no. of cells seeded x PE

PE ratio for BEAS-2B cells calculated from three independent experiments was above 60%.

2.6. Data analysis

Three separate *in vitro* cytotoxicity experiments were conducted in which all samples of compounds were tested simultaneously. Each dose in separate experiment was tested in 9 replications (n = 9 wells per treatment). Replicates from 3 experiments were averaged. The CFEA test was carried out in the three separated experiments with three replications for each concentration of MoS_2-NPs or MoS_2-MPs. The results were presented as surviving fraction ratio (SF) $\pm$ standard deviation (SD). SF = 1 was set for the control. CFEA data were analysed by Student's test for comparison between two groups.

3. Results

3.1. Characterization of MoS_2-NPs and MoS_2-MPs

Before starting the cytotoxicity assessment of MoS_2-NPs and MoS_2-MPs, their properties were characterized in terms of morphology, particle sizes and size distribution. SEM analysis showed that MoS_2-NPs was a homogeneous material in its whole volume and consisted of disk-shaped particles (Fig. 1a). In the case of MoS_2-MPs, the SEM analysis showed that it was in the shape of multilayer hexagonal plates (Fig. 1b).

The histograms of particle size distribution indicated that the diameter of the disks of MoS_2-NPs was 97 $\pm$ 32 nm (the average of 500 counts) (Fig. 2a), while the thickness of the disks evaluated on the basis of XRD results was 8.5 nm $\pm$ 1.5 nm (data no shown). The size of MoS_2-MPs plates was 1.92 $\pm$ 0.64 μm (mean from 500 counts) (Fig. 2b), while the thickness evaluated on the basis of XRD results was about 10 nm (data no shown).

DLS analysis (in the natural colloid environment) indicated that the hydrodynamic diameter of MoS_2-NPs coated with PVP was d = 251 $\pm$ 94 nm, while MoS_2-MPs d = 0.7 $\pm$ 0.3 μm (data not shown). No peaks from large agglomerates were observed.
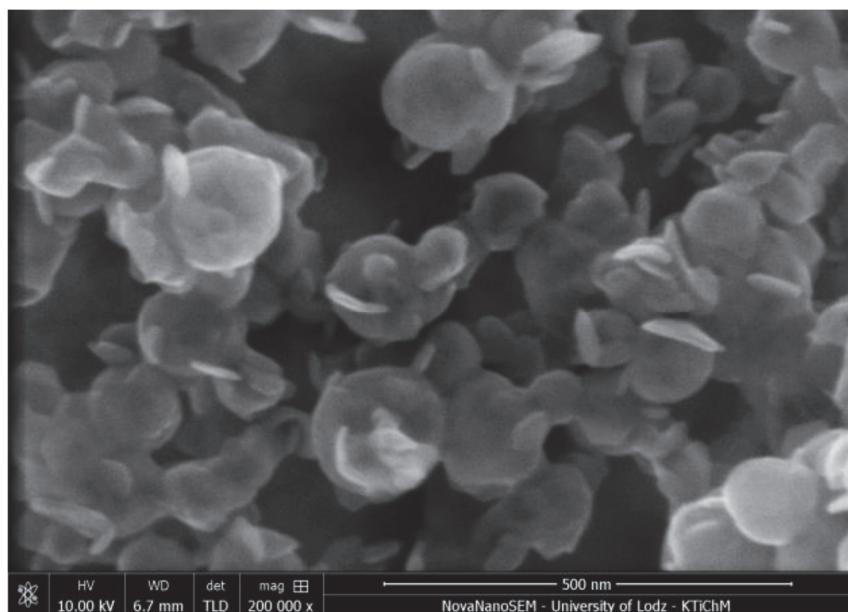
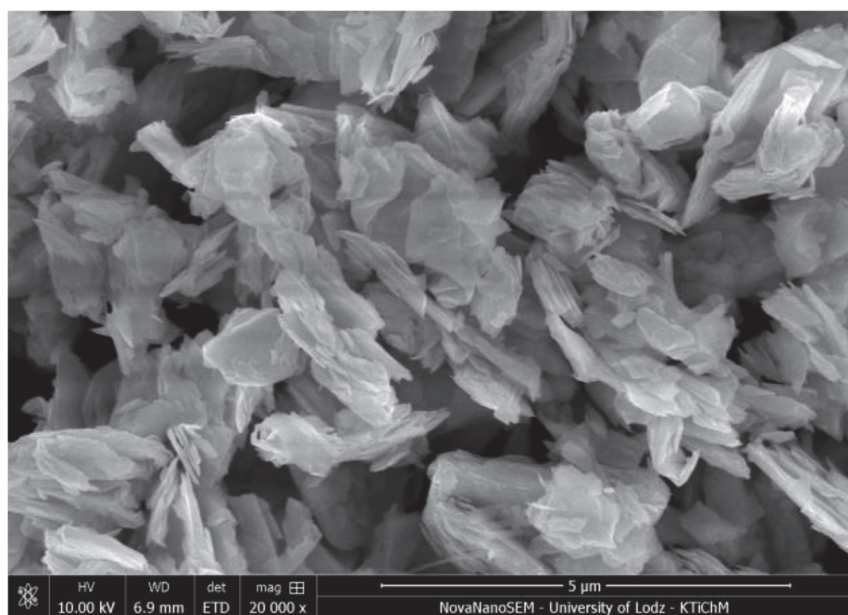**Fig. 1a.** SEM image of MoS$_2$-NPs. Magnification and scale are present on the picture

