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BIOLOGICAL EFFECTS OF MOLYBDENUM COMPOUNDS IN NANOSIZED FORMS UNDER *IN VITRO* AND *IN VIVO* CONDITIONS

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Abstract
Nanoparticles of transition metal dichalcogenides, particularly of molybdenum (Mo), have gained a lot of focus due to their exceptional physicochemical properties and the growing number of technological applications. These nanoparticles are also considered as potential therapeutic tools, biosensors or drug carriers. It is crucial to thoroughly examine their biocompatibility and ensure safe usage. The aim of this review is to analyze the available data on the biological effects of different nanoforms of elemental Mo and its compounds. In the reviewed publications, different conditions were described, including different experimental models, examined nanoforms, and their used concentrations. Due to these differences, the results are rather difficult to compare. Various studies classify Mo related nanomaterials as very toxic, mildly toxic or non-toxic. Similarly, the mechanisms of toxicity proposed in some studies are different, including oxidative stress induction, physical membrane disruption or DNA damage. Quite promising, however, are the potential medical applications of MoS_2 nanoparticles in therapy of cancer and Alzheimer's disease. Further studies on biocompatibility of nanomaterials based on Mo compounds are warranted. Int J Occup Med Environ Health. 2020;33(1):1–19

Key words:
***in vitro*, nanoparticles, *in vivo*, molybdenum disulphide, biological effects, molybdenum compounds**

NANOMATERIALS NOMENCLATURE

The expanding use of nanomaterials in many branches of industry or medical applications makes them a focus of interest not only from the technical point of view, but also for safety reasons. The most essential matter, which is currently being debated, is a precise definition of the material, including at least most of the possible variations and modifications. According to the EU Commission Recommendation 2011/696/EU [1], a nanomaterial can be defined as consisting of particles, of which ≥50% have at least 1 dimension in the size 1–100 nm. However, it is possible, in specific cases, to classify a material having <50% particles with a dimension in the size 1–100 nm as a nanomaterial. As another exception, materials with the external dimension <1 nm, like graphene flakes, fullerens or carbon nanotubes, can be considered nanomaterials. Additional

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definitions cover the aggregate or agglomerate concept. A more precise term, the nanoform, covers nanoparticles of different shapes, including chemical surface modification [2]. At least 3 features should be taken into account while characterizing nanoforms, these being their shape, size and surface modification. Hence, examples of different nanoforms include nanotubes, nanosheets, nanorods, nanoflakes, nanospheres (even if they were made of the same compound), untreated or modified with chemicals.

As regards nanoparticles with 1 dimension significantly smaller than the other, such as nanoflakes or nanosheets, an additional challenge is posed by the exfoliation method, influencing the number of layers, which can determine the properties of the material [3]. The most popular 2-dimensional (2D) nanomaterial, graphene, is basically a sheet of atoms arranged in the hexagonal lattice. Hence, the term "graphene" is connected with a carbon nanomaterial of atomic thickness. It is possible to produce more complicated structures, like bi- or tri-layer graphene. When it comes to multi-layer materials, the nomenclature gets more complicated [4]. There is no clear definition, or even an estimated size range, so such terms as nanosheet, nanoflake or nanoplate are used interchangeably and rather intuitively to describe particles with 1 dimension distinctly smaller than the other (so, basically, flat). The issue obviously concerns not only graphene but also other 2D nanomaterials. The lack of uniform guidelines makes the analysis of literature data rather difficult, as the interpretation of a given nanoform (whether it is a plate, a sheet, a platelet or a flake) and the usage of the same nomenclature can vary.

APPLICATIONS OF MOLYBDENUM AND ITS COMPOUNDS IN NANOSIZED FORMS

Elemental molybdenum

Molybdenum (Mo) is a transition metal with a body centered cubic crystal lattice from spatially centered cubic cells, with the Im3m crystallographic system [5]. Molybdenum nanoparticles (MoNPs) are characterized by increased tensile strength [6], and increased hardness and plasticity at high temperatures; they are almost insoluble in water, and have excellent thermal and electrical conductivity [7].

Inter alia, MoNPs are obtained by plasma physical deposition from the gas phase, a reduction of its oxide and chloride vapors. They can be obtained from the ^{99}Mo isotope by the $Mo(NH_4)_6$ radiolysis with Mo ions.

The nanoparticles and compounds of Mo find many applications in various industries. The most common application is the strengthening of metal structures, high temperature and high pressure elements. When added at 1–4% by weight to stainless steel, Mo increases its corrosion resistance.

Molybdenum is widely used in tribology. It is believed that the addition of only 0.25% of this element and its compounds results in a reduction of the coefficient of friction by at least 5% [6]. Thanks to high electrical conductivity, it is actively used in electrochemistry and electronics, combining electronic components, and as an addition to electrodes, mainly in the form of oxides.

Molybdenum oxides

Nanoparticles of molybdenum oxides (MoO_xNPs) can be obtained by pulsed laser ablation in water [8], and trioxide can additionally be obtained with the hydrothermal method [9]. These nanoparticles can be used in the degradation of polymeric materials, as well as in gas sensors in cars, and as anodes in Li-ion batteries [7,8,10]. In agriculture, MoO_xNPs have been shown to improve microbiological activity in the rhizosphere of chickpeas, and to increase its length, diameter, and root circumference [11]. What is more, MoO_xNPs display unique catalytic and electronic properties and have potential applications in chemical synthesis and in the refining of crude oil. They are also used in optical devices and constitute a promising material for the production of photoelectrochemical energy with

higher efficiency. In addition, MoOxNPs are used in electrochemical capacitors, coatings, nanowires, nanofibers, plastics and textiles, in specific applications of alloys and catalysts, as oxidation catalysts, cracking catalysts, hydrogenation catalysts and pigments (similar to Mo itself), in ceramics and glass production, and as a raw material for the production of molybdenum metal [12].

