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Evaluation of biological response induced by molybdenum oxide nanocolloids on *in vitro* cultured NIH/3T3 fibroblast cells by micro-Raman spectroscopy

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ABSTRACT

Tailored colloids of uniformly sized and engineered molybdenum oxide nanoparticles were produced, *for the first time,* by pulsed laser ablation in water. This green technique ensures the formation of contaminant-free nanostructures and the absence of by-products, very useful issues in biological applications. A selective tuning of Mo−O chemical bonding configurations and a suitable control of nanoparticles size distributions were achieved during the ablation processes by varying the water temperature and by applying an external electric field. The metal redox properties are fundamental factors governing both cell uptake and interaction mode with Mo oxide nanoparticles. Micro-Raman spectroscopy was used to investigate the existence of cellular changes induced by Mo oxide colloids on the fibroblast cell line NIH/3T3 in relation to the molecular vibrations due to proteins, lipids and nucleic acids. The label-free micro-Raman spectroscopy provides an easy and noninvasive method to monitor the harmful effect of toxic agents on cells through ROS production or redox-dependent mechanisms. In view of potential biological applications, molybdenum oxide nanoparticles cytotoxicity towards NIH/3T3 cells was also investigated. A statistical analysis shows that, in the 10–100 μg/mL Mo concentration range, all the colloids are cytotoxic, progressively reducing the cell viability down to 75% upon increasing the concentration. The effect is less pronounced for the oxygen deficient MoO_3 samples where cell viability does not fall below 85%. These results open the way to identify potential bioactive products affecting cellular redox status, by using only the Raman spectral data, even before performing lengthy and expensive specific clinical analyses.

1. Introduction

Metal oxide nanostructures can act as nanoprobes for biomedical imaging, drug delivery carriers or as therapeutic agents by themselves. Moreover, they have shown to be excellent antioxidants *in vitro* and *in vivo* models, even if their toxicity emerges in some conditions. This is determined by the chemical nature of the metal oxide nanostructures, able to switch between different oxidation states, the surface-to-volume ratio and by the testing conditions (i.e. the experimental environment or the cell lines). Among the investigated metal oxide nanomaterials, molybdenum oxide nanostructures have become an attractive material as potential bioactive products. Recent studies show that Mo oxide nanostructures are effective against cytotoxicity and oxidative stress induced by H_2O_2 in human breast MCF-7 and fibrosarcoma HT-1080 cells [1]. On the other hand, Mo oxide nanoparticles (NPs) exhibit excellent antimicrobial activity against *Candida albicans* and *Aspergillus niger* and potent cytotoxicity against lung and breast cancer cells (MCF-7 and HEP G2 cell lines) [2]. Moreover, it is known that Mo is incorporated into the pterin-derived cofactor MoCo required for the catalytic functions of several redox enzymes present in many types of cells, including human cells [3]. In biological systems, as part of redox enzymes activity, Mo acts by shuttling itself between three different oxidation states and it results a key factor in the reduction of chemicals at the surfaces of Mo based sulfides or oxides NPs.

Despite these unique properties, which make its use very attractive, Mo oxide nanostructures are reactive or catalytic, and thus potentially

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toxic. Hence, for their safe use, investigations about the cellular toxicity in relation with Mo dose, selective Mo-O chemical bonding configurations, NP size and their purity are yet needed. Since the mice fibroblast NIH/3T3 cell line is one of the widely employed cell lines most sensitive to chemical-induced cytotoxicity in toxicity tests of biomaterials or drugs in medicine, a systematic study to understand the interactions of Mo oxide nanomaterials with NIH/3T3 cells is a particularly important scientific issue.

In this work, *for the first time to our knowledge*, physical-chemical properties of Mo oxide nanocolloids were controlled during the production process, namely the laser ablation in liquids technique, by varying the water temperature and by applying an external electric field. In particular, we obtained two types of nanocolloids: one with spherical shape, size less than 50 nm and a dominant MoO_2 surface chemical configuration, and another with oblong structure where the oxygen deficient MoO_3 phase increases at expense of the MoO_2 one. Both nanocolloids induce metal redox and antioxidant properties which strongly depend on the Mo—O surface chemical bonding configurations and NPs morphology. Compared to the literature data, our result is innovative in terms of: i) the possibility of tuning NPs surface composition and surface/volume ratio directly in the production phase, without any post-synthesis treatment, ii) the ability to modulate their bio-properties as the production of Reactive Oxygen Species (ROS) and antioxidant capability, which affect cell-signaling mechanisms. We showed also that detailed indications of cellular changes can be obtained by using the spectroscopic data coming from optical Raman scattering experiments, i.e. even before performing lengthy and expensive specific biochemical analyses. These evidences are in good agreement with the variations of cytotoxicity and antioxidant properties carried out with standard procedures. Thus, Raman spectroscopy revealed to be a powerful clinical tool to monitor potential DNA or lipid damage induced by Mo oxide on the cells, i.e. useful to identify potential anticancer complexes acting through ROS production or redox-dependent mechanisms.