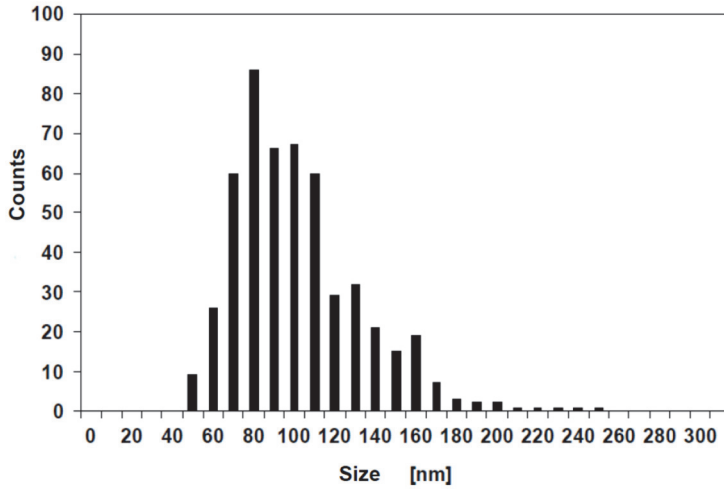**Fig. 1b.** SEM image of MoS$_2$-MPs. Magnification and scale are present on the picture

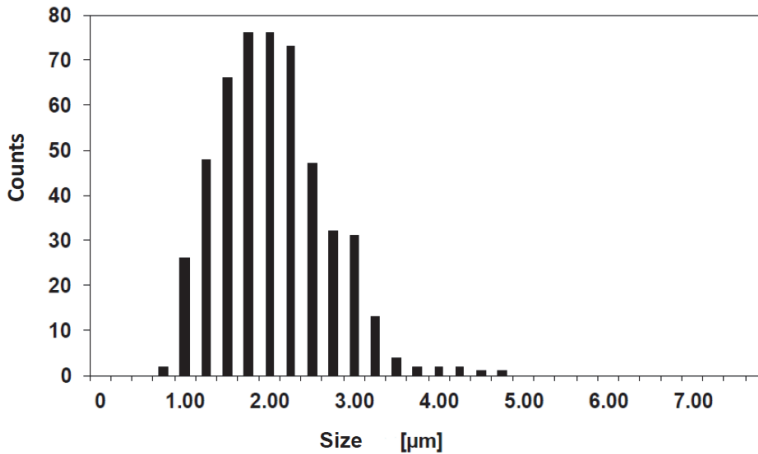**Fig. 2a.** The histogram of particle size distribution of MoS$_2$-NPs