Molybdenum disulphide

Molybdenum disulphide (MoS_2) belongs to a class of transition metal dichalcogenides (TMDs), generally described by formula MX_2, wherein M represents a transition metal from groups 4–10 of the periodic table and X represents a chalcogen (S, Se, or Te). It exists in 2 crystalline forms: rhombohedral and hexagonal, the latter being more common. The hexagonal form is characterized by a multi-layer structure (platelets) in which the individual layers are loosely bound together with weak van der Waals forces, which results in effortless sliding between 2 S-Mo-S layers. This phenomenon, coupled with a lot of unique properties such as a low coefficient of friction, strong affinity for metallic surfaces, a film forming structure, and stability in the presence of most solvents, determines the excellent lubricating properties of MoS_2. In addition, MoS_2 retains lubricating properties *in vacuo*, and at extreme temperatures, from cryogenic temperatures to about 350°C in air. This is why MoS_2 in the bulk form is mainly used in the tribology area, as a dry and solid lubricant in, e.g., greases, dispersions, friction materials and bonded coatings.

Molybdenum disulphide 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 >80% of the mass of lubricants that exist in the form of powders or aerosols, and which are available on the consumer market. The use of MoS_2 in dry lubricants is of particular importance in aviation, aerospace, automotive, transportation, plastics, composites and various other industries (the International Molybdenum Association [IMOA]).

In recent years, an increase in the use and applications of the nanoform of MoS_2 (<100 nm) has been observed. The presence of weak van der Waals bonds allows obtaining single-layer and few-layer structures from the multi-layer MoS_2, which exhibit unique electronic, optical, mechanical, structural and chemical properties, distinct from the bulk form of MoS_2 [13–15]. The most prospective applications are foreseen for structures similar to nanotubes (an inorganic nanotube [INT]), fullerenes (e.g., an inorganic fullerene-like material [IF-MoS_2]) and 2D plates (2D)MoS_2. These structures are widely used for the synthesis of analogs of carbon nanotubes, fullerenes or graphene, in order to replace them in many consumer products [5,16–20].

Structures such as IF-MoS_2 and INT are mainly used in the production of advanced polymer nanocomposites, lubricants and self-lubricating coatings [16]. Their enhanced tribological parameters favor their frequent applications in the automotive industry and transportation as additives in engine oil and brake pads formulation. Their crucial role for friction and wear reduction has been described in several review articles [20–22]. The tribological properties of IF-MoS_2 have also been used in the field of biomedicine to reduce friction in some operations, e.g., in endoscopic procedures and bone tissue engineering [23].

Ultrathin (2D)MoS_2 also exhibits unusual properties, including photoluminescence, high lubricity, flexibility, and catalytic activity. The capabilities offered by (2D)MoS_2 with atomic-scale thickness are so wide that it is anticipated to eventually replace graphene and silicon in electronics, optoelectronics, photovoltaics, spintronics, sensing and energy storage in the future [6,14,15,18].

Both (2D)MoS_2 and IF-MoS_2 have huge prospects of wide application in biomedicine. Sensitive biosensors based on nanosized MoS_2 could serve as a diagnostic tool for cancer, detecting biomarkers like the prostate specific antigen (PSA) for prostate cancer or breast cancer miRNA [24,25], along with other molecules playing an

important role in organism homeostasis, e.g., H_2O_2 or glutathione [26,27]. Singh et al. [28] have created a biosensor allowing fluorescent pathogen detection. In comparison with the recently reported methods, this device is very sensitive, detecting *Salmonella typhimurium* at the 10 CFU×ml^{-1} level.

Another potential medical application for MoS_2 nanoparticles is biomedical imaging. Due to its unique properties, it could be applied in many diagnostic techniques, like computer tomography or magnetic resonance, thus replacing the toxic and potentially harmful contrasting agents such as gadolinium [29,30]. It has been shown that MoS_2 nanosheets display high absorbance in the near infrared (NIR) region, which makes them a potential NIR absorbing agent for cancer phototherapy [31,32]. An innovative method reported by Yin et al. [33] combines photo- and chemotherapy of cancer, using polyethylene glycol (PEG)-coated MoS_2 nanosheets as both photothermal agents and drug carriers [33]. Few reports have revealed certain antibacterial capabilities of different MoS_2 nanoforms, affecting pathogen-specific metabolic pathways, causing oxidative and membrane stress [34–36].

In conclusion, due to such a wide range of applications, mass production and commercialization of MoS_2, especially its nanoforms, is expected.

ENVIRONMENTAL ASPECTS OF THE APPLICATION OF MoS_2 NANOPARTICLES

Any compound that is widely used in the industry should be analyzed as potentially hazardous, due to the risk of accidental release to the environment. Considered factors should include, on the one hand, the persistence and accumulation of non-degradable particles and, on the other hand, solubility and reactivity which result in releasing the potentially toxic products of redox processes.

While MoS_2 in the bulk form is considered a stable compound, it can be oxidized to molybdenum oxide (considered low-toxic) [37]. A study conducted on chemically exfoliated MoS_2 nanosheets has shown an oxidative dissolution process in aqueous solutions, dependent on pH and the composition of this solution suggesting a non-persistent character of the nanoform in question [38]. Another study has characterized transport mechanisms in an aquatic environment for 2 types of MoS_2 nanoparticles. In porous media, simulated by sand columns, the more mobile type of the particles was MoS_2-Li (lithiated, compared with pluronic MoS_2-PL). The less mobile MoS_2-PL showed a tendency to aggregate and deposit on quartz media (sand), which may influence benthic organisms [39]. Taken together, limited data suggest that although clearance pathways depend on the nanoform and manufacturing method, MoS_2 should not be considered a putative environmental hazard.