2. Materials and methods

2.1. Molybdenum oxide NPs synthesis and characterization

The second harmonic (532 nm) of a laser operating at 100 kHz repetition rate with a pulse width of 6–8 ps was used for the ablation experiments. Four colloids were prepared varying the water temperature and applying an external electric field (EF) during the ablation processes. Here, the colloids prepared at room temperature (RT) and 80 °C, without and applying the electric voltage, have been named Mo_RT_0 V, Mo_RT_100 V, Mo_80C_0 V and Mo_80C_100 V, respectively. More details about the samples preparation procedure are reported in Ref. [4].

Mo content in colloids was determined by GF-AAS using a Varian 220/Zeeman atomic absorption spectrometer (AAS Mulgrade, Victoria, Australia), equipped with a single-element hollow cathode lamp and a Varian PSD autosampler. For the analysis, samples were diluted 1:5000 (v:v) with 0.2% HNO_3 (AAS grade). The quantification of Mo in the colloids was carried out using the external standard method; the reported data are the average of three determinations. The analytical method was validated according to the ICH guidelines (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005). The linearity was > 0.999, the precision (expressed as relative standard deviation) 0.552, and the detection limit 5.0 ng/mL.

X-ray photoelectron spectroscopy (XPS) spectra were acquired by an apparatus equipped with a monochromated Kα radiation as exciting source while a flood electron gun was used to avoid sample charging. The surface status was checked not to vary after some mild argon ions sputtering cycles, carried out to remove contamination from chemisorbed species. The samples chemical composition was determined using Scofield's sensitivity factors supplied with the Thermo-Avantage analysis software. X-ray diffraction (XRD) patterns were recorded in the 2Θ range from 20 °C to 80 °C using a Bruker D8 Advance X-ray diffractometer with CuKα radiation (1.5406 Å). The Fourier transform infrared (FTIR) spectra were recorded, in the 4000–600 cm^{-1} range, using a Spectrum 100 Perkin-Elmer spectrometer equipped with a microscope which allows single-point infrared spectroscopy. The Raman spectra were acquired using a Jobin-Yvon XploRA apparatus. The excitation wavelength used was the 532 nm solid state semiconductor laser. The 5-mW laser beam was focused by the optics of an Olympus BX40 confocal microscope onto an area of 1.8 μm^2 on the sample surface. The backscattered radiation was collected by the same optics of the microscope and dispersed by a monochromator equipped with two gratings which allow investigation in the spectral range 600-1800 cm^{-1}, with a spectral resolution of about 1.0 and 2.5 cm^{-1}, respectively. The elastically scattered radiation was rejected by the use of an edge filter. Finally, a cooled CCD sensor was used to record the spectra, usually averaged for a period of 80 s.

In vitro ABTS assay, carried out as described in a previous paper [5], determines the capacity of the colloidal systems Mo_80C_0 V and Mo_80C_100 V (Mo 0–100 μg/mL) to quench the stable radical $ABTS^{\cdot+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation]. Values obtained for each sample were compared with the concentration–response curve of a standard Trolox solution, and expressed as μmoles of Trolox Equivalents (TE) for Mo mg. Each determination was carried out in triplicate.

2.2. Cell cultures

In these experiments we employed the fibroblast cell line NIH/3T3 (American Type Culture Collection, Rockville, MD, USA). Cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, streptomycin, and penicillin and maintained in an incubator with humidified atmosphere containing 5% CO_2 at 37 °C. For the experiments, cells were plated in 24-wells cell plates at an initial density of 7.5×10^4 cells/well.

2.3. Citotoxicity assay

The biocompatibility of the colloids Mo_80C_0 V and Mo_80C_100 V on NIH/3T3 cells was investigated using sulforhodamine B (SRB; a dye binding to cellular proteins) assay, as described by Vichai et al. [6] with some modifications. For the SRB assay, cells (7.5×10^4 cells/well) were plated in 24-wells cell plates and, after 24 h, semi-confluent monolayers were treated for 24 h with the colloid solutions added to the cell culture medium (up to 100 μg/mL Mo for Mo_80C_0 V and up to 70 μg/mL Mo for Mo_80C_100 V). Since the investigated samples have a different Mo content, the concentration range used for each colloid was chosen so to avoid the harmful effects on cell growth that might be due to a greater solvent amount added to the cultures. Control cells (CTR) were exposed to the same volumes of the vehicle alone (water). Then, cells were fixed using 10% trichloroacetic acid for 1 h at 4 °C. After fixation, cells were washed twice with water and incubated with SRB (0.4% w/v in 1% acetic acid) for 30 min at RT, followed by four washes with 1% acetic acid. The bound dye was solubilized in 1 mL of 10 mM Tris base solution and the absorbance was measured at 565 nm. Cell viability results are expressed as percentage of viable cells in treated samples with respect to controls. All the experiments were performed in triplicate and repeated three times.

2.4. Cell TAA and ROS accumulation

Cells exposed for 24 h to Mo_80C_0 V and Mo_80C_100 V (20, 40 and 100 μg/mL Mo for Mo_80C_0 V; 20 and 40 μg/mL Mo for Mo_80C_100 V) were lysed with 0.05% Triton X-100. The