Fig. 2b. The histogram of particle size distribution of MoS$_2$-MPs

3.2. Effects of MoS$_2$-NPs and MoS$_2$-MPs on cell viability and proliferation

The effects of both MoS$_2$ on the viability of A549 and BEAS-2B cells assessed by MTT and NRU tests after 24, 48 and 72 h exposure are presented in Figures 3-6. The assessment of cytotoxicity performed in preliminary experiments did not show the effect of PVP on BEAS-2B and A549 cells in the range of concentrations used.
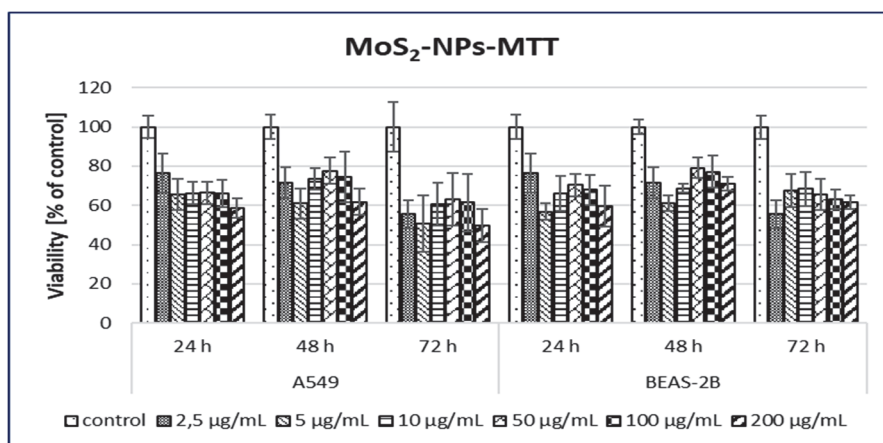
Fig. 3. Cytotoxic effect of MoS₂-NPs in A549 or BEAS-2B cells after 24 h, 48 h and 72 h exposure of the cells, assessed by MTT assay

MoS₂-NPs at a concentration of 2.5 µg/mL reduced the viability of both cell types assessed by the MTT reduction assay to about 70% after 24 and 48 h of exposure and to about 50% after 72 h compared to the control. Exposure of cells to higher concentrations of the substance did not result in a further decrease in their viability (Fig. 3). MoS₂-MPs had a similar effect. After 24 hours of exposure to the compound at a concentration of 5 µg/mL, cell viability was reduced to about 70% (A549) and about 60% (BEAS-2B). Higher concentrations and longer exposure time did not cause further decrease in cell viability (Fig. 4).
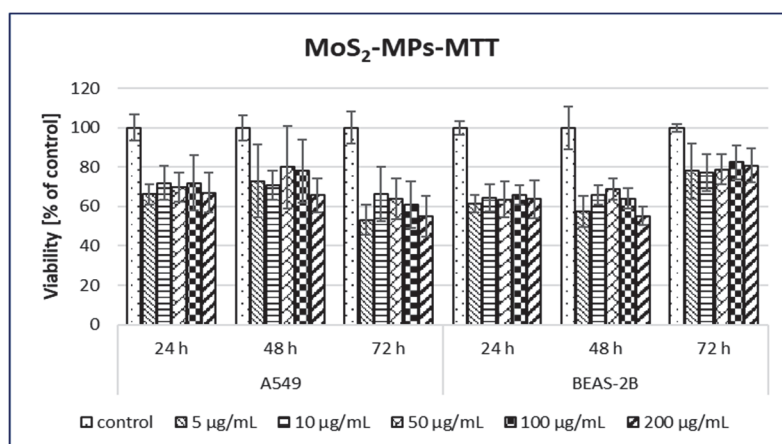

Fig. 4. Cytotoxic effect of MoS₂-MPs in A549 or BEAS-2B cells after 24 h, 48 h and 72 h exposure of the cells, assessed by MTT assay

Both MoS$_2$-NPs and MoS$_2$-MPs assessed by the NRU assay did not cause any decrease in cell viability relative to the control (on the contrary some of the measurements were above control values) (Fig. 5-6).
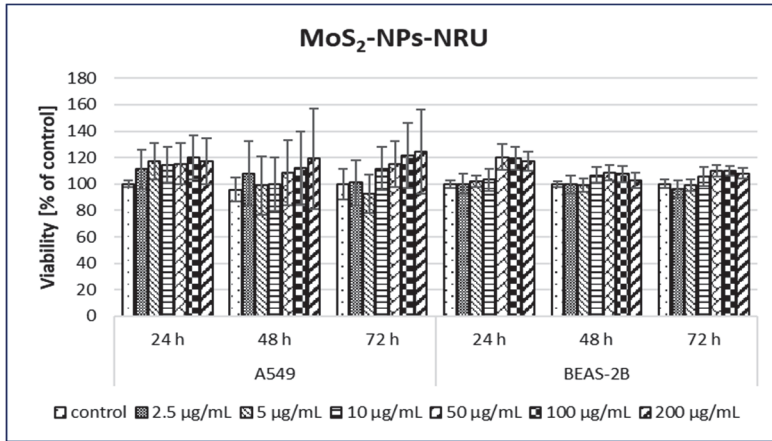