Another aspect of nanosized Mo compounds is their environmental application. In general, MoS_2 exhibits a good adsorption capability, both for organic compounds and for heavy metal ions [40,41]. In addition, MoS_2 nanosheets are considered as a base for nanoporous or layer-stacked membranes. Currently, the development of such membranes is at early stages, but computer simulation and simple experiments with organic dyes suggest that they could be even more effective than graphene-based membranes. Potential applications of such devices (after up-scaling) would be in water treatment. Due to exceptional physical properties, sensors based on MoS_2 show high sensitivity towards biomolecules and metal ions. The reported compounds which could be detected with MoS_2-based field-effect transistor-based (FET) sensors include volatile organic compounds (hexane, toluene) [42], gases (NH_3, H_2) [43] or mercury ions [44].

BIOLOGICAL EFFECTS OF NANOPARTICLES OF Mo COMPOUNDS

Basic cytotoxicity testing on *in vitro* models

Different Mo compounds in nanosized forms were tested on mammalian cell lines in various *in vitro* experimental setups. Human cell lines, such as MCF-7 (breast cancer)

and HepG2 (hepatoma), when treated with a range of molybdenum trioxide (MoO_3) nanoparticles concentrations (25–0.625 μg/ml), showed a dose-dependent decrease in viability in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay [9]. The nanomaterial was manufactured using a hydrothermal method, resulting mostly in hexagonal nanorods of a mean size of 75 nm. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction test, performed on the C18-4 spermatogonial stem cell line treated with MoO_3 nanoparticles for 48 h, indicated a statistically significant influence of the NPs at concentrations of ≥50 μg/ml. Even lower concentrations (5–100 μg/ml) resulted in lactate dehydrogenase (LDH) leakage, which suggested cell membrane integrity disruption [45].

Nanoparticles of MoO_3 were considered mildly toxic in a study conducted on BRL 3A cells (immortalized rat liver cells) which analyzed 2 cytotoxicity endpoints, i.e., LDH leakage and mitochondrial function [46]. After 24-h incubation with NPs, the cell viability assessed by means of the MTT test suggested the toxic effect at 250 μg/ml; however, significant LDH leakage was present already at the concentration of ≥100 μg/ml. As for MoS_2, a study conducted on 2 cell lines, i.e., the NIH-3T3 murine embryo fibroblast cells and the human adipose derived mesenchymal stem cells (MSCs), led to similar conclusions. For the NIH-3T3 line, the concentration of 50 μg/ml of the NPs seemed to have a negative effect on cell viability. Hexagonal nanoplatelets of MoS_2 coated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (DSPE-PEG) showed no toxic effect at <300 μg/ml on human adipose derived mesenchymal stem cells [47].

Studies on cytotoxicity mechanisms

More detailed studies provide some possible mechanisms underlying the cytotoxic effect of MoNPs. Some of the investigated endpoints are oxidative stress, evaluated as the reactive oxygen species (ROS) production or glutathione depletion in cells. Fazio et al. [48] investigated the influence of MoO_2 and MoO_3 nanocolloids, produced using the laser ablation method, on the NIH/3T3 cell line. The cells were treated with the NPs in the concentration range of 10–100 μg/ml for 24 h, following which they were assayed with sulforhodamine B to measure viability. Additionally, total antioxidative activity (TAA) and ROS generation were measured using fluorescence-based methods (the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation decolorization and dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe, respectively). Treatment with both nanocolloids resulted in decreased viability (in a concentration-dependent manner). Moreover, ROS production was correlated with the NP size – smaller particles induced ROS accumulation; however, in a higher concentration (100 μg/ml) the effect was balanced by the antioxidative activity of the nanocolloids. According to the authors, the cytotoxic effect of the NPs was associated with a change in the redox status of the cell, resulting in oxidative stress induction.

In another study, MoO_3 nanoplates were tested on human breast cancer cells (MCF-7 and the invasive type MCF-7 with the $CD44_{high}/CD24_{low}$ phenotype) and the human keratinocyte HaCaT line in a broad concentration range – 50–400 μg/ml [49]. Cell viability was assessed using the MTT reduction test, while apoptosis, changes in the mitochondrial membrane potential and ROS production were measured using flow cytometry. Additionally, the western blotting method was employed to analyze the proteins connected with apoptosis. The HaCaT cells did not show decreased viability, but both MCF-7 lines were susceptible to the NPs. The cytometric analysis suggested that the decrease in viability was connected with apoptosis, probably involving the loss of the mitochondrial membrane potential and an induction of oxidative stress. The western blot analysis, showing elevated levels of cleaved caspases 8 and 9, and of Bax and Bcl-2 proteins, supported this theory.

In addition, ROS production seems to be a possible factor of the MoS_2 influence. In the study by Zou et al. [50], single-layer nanosheets reduced the viability of human embryonic lung fibroblast (HELF) cells, unless coated with bovine serum albumin (BSA), which diminished the toxic effect. In parallel, both coated and uncoated NPs promoted the proliferation of HELF cells, which, as a pathological process, can lead to idiopathic pulmonary fibrosis. The enhanced proliferation level might be in connection with the elevated ROS level, quantified with the DCFH-DA probe. Additionally, the western blot analysis revealed increased levels of the phosphorylated forms of PI3K (phosphoinositide 3-kinase), Akt and mTOR (the mammalian target of rapamycin kinase) after treatment with the uncoated NPs, which suggested the activation of the Akt signaling pathway. This protein cascade is engaged in the regulation of death and proliferation, and can underlie the abnormal growth of HELF cells. Activation of the signaling pathway can be induced as a result of an elevated ROS level in the cell, which points to a possible mechanism connecting the MoS_2-related oxidative stress and the increased proliferation of cells.

Liu et al. [51] investigated the influence of MoS_2 nanosheets on HepG2 cells, with special focus on the ATP-binding cassette (ABC) transporter. The viability assessed using the CCK-8 (cell counting kit) test was reduced at concentrations of 30 μg/ml and higher, and LDH leakage, suggesting membrane disruption, occurred at concentrations of 8 and 15 μg/ml. The use of a fluorescent trimethylammonium diphenylhexatriene (TMA-DPH) probe made it possible to analyze membrane features; its fluidity was found to have increased, and the phospholipid bi-layer was more chaotic in the treated cells, compared to the control group, supporting the observation that contact with the NPs influences the membranes in a negative manner. Also in this case, the ROS level and the mitochondrial membrane potential were altered in the treated cells, suggesting oxidative stress and apoptosis induction. Furthermore, the increased accumulation of the Calcein AM dye in the cells after contact with the NPs suggests an inhibition of the ABC transporter.

The ineffective mechanism of xenobiotic removal resulted in an increased chemosensitivity of the cells, evaluated by the ROS level measurement in response to arsenic treatment. Arsenic combined with NPs caused a higher level of oxidative stress than when applied alone, suggesting the xenobiotic accumulation in the cell. One of the possible mechanisms behind the impaired ABC transporter activity might be the soluble Mo ions released inside the cell from NPs. Molybdenum itself, or its derivative, could be a competitive inhibitor of the pump. Another probable explanation is damage to the membrane, influencing the ABC function.

In another study, the L929 murine fibroblast cell line treated with MoNPs showed a dose- and time-dependent decrease in viability in the concentration range of 1–100 μg/ml, measured with MTT and neutral red uptake (NRU) tests. The treatment-induced oxidative stress was analyzed using 3 endpoints: the lipid peroxidation level, i.e., the thiobarbituric acid reactive substances (TBARS) concentration measurement, glutathione (GSH) depletion and catalase activity of the cells. The NPs influence was observed at the concentration of 25 μg/ml, and for catalase even at 10 μg/ml, resulting in increasing lipid peroxidation, and GSH and catalase activity reduction. Also, the ROS production and mitochondrial membrane potential values were affected by the treatment, which suggests an elevated level of oxidative stress in the cells. Interestingly, MoNPs seemed to have a significant genotoxic influence on DNA, which was assessed using the comet assay. The possible mechanism of DNA breakage might be connected not only with ROS induction but also with the interaction of MoNPs with the enzymes involved in DNA processing and gene expression [52]. The production of singlet oxygen species might also be involved in the antibacterial activity of MoS_2 nanoflakes.

In the study by Shin et al. [53], MoS_2 nanoflakes were used to coat titanium (Ti) dental mini-implants. The aim of such an additional coating of Ti implants was to minimize the risk of bacterial infections and bacteria growth on the implant surface. In the experiment, a suspension of *Escherichia coli* was dispersed on Ti plates with MoS_2 NP coating. The analysis included bacteria viability and the measurement of ROS production, using a singlet oxygen sensor green (SOSG) reagent, as well as scanning electron imaging (SEM) of the plate surface. The SEM images indicated a destruction of the bacterial cell membrane on plates with MoS_2 coating, which could be explained by the elevated singlet oxygen level, correlated with the loss of the colony-forming unit (CFU) and the decreased viability of bacteria. On the other hand, the test with the MC3T3-E1 murine osteoblast cell line, suggested an increased proliferation of the cells, which is a positive factor for dental implants. The surface promoting cell growth determines a good biocompatibility and successful implantation. It is, however, possible that such an intensified proliferation in a longer time perspective may shift to a pathological process.

The so-called nanoknife effect is an interesting concept of the nanoparticles cytotoxicity mechanism. It has been studied on graphene nanoparticles which are usually very thin (even of atomic-scale thickness). The shape of the particles, and in particular the sharp, thin edges, can act like a blade, mechanically damaging the membrane. Graphene NPs induce pores in the cell membrane, leading to cytoplasm leakage and cell death. This effect was observed both on bacteria and cell lines [54,55].

A similar observation was made by Chng et al. [56], investigating the influence of the MoS_2 nanoparticles exfoliation level (meaning the particle thickness) on the cytotoxic effect. The MoS_2 nanosheets used for this purpose were made of bulk forms using 3 different techniques, resulting in 3 types of particles, distinguished by the number of layers. The A549 human lung cancer cells were then treated with varying concentrations of NPs (3.125–400 μg/ml) for 24 h. The toxic effect, measured with the MTT and WST-8 (water-soluble tetrazolium salts) assays, was stronger for the most effectively exfoliated nanosheets, and could be correlated with NPs thickness. A possible explanation stems from the nanoknife theory – thinner nanosheets have sharper edges, hence resulting in bigger membrane damage. Mechanical interaction between the cell membrane and MoS_2 nanoparticles can also be dangerous due to the phospholipid extraction, destroying the integrity of the membrane and leading to the same results: cytoplasm leakage and finally cell death.

This mechanism was examined considering the antibacterial potential of MoS_2 nanosheets, using *E. coli* and *Staphylococcus aureus* as a model of Gram-negative and -positive bacteria, respectively [57]. The experiment was supported by a computer simulation, showing an interaction between the MoS_2 nanosheet surface and membrane phospholipids. The simulation as well as SEM images of the bacteria showed the presence of dents on the membrane surface.

Not only lipids might be a target for MoS_2 nanoparticles. A simulation performed by Gu et al. [58] investigated the influence of MoS_2 nanosheets on 4 α-helical polyalanine peptides with different lengths. The model predicted structural changes of the peptides as soon as they touched the MoS_2 surface, which suggested the formation of a really strong bond. After some time, the interaction was quite broad and led to weakening the intra-peptide hydrogen bonds. As a result, the secondary structure of the peptides was significantly affected and finally destroyed.