Fig. 5. Cytotoxic effect of MoS$_2$-NPs in A549 or BEAS-2B cells after 24 h, 48 h and 72 h exposure of the cells, assessed by NRU assay
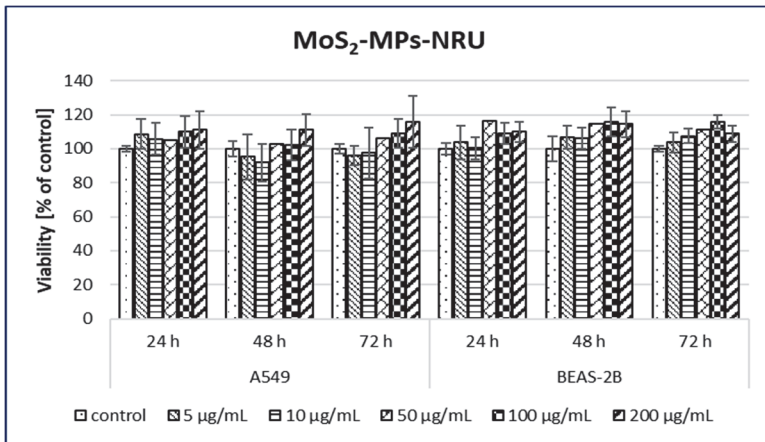

Fig. 6. Cytotoxic effect of MoS$_2$-MPs in A549 or BEAS-2B cells after 24 h, 48 h and 72 h exposure of the cells, assessed by NRU assay

3.3. Effects of MoS₂-NPs and MoS₂-MPs on colony formation ability of the cells

The Colony Forming Efficiency Assay (CFEA) was used to assess the toxic effects of the MoS₂-NPs and MoS₂-MPs, after long-term cell exposure. CFEA is based on ability of a single cell to grow into a colony (Puck & Marcus 1956). A colony being defined to consist as at least 50 clones of one cell (which corresponds to 6 mitotic divisions). This test is used to detect cells that retained the capacity for producing a large number of progeny after treatments that can cause reproductive death as result of damage to chromosomes, apoptosis, etc. (Herzog et al. 2007).

MoS₂-NPs at the lowest of the concentrations used (25 and 50 µg/mL) limited the proliferation of BEAS-2B cells to about 80%. Higher concentration (100 µg/mL) resulted in inhibition of growth of the culture to approx. 30%. The toxic effect of MoS2-NPs was also seen on A549 cells. However, the reduction of A549 culture growth to 80% occurred only in the highest concentrations of the compound (100 and 200 µg/mL) (Fig. 7).
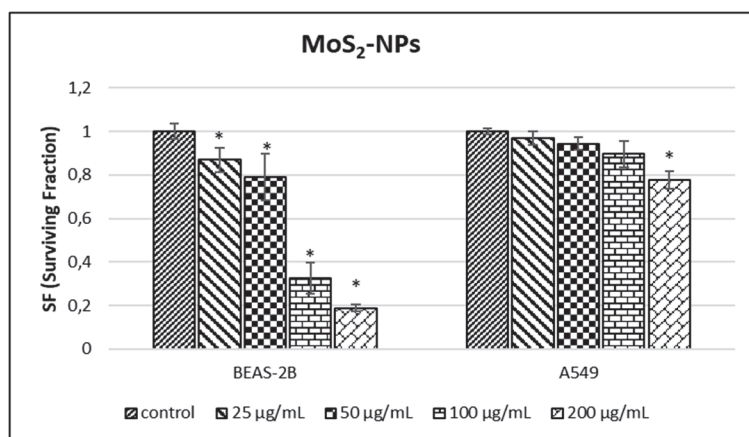

Fig. 7. Colony forming ability of BEAS-2B or A549 cells treated with different concentrations of MoS₂-NPs. Each bar represents the mean ± SD of 3 independent experiments. * $p < 0.05$ vs. control