Studies showing negligible or no cytotoxicity of the nanoparticles of Mo compounds – possible medical applications

In contrast to the above mentioned studies showing different cytotoxic effects, there are studies indicating a low and negligible cytotoxicity of Mo nanoforms, and the possibilities for their use as therapeutic tools.

The PC12 rat pheochromocytoma cells and the rat adrenal medulla endothelial cells (RAMEC) treated with a 1 ng – 100 μg/ml MoS_2 nanosheets suspension showed no deviation in viability when assayed with sulforhodamine B, compared to the untreated group. Abnormalities were neither observed in microscopic analysis nor with the electrical impedance sensing method [59]. In the study by Appel et al. [60], nanoflakes and nanosheets of MoS_2, immobilized on poly-dimethylsiloxane, were used as a base for the human epithelial kidney cells (HEK293f) growth. The cells were kept in contact with the nanomaterial for 4, 12, 24 and 48 h. Cell viability was estimated using fluorescent dyes – Calcein AM, live cell labeling, and ethidium homodimer-1, which enters dead cells only. Both the nanomaterials – mechanically exfoliated and produced with chemical vapor deposition – presented no toxic effect. Also, ROS production, measured after 24 h with a fluorogenic probe, was not elevated compared to the control group. Interestingly, the nanomaterials showed no mutagenic effect. The Ames test conducted on the TA100 strain of *S. typhimurium* did not show any significant increase in the mutation rate after 3 days of exposure to the NPs.

Results of the study comparing 2 nanoforms of MoS_2 (Pluronic F87 dispersed MoS_2 (PF87-MoS_2) and lithiated MoS_2 (Lit-MoS_2)) with an aggregated form (Agg-MoS_2) suggested that the aggregated form was more hazardous, leading to an increased inflammatory response where both nanoforms showed a negligible effect. The THP-1 (human monocytic cell line) and BEAS-2B (immortalized human bronchial epithelial cell line) cells were used as representative models of the response during inhalatory exposure to MoS_2. The MTT assay revealed that cell viability was not affected by any of the MoS_2 materials. Due to the macrophage-like and epithelial properties of THP-1 and BEAS-2B cells, respectively, it was possible to assess the inflammatory response under *in vitro* conditions by measuring the levels of interleukin 8 (IL-8) and 1β (IL-1β) and tumor necrosis factor α (TNF-α), using the enzyme-linked immunosorbent assay (ELISA) test. These chemo- and cytokines play an important role in the lungs during the immunological response to inhaled particles, attracting neutrophils to the inflammation site and mediating inflammatory reaction. The aggregated MoS_2 caused a significant release of IL-8, IL-1β and TNF-α, which was lower for Lit-MoS_2 and PF87-MoS_2. The results suggest not only a low cytotoxicity of MoS_2 NPs, but also a low pro-inflammatory effect, especially in comparison with the aggregated form [61].

Another study using immunotechniques was conducted by Pardo et al. [62] with fullerene-like MoS_2 nanoparticles. In this experiment, 3 cell lines, i.e., HepG2 human hepatoma, RAW264.7 murine macrophage-like and NL-20 human non-tumorigenic human bronchial epithelial cell lines, were used. All the 3 lines did not show decreased viability after 24-h exposure to NPs concentrations of 10, 50 and 100 μg/ml. The secretion of IL-1β, IL-6, IL-8 and TNF-α by the NL-20 line was assessed using the ELISA method, and mRNA for these cytokines was isolated and used in the real time polymerase chain reaction (RT-PCR) to estimate gene expression. Compared to the controls, only the TNF-α mRNA level was increased; however, the protein level was not elevated, which suggested that the NPs did not stimulate cytokine production. Similar results were obtained for the RAW264.7 cells, when the expression levels of genes for IL-1β, IL-8 and TNF-α were measured. The analysis of genes connected with the antioxidative capacity of the cell (hemeoxygenase-1, catalase, superoxide dismutase) suggests that the NPs might induce a protective response in the cells, stimulating the antioxidative response and, therefore, reducing its toxic effect. Hence, the low cytotoxicity would be a matter of complicated cellular balance.

Akhtar et al. [63] studied the effect of MoNPs as potential antioxidative agents. The MCF-7 and HT-1080 (human fibrosarcoma) cell lines, treated with different concentra-

tions of MoNPs, >200 μg/ml, showed no decreased viability in 2 assays, MTT and NRU. The LDH release test and the TBARS level measurement showed no difference between the control and Mo-treated cells. Comparable were also the ROS levels, measured with the DCFH-DA probe. Moreover, MoNPs significantly stimulated intracellular glutathione production, assessed with fluorescence probe o-phthalaldehyde, suggesting the antioxidative potential of the NPs. These nanoparticles were further tested in an experiment with hydrogen peroxide, revealing cytoprotective properties and maintaining the viability level despite the treatment. The results indicate that MoNPs are rather safe and can also have the ability to protect cells against oxidants.

The low cytotoxicity of nanoparticles of Mo compounds allows using them as phototermal agents. Photothermal therapy (PTT) is an evolving cancer therapy method, based on photo-absorbing nanomaterials, which can induce hyperthermia in the tumor, saving surrounding tissues. Nanoparticles of MoS_2 are useful due to their good absorbance of NIR light. Feng et al. [64] investigated the capabilities of flower-like nanoflakes, coated with PEG. In their study, the 4T1 murine mammary carcinoma cells and HeLa human cervical cancer cells were treated with the NPs themselves or with a NIR 808-nm laser irradiation. The CCK-8 assay indicated a rather good biocompatibility of the nanoflakes in the concentration range of 3.6–100 μg/ml. Cell viability was influenced by adding laser irradiation and decreased in a dose-dependent manner, both with the increasing MoS_2 concentration and the increasing laser power density. The confocal imaging of the cells revealed a damaged cytoskeleton and lysosome membrane, leading to cell death.

A similar study was performed on L929 murine fibroblasts and 4T1 cells, treated with MoS_2 nanosheets, plain or PEG-coated, in the concentration of >0.5 mg/ml. The cytotoxicity of nanoparticles was assessed using the CCK-8 kit. After 24-h incubation, no cytotoxic effect was observed, and cell morphology was unaltered. The NP-based treatment was, however, effective when combined with laser irradiation, showing a dose-dependent relationship, where cell viability decreased with the increasing NPs concentration. *In vitro* hemocompatibility tests on human red blood cells (HRBCs), measuring the hemolysis and coagulation levels, did not show any significant differences between the treated HRBCs and control cells [65].

A very interesting medical application of MoS_2 was proposed by Han et al. [66], who investigated the potential of polyvinylpyrrolidone-coated NPs in Alzheimer's disease (AD) therapy. The current hypothesis for the pathomechanism of AD is connected with the β-amyloid peptides (Aβ) forming aggregates and fibrils, and mediating oxidative stress, leading to the loss of neural connections and cell death. The most desired therapy should, therefore, inhibit the agglomeration of Aβ, dissociate the already existing agglomerates and, ideally, counteract ROS. The MoS_2 nanoparticles incubated with the Aβ precursor, the Aβ42 peptide, significantly inhibited its agglomeration; interacting with Aβ fibrils caused its destabilization in a time- and concentration-dependent manner. The SH-SY5Y human neuroblastoma or BV2 murine microglia cells incubated with Aβ42 showed viability reduced up to 40%, while the coincubation with MoS_2 nanoparticles showed a cytoprotective effect, increasing the viability level even for low NPs concentrations (2 μg/ml). The nanoparticles alone exhibited no toxic effect for both cell lines. The coincubation with MoS_2 nanoparticles also reduced the ROS level, especially for •OH radical.

Further results suggest that NPs can act as an inhibitor for calcium channels, maintaining calcium homeostasis in the cell. The lack of balance in the calcium levels can be a factor activating ROS production and increasing oxidative stress. Taken together, MoS_2 nanoparticles have a great potential against Aβ aggregation, which may lead to the development of new therapeutic applications.

Toxicity evaluation on *in vivo* models

Nanoparticles of Mo compounds were also tested on various *in vivo* models. Hao et al. [67] conducted a study investigating not only the toxicity but also biodistribution of PEG-coated MoS_2 nanosheets in Balb/c mice. The animals were administered with NPs intravenously (i.v.) (a dose of 10 mg/kg). Then, after 1, 7, 14, 30 and 60 days, they were sacrificed to collect major organs, *inter alia*, the heart, skin, bone and liver, and blood. The presence of MoS_2 NPs was traced after 1 day mostly in the mononuclear phagocyte system of the spleen and liver. After 30 days, the NPs were almost completely excreted from the body. High levels of Mo were found in the urine and feces of the animals, suggesting that both the renal and fecal pathways are engaged in Mo clearance, probably after MoS_2 oxidization and transformation into soluble oxide species, (e.g., MoO_4^{2-}). The serum biochemical indicators (e.g., aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase) and blood parameters were within the normal ranges, showing no significant differences. The results suggest no long-term toxicity of MoS_2, mainly due to its fast excretion.

Wang et al. [61] investigated the influence of MoS_2 (in the nano- and aggregated form) on the lung tissue of C57Bl/6 mice. Fourty hours or 21 days after oropharyngeal instillation (2 mg/kg), the mice were sacrificed, and the lung tissue and bronchoalveolar lavage fluid (BALF) were collected for further tests. A histological analysis of the lung tissue showed that only the aggregated MoS_2 form induced significant inflammatory reaction, whereas the NPs did not stimulate inflammation or increase the cyto- and chemokine level. A patch testing study conducted by Chen et al. [68] assessed the allergenic potential of MoS_2. Guinea pigs were exposed dermally to the MoS_2 thin film and nanoparticles (at the concentration of 1.6 and 0.16 mg/ml) for 24 or 48 h. The skin was observed for the induction of edema, erythema or ulceration. No allergic reaction was observed up to 48 h after the exposure.

Asadi et al. [69] investigated the influence of MoNPs (no physicochemical characteristics of the nanoparticles was provided) on male Sprague-Dawley rats. The NPs were administered intraperitoneally in three concentrations: 5, 10 and 15 mg/kg daily for 28 days. The rats were sacrificed and the liver, testes and blood samples were collected. The blood parameters (e.g., the number of red and white blood cells, the hemoglobin level, and hematocrit) were in the normal ranges. A microscopic analysis of the tissues revealed inflammatory infiltrates in the liver and a reduced diameter of seminiferous tubules. The histopathological changes correlated with enzyme or hormone levels, which were decreased for both the liver (aspartate transaminase [AST], LDH) and testes (testosterone).

The potential application of MoS_2 nanoparticles in photothermal therapy, as mentioned earlier, was investigated also on an *in vivo* model. Feng et al. [64] analyzed tumor tissues from Balb/c female mice after NIR laser irradiation, with or without MoS_2 nanoparticles administration. The tumors treated with MoS_2+NIR showed significant damage, characteristic of thermal therapy, i.e., shrunken cells, pyknotic nuclei, or DNA fragmentation. In a similar study, Wang et al. [65] investigated the effects of PTT on Balb/c mice bearing 4T1 cell-induced tumors. The analysis of Mo biodistribution after *i.v.* administration of PEG-coated NPs was in line with other published data, revealing the accumulation in the liver and spleen after 24 h and a time-dependent clearance within 40 days. During histological evaluation of the tissues, no abnormalities were observed. Also, blood parameters were comparable for the MoS_2-treated and control mice. After laser irradiation, only the group treated with the NPs showed tumor growth inhibition, and the animals were clinically normal >40 days after the treatment. In this combined therapy group (NIR laser + MoS_2 nanoparticles), a prolonged lifespan of tumor-bearing mice was observed (Table 1).