MoS₂-MPs caused a greater (as compared to MoS₂-NPs) inhibition of BEAS-2B cell proliferation, however, the obtained results were very heterogeneous (high values of standard deviations). There was no toxic effect of the compound on the A549 cells (Fig. 8).
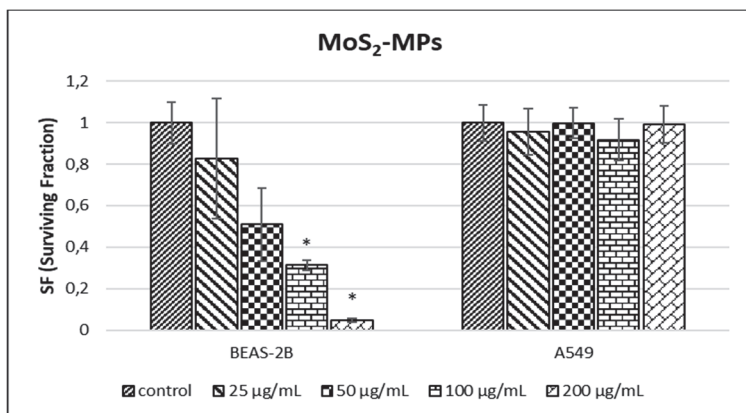
Fig. 8. Colony forming ability of BEAS-2B or A549 cells treated with different concentrations of MoS$_2$-MPs. Each bar represents the mean ± SD of 3 independent experiments. * p < 0.05 vs. control

4. Discussion

A wide range of applications of MoS$_2$-NPs in many industries, causes the possibility of occupational and environmental health implications of humans exposed to them. In the present study the comparison of the ability of MoS$_2$-NPs and MoS$_2$-MPs to induce cytotoxicity in two human lung epithelial cell models (BEAS-2B and A549) was carried out. The assessment of the cytotoxicity of nanomaterials is an essential tool in determining their potential effects at cellular level. It is the basis for the extrapolation of acute toxicity in animals and the determination of sub-lethal doses for in-depth toxicity studies (e.g. oxidative stress, inflammatory reactions, genotoxicity, etc.) (Stone et al. 2009). The studies of cytotoxicity *in vitro* are among the basic steps in Integrated Testing Strategy (ITS) for nanomaterials to predict their toxicity (Combes & Balls 2011; Stone et al. 2014). The results of the assessment of cytotoxicity often depend on the model used and applied research methods to assess cytotoxicity. In the studies carried out, BEAS-2B cells showed slightly higher sensitivity to the tested substances, while large differences in the levels of cytotoxic doses associated with the applied test method were observed. The test assessing the integrity of cell membranes and lysosomal activity (NRU) practically did not give any cytotoxic response, on the contrary, some results indicate a higher viability of exposed cells. It is difficult to explain such a phenomenon. Perhaps the smallest of the particles were accumulated in lysosomes and interfered with NR dye. This may be evidenced by large scatter of results, especially on A549 cells.

In MTT reduction assays the little trend in cytotoxicity profiles of MoS$_2$ was found: both sulphides at low concentrations (2.5 and 5 µg/mL) reduced the

viability of the cells to about 60%, after which there was no further decrease in cell viability, despite being exposed to higher concentrations. Very similar results of MoS_2-NPs cytotoxicity received Chng et al. (2014) in MTT assay on A549 cells. Pristine MoS_2-NPs decreased viability of the cells in doses 3.125 and 6.25 µg/mL whereas higher concentrations did not cause changes in viability of the cells. Also, Wang et al. (2015) showed a slight (up to 80%) decrease in the viability of THP-1 cells and no cytotoxic effect in BEAS-2B cells exposed to various structural forms of MoS_2-NPs in concentrations of 10-50 µg/mL. It should be emphasised that MoS_2-NPs, despite the lack of cytotoxic effects, caused the release of pro-inflammatory mediators in cells and, in vivo inflammation and fibrosis. In turn, Quereshi et al. (2015) observed a minimal decrease in the survival rate of HeLa cells with increase of concentration of MoS_2-NPs, but they use higher doses, i.e. from 32.5 to 300 µg /mL. Likewise, Corazzari et al. (2014) and Theo et al. (2014) in the studies on A549 cells demonstrated cytotoxic activity of MoS2-NPs only in high concentrations (400 mg/mL).

In order to study possible long-term toxic effects of MoS_2-NPs and MoS_2-MPs the clonogenic assay was used, which is performed within 7-10 days. Recently, the clonogenic assay is considered a promising test to study toxicity of nanomaterials, as it makes use of no cellular dyes, that have been demonstrated to be a possible reason for invalid results due to their biochemical interaction with the nanomaterials tested (Casey et al. 2007, Herzog et al. 2007, Ponti et al. 2010,). The results from an interlaboratory comparison study performed in the frame of OECD's Working Party of Manufactured Nanomaterials (WPMN) showed that the CFE assay is a suitable and robust *in vitro* method to assess cytotoxicity of nanomaterials (JRC Report 2014). In this experiment both, MoS_2-NPs and MoS_2-MPs were able to significantly decrease the clonogenic survival and cell proliferation in a dose-dependent fashion in a wide range of concentrations to 200 µg/mL, when exposed constantly over 10 days, but this effect was visible mainly in BEAS-2B cells. The MoS_2-NPs were slightly more cytotoxic than MoS_2-MPs. Both MoS_2 did not cause inhibition of colony forming ability of A549 cells.