Table 1. Summary of *in vitro* and *in vivo* studies from Europe, Asia and the USA, performed on different nanoforms of molybdenum (Mo) compounds (a review conducted between 2005–2018)

MoX nanoform, method of NP production	*In vitro*/ *in vivo*	Type of cells/animals	Concentration used	Exposure time	Results	Comment	Reference
Elemental Mo nanoparticles ø40 nm	*in vitro*	L929 murine fibroblast cells	1–100 μg/ml	24, 48 h	concentration-dependent decrease in viability; induction of oxidative stress	positive genotoxic effect by comet assay	Siddiqui et al., 2015 [52]
Elemental Mo nanoparticles ø34 nm;	*in vitro*	MCF-7 human breast cancer cells; HT-1080 human fibrosarcoma cells	25–200 μg/ml	24 h	cell viability unaltered; the ROS level unaltered; no increase in LDH leakage	NPs showing antioxidative capabilities	Akhtar et al., 2015 [63]
Elemental Mo nanoparticles	*in vivo*	Sprague-Dawley rats	5–15 mg/kg daily	28 day	blood parameters unaltered; decreased testosterone, LDH and AST levels; inflammatory infiltrations in the liver; pathological changes in the testes		Asadi et al., 2017 [69]
MoO_3 nanorods; hydrothermal method ø75 nm	*in vitro*	MCF-7 human breast cancer cells; HepG2 human hepatoma cells	0.625–25 μg/ml		dose-dependent decrease in viability		Fakhri et al., 2016 [9]
MoO_3 nanoparticles; pulsed-plasma reactor ø30 nm	*in vitro*	C18-4 murine spermatogonial cells	5–100 μg/ml	48 h	decrease in viability from 50 μg/ml; LDH leakage from 5 μg/ml		Braydich-Stolle et al., 2005 [45]
MoO_2/MoO_3 nanocolloids; laser ablation ø50 nm	*in vitro*	NIH/3T3 murine fibroblast cells	10–100 μg/ml	24 h	concentration-dependent decrease in viability; induction of oxidative stress	NPs showing antioxidative capabilities	Fazio et al., 2018 [48]
MoO_3 nanoparticles ø30, 150 nm	*in vitro*	BRL 3A (rat liver cells)	50–250 μg/ml	24 h	decrease in viability at 250 μg/ml; LDH leakage from 100 μg/ml		Hussain et al., 2005 [46]
MoO_3 nanoplates 200×400 nm	*in vitro*	MCF human breast cancer cells; iMCF-7 human invasive breast cancer cells; HaCaT human keratinocytes	50–400 μg ml	48 h	concentration-dependent decrease in viability of MCF and iMCF; no alterations for HaCaT cells; induction of oxidative stress	apoptosis pathway activated; cleaved caspase 8 and 9	Anh Tran et al., 2014 [49]

Table 1. Summary of *in vitro* and *in vivo* studies from Europe, Asia and the USA, performed on different nanoforms of molybdenum (Mo) compounds (a review conducted between 2005–2018) – cont.

MoX nanoform, method of NP production	*In vitro*/ *in vivo*	Type of cells/animals	Concentration used	Exposure time	Results	Comment	Reference
MoS_2 nanoflakes (coating on the Ti surface)	*in vitro*	MC3T3-E1 murine osteoblast precursor cells; *E. coli*		6 h	antibacterial capacity; induction of oxidative stress; membrane disruption; enhanced proliferation of MC3T3-E1 cells		Shin et al., 2018 [53]
MoS_2 nanosheets; liquid exfoliation	*in vitro*	PC12 rat pheochromocytoma cells; rat adrenal medulla endothelial cells (RAMEC)	0.01–100 μg/ml	24 h	cell viability unaltered		Shah et al., 2015 [59]
Single-layer MoS_2 nanosheets, plain or coated with BSA ø1–2.5 μm	*in vitro*	human embryonic lung fibroblasts (HELF)	0.1–25 μg/ml	24 h	decrease in viability; enhanced proliferation rate; induction of oxidative stress	BSA coating diminished toxic effect	Zou et al., 2017 [50]
MoS_2 nanoflakes and nanosheets prepared by scotch tape exfoliation/chemical vapor deposition +sonication ø200 nm	*in vitro*	HEK293f human epithelial kidney cells; *S. typhimurium*	0.1–100 μg/ml	4, 12, 24 and 48 h	cell viability and the ROS level unaltered; no increase in the mutation rate for *S. typhimurium*	NPs immobilized on the PDMS plate	Appel et al., 2016 [60]
MoS_2 nanosheets ø441 nm	*in vitro*	HepG2 human hepatoma cells	0.5–30 μg/ml	24 h	decrease in viability from 30 μg/ml; induction of oxidative stress; LDH leakage from 8 μg/ml	inhibition of the ABC transporter – probably by competitive inhibition or membrane disruption	Liu et al., 2017 [51]
MoS_2 nanoparticles coated with PVP; laser ablation ø100 nm	*in vitro*	SH-SY5Y human neuroblastoma; BV2 murine microglial cells	1–10 μg/ml	48h	cell viability unaltered	application in AD treatment; inhibition of β-amyloid peptide aggregation; antioxidative capabilities	Han et al., 2017 [66]

MoS_2 nanosheets; chemical intercalation	*in vitro*	A549 human lung cancer cells	3.125–400 μg/ml	24 h	exfoliation level-dependent decrease in viability (thinner NPs had enhanced cytotoxic effect)	induction of the nanoblade effect	Chng et al., 2014 [56]
Flower-like MoS_2 nanoflakes coated with PEG hydrothermal method ø90 nm	*in vitro* + *in vivo*	4T1 murine mammary carcinoma cells; HeLa human cervical cancer cells; Balb/c mice	3.6–200 μg/ml	48 h	cell viability unaltered	application in photothermal therapy; anti-tumor capabilities in combination with NIR irradiation	Feng et al., 2015 [64]
MoS_2 nanosheets ø100 nm	*in vitro* + computer simulation	*E. coli*; *S. aureus*	5–60 μg/ml		antibacterial capacity; membrane disruption	phospholipid extraction	Wu et al., 2013 [57]
MoS_2 nanoplatelets, coated with DSPE-PEG ø90 nm	*in vitro*	NIH-3T3 murine embryo fibroblast cells; MCS human adipose derived mesenchymal stem cells	5–300 μg/ml	6, 12, 24 h	decrease in viability of NIH-3T3 cells from 50 μg/ml; no alterations in MCS cells		Rashkow et al., 2015 [47]
MoS_2 nanosheets and aggregates by ultrasonication/ chemical intercalation/ floculation ø27.6 nm/ 187.5 nm	*in vitro* + *in vivo*	BEAS-2B immortalized human bronchial epithelial cells; THP-1 human monocytic cells C57BL/6 mice	6.25–50 μg/ml	24 h	cell viability unaltered *in vitro*; for nanoforms: low proinflammatory effect *in vitro* and *in vivo*; for aggregates: inflammatory reaction *in vitro* and *in vivo*		Wang et al., 2015 [61]
MoS_2 nanofilm and microparticles; direct sulfurization	*in vitro* + *in vivo*	PANC1 human pancreatic cancer cells; 293 human embryonic kidney cells; human pancreatic duct epithelial (HPDE) cells; human mammary epithelial (HMLE) cells; SUM159 mesenchymal triple-negative breast cancer cells; MDA-MB-231 invasive ductal breast carcinoma cells; guinea pigs	0.0016–16 mg/ml	24 h	cell viability unaltered up to 0.016 mg/ml, slight decrease in viability for higher concentrations; no skin allergy symptoms *in vivo*		Chen et al., 2018 [68]

Table 1. Summary of *in vitro* and *in vivo* studies from Europe, Asia and the USA, performed on different nanoforms of molybdenum (Mo) compounds (a review conducted between 2005–2018) – cont.

MoX nanoform, method of NP production	*In vitro*/ *in vivo*	Type of cells/animals	Concentration used	Exposure time	Results	Comment	Reference
MoS_2 fullerene-like nanoparticles ø80 nm	*in vitro*	NL-20 human non-tumorigenic human bronchial epithelial cells; HepG2 human hepatoma cells; Raw264.7 murine macrophage-like cells	10–100 µg/ml	24 h	cell viability unaltered; cytokines production unaltered	stimulation of antioxidative enzyme genes (SOD, HO-1, catalase)	Pardo et al., 2014 [62]
MoS_2 coated with PEG prepared with a high-temperature solution-phase method and sonication ø91 nm	*in vitro* + *in vivo*	RAW 264.7 murine macrophage-like cells; 4T1 murine mammary carcinoma cells; 293T human embryonic kidney cells; Balb/c mice	*in vitro*: 25–200 µg/ml *in vivo*: 10 mg/kg		slight decrease in cell viability; ROS level unaltered; slightly increased LDH leakage; blood and biochemical parameters unaltered; effective excretion of Mo from the organism		Hao et al., 2016 [67]
MoS_2 nanosheets coated with PEG; hydro- or solvothermal method ø50, 80, 100 nm	*in vitro* + *in vivo*	L929 murine fibroblasts; 4T1 murine mammary carcinoma cells; Balb/c mice	0.125–0.5 mg/ml	24 h	cell viability unaltered; no hemolytic effect *in vitro* and *in vivo*	application in photothermal therapy; anti-tumor capabilities in combination with NIR irradiation	Wang et al., 2015 [65]
MoS_2 nanosheets	computer simulation				membrane disruption	degradation of the α-helical peptides secondary structure	Gu et al., 2016 [58]

AD – Alzheimer's disease; AST – aspartate transaminase; BSA – bovine serum albumin; DSPE-PEG – 1,2-distearoyl-snglycero-3-phosphoethanolamine conjugated with polyethylene glycol; HO-1 – heme oxygenase-1; LDH – lactate dehydrogenase; NIR – near infrared; NP – nanoparticle; PDMS – polydimethylsiloxane; PEG – polyethylene glycol; PVP – polyvinylpyrrolidone; ROS – reactive oxugen species; SOD – superoxide dismutase.

CONCLUSIONS

The analysis of the available data indicates that there is no coherent mode of action of the nanoparticles of molybdenum compounds in biological systems. The experimental conditions as well as the nanomaterials used vary considerably. Usually, the physicochemical characterization of the NPs is not sufficient, different experimental models are employed, and the concentrations/doses of the NPs used for exposure range from ng/ml to mg/ml. The antibacterial activity of the NPs seems to be confirmed, but the mechanism is not clearly defined. Moreover, different cellular studies indicate divergent mechanisms, from increased ROS production to antioxidative and cytoprotective activity. Some nanoforms can be clearly cytotoxic, while others can promote the proliferation of normal and cancer cells. *In vivo* experiments indicate a rather good biocompatibility of molybdenum nanoforms and their fast excretion in the water-soluble form. Published data on the possible medical applications of molybdenum nanoforms, especially MoS_2, warrant further studies in this direction.

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