Inhibition of the ability of BEAS-2B cells to proliferate under the influence of both MoS_2 particles may be an unfavourable phenomenon for predicting their long-term effects of exposure. At the body level, these cells are a kind of barrier to xenobiotics. Thanks to the ability to create tight junctions and the ability to adhere "foreign" particles on the surface of cell membranes, these cells form the body's defence line against the penetration of particles into cells (Heijink et al. 2010).

5. Conclusion

The obtained data with regards cytotoxic and clonogenic effects of MoS_2-NPs and MoS_2-MPs suggest rather their low hazardous potency with an indication of higher toxicity of MoS_2-NPs. Such result, although it needs to be

confirmed in *in vivo* studies, is promising due to the predicted wide use of molybdenum disulfide nanoparticles. Especially that molybdenum disulphide is one of the most promising post-graphene nanomaterials.

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Abstract

The present investigation was aimed to study the cytotoxic response induced by molybdenum disulfide in nano- (MoS$_2$-NPs) and micro- (MoS$_2$-MPs) size of particle in human bronchial (BEAS-2B) and alveolar (A549) cells. The cells were exposed with different particle size of MoS$_2$ in concentrations range 1-200 μg/mL for 24, 48, and 72 h, and then the cytotoxicity assays (MTT and NRU) was performed. Afterwards, long-term toxicity was assessed by colony forming efficiency assay (CFEA) during 10 days exposure of the cells.

Both MoS$_2$-NPs and MoS$_2$-MPs showed similar, weak cytotoxic effects on BEAS-2B and A549 cells assessed by MTT assay, that is reduction of cell viability to approx. 60-70% at concentrations of 2.5 and 5 μg/mL. The percentage viability remained relatively constant at this level across all concentrations above 5 μg/mL. In long-term exposure, both MoS$_2$ inhibited colony formation in a wider range of concentrations to 200 μg/mL. MoS$_2$-NPs were slightly more cytotoxic than MoS$_2$-MPs. The data suggest the low potential hazardous nature of both MoS$_2$ tested with an indication of higher toxicity of MoS$_2$-NPs.

Keywords:
molybdenum disulphide, nanoparticles, cytotoxicity

Ocena cytotoksycznego działania cząstek disiarczku molibdenu na komórki układu oddechowego

Streszczenie

Celem badań była ocena cytotoksycznego działania nano- (MoS$_2$-NPs) i mikrometrycznego (MoS$_2$-MPs) disiarczku molibdenu na ludzkie komórki nabłonka oskrzelików (BEAS-2B) i pęcherzyków (A549) płuc. Komórki narażano na różnej wielkości cząstki MoS$_2$ w zakresie stężeń 1-200 μg/ml przez 24, 48 i 72 h. Badano wpływ obu rodzaju cząstek MoS$_2$ na integralność błon komórkowych (test NRU) oraz aktywność metaboliczną komórek (test MTT). Oceniano również zdolność komórek do proliferacji po długotrwałym (10 dni) narażeniu na podstawie testu efektywności formowania kolonii (CFEA).

Oba MoS$_2$ powodowały podobne, słabe działanie cytotoksyczne na komórki oceniane testem MTT, tj. obniżenie przeżywalności komórek do ok. 60-70% w stężeniach of 2,5 and 5 μg/mL po 24 h, które w niewielkim stopniu nasilało się z czasem narażenia, natomiast nie obserwowano wzrostu cytotoksyczności ze wzrostem stężenia. Po długotrwałej ekspozycji (10 dni) oba MoS$_2$ hamowały zdolność tworzenia kolonii w szerszym zakresie stężeń tj. do 200 μg/mL, przy czym MoS$_2$-NPs wykazywały silniejsze działanie ograniczające zdolność komórek do proliferacji niż MoS$_2$-MPs. Dane wskazują na niski potencjał toksyczny obu badanych MoS$_2$ ze wskazaniem wyższej toksyczności MoS$_2$-NP.

Słowa kluczowe:
disiarczek molibdenu, nanocząstki, cytotoksyczność