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Abstract

Context: as a kind of non-metal oxide SiO_2 NPs have been extensively used in biomedicine, pharmaceuticals and other industrial manufacturing fields, such as DNA delivery, cancer therapy… Our group had developed a method based on microemulsion process to prepare SiO_2 NPs incorporating photonic or magnetic nanocrystals and luminescent nanosized inorganic metal atom clusters. However, the toxicity of nanoparticles is known to be closely related to their physico-chemical characteristics and chemical composition.

Object: it is therefore of interest to investigate the toxicity of these novel SiO_2 NPs to the cells that may come in contact.

Materials and methods: the potential toxic effect of the functional @SiO_2 NPs containing Mo_6 clusters with or without gold nanoparticles was investigated, at concentrations 1 µg/mL, 10 µg/mL and 100 µg/mL each, on three different cell lines. Cell viability was measured by the MTT test in monolayer's culture whereas the cytotoxicity in spheroid model was examined by the APH assay. In a second time, oxidative-stress-induced cytotoxicity was investigated through glutathione levels dosages.

Results: the results indicated that both A549 and L929 cell lines did not exhibit susceptibility to functional @SiO_2 NPs-induced oxidative stress unlike KB cells.

Discussion: SiO_2 NPs containing CMB may become toxic to cultured cells but only at a very high dosage level. Therefore, this toxicity depends on cell lines and more, on the model of cell cultures. The selection of appropriate cell line remains a critical component in nanotoxicology.

Conclusion: these results are relevant to future applications of SiO_2 gold-cluster NPs in controlled release applications.

Introduction

The rapid development of nanotechnology in the last decade has created a myriad of engineered nanomaterials. In recent years, the use of functional SiO_2 NPs (classically noted @SiO_2) has been extended to biomedical and biotechnological fields such as biosensors and biomarkers [1-3]. More, novel procedures, like microemulsion, for preparing nanocomposites @SiO_2 with complex architectures were developed in the last decade [4-7]. In a biomedical context, @SiO_2 easily leads to surface modification and the ability to intercalate fluorescent probes into the particle structure. For example, neutral-red@SiO_2 NPs were utilized to monitor a variety of intracellular nutrients level [8]. Similarly, treatment of breast cancer using gold@SiO_2 NPs has been suggested [9].

In a same way, since 2008, our group develops a simple, versatile, highly reproducible and efficient method based on microemulsion process to prepare large amount of @SiO_2 NPs incorporating photonic or magnetic nanocrystals and luminescent nanosized inorganic metal atom cluster based on Molybdenum (Mo) or Rhenium (Re) compounds [10-13]. These metal atom cluster salts, as for instance $Cs_2Mo_6Br_{14}$ (noted CMB) used in this work, are synthesized by a solid state chemistry route at high temperature [14]. These compounds are built up from Cs cations and discrete nanosized cluster units $[Mo_6Br^i_8Br^a_6]^{2-}$ wherein the Mo_6 cluster is face-capped by eight inner bromine ligands (Br^i) and additionally bonded to six bromine apical ligands (Br^a). Owing to the ionic nature of the interaction between the cluster units and the cations, $Cs_2Mo_6Br_{14}$ solid state powder can be dispersed at nanosized level in solution [11]. Even after high dispersion in organic or inorganic matrix, these metal atom clusters exhibit a broad emission band in the red and NIR (550–900 nm), centred around 700 nm [15,16], which is particularly interesting for biotechnology applications as it corresponds to a low absorption of human tissues at these wavelengths. Moreover, these metal clusters could generate singlet oxygen under irradiation, what is of particular interest for photodynamic therapy applications

(PDT) [17]. Thus all these works already demonstrated that Mo_6 or Re_6 cluster units may represent a complementary alternative to traditional luminophores (organic dyes, QDs or lanthanide based nanocrystals) developed for theranostic applications. In this work, three different types of nanoparticles were used: SiO_2, $Cs_2Mo_6Br_{14}@SiO_2$ (noted CMB@SiO_2) and [$Cs_2Mo_6Br_{14}$-Gold]@SiO_2 (noted Au-CMB@SiO2).

However, the toxicity of nanoparticles is known to be closely related to their physico-chemical characteristics and among them the size, shape, specific surface area, surface charge and chemical composition [18]. So, these attractive composite nanoparticles may have new toxicity profiles due to their increased reactivity and must be approached carefully [19]. It is therefore of interest to investigate the toxicity of these novel silica NPs to different exposed cells (mouth and lung epithelial cells and fibroblasts).

Previous studies have shown possible hazardous effects of some nanoparticles on mammalian cells grown in two-dimensional (2D) cultures. However, 2D in vitro cell cultures display several disadvantages such as changes in cell shape, cell function, cell responses and lack of cell-cell contacts. There is however a lack of suitable in vitro systems to study the tissue damage of nanoparticles. For this reason, the development of better models that planar cell culture for mimicking in vivo conditions is essential. Cells growing in spheroids show higher degree of morphological and functional differentiation than monolayer cells, due to oxygen/nutrients' gradients, accumulation of catabolites, cell junctions development, matrix extra cellular synthesis…The potential of this model system in cell and tissue research and drug delivery has been stressed previously [20].

Nanoparticle penetration through tissue after extravasation is considered as a key issue for their distribution and therapeutic or pathologic effects. In the present study, we developed and thoroughly characterized a 3D spheroidal cell culture to mimic natural tissue and investigated

the nanoparticles cytotoxic effects. The results were compared to cultivation in 2D monolayer culture.

The potential toxic effect of the functional @SiO_2 NPs containing Mo_6 clusters with or without gold nanoparticles was investigated on three different cell lines; human lung tumor epithelial cell (A549), human oral cancerous keratinocyte cell (KB) and fibroblast murine cell L929. In a second time, as data on silica NPs showed oxidative-stress-induced cytotoxicity in different types of cultured mammalian cell lines [21], this possible mechanism of toxic action was investigated through glutathione levels dosages.

3D culture that mimics the tissue morphology, tumor stroma, is ideally suited to systematically investigate the factors influencing the penetration characteristics of newly developed nanomedicines to allow the design of nanoparticles with optimal penetration characteristics.

MATERIALS AND METHODS

Nanomaterials

Polyoxyethylene (4) lauryl ether (Brij30) and tetraethoxysilane (TEOS, 99.00%) were purchased from Sigma-Aldrich. Ammonia (28 wt % in water) and n-heptane (99.00%) were purchased from VWR. Ethanol (99.80%) was purchased from Fluka. The $Cs_2Mo_6Br_{14}$ cluster compound was prepared according to a published procedure [14]. All the silica NPs have been prepared using a water-in-oil (W/O) microemulsion process developed by our group since the earlier 2000 [5]. Microemulsions are thermodynamically stable dispersions of two immiscible fluids (i.e. n-heptane and complex water phase) stabilized by the arrangement of surfactant molecules (i.e. Brij30) at the interface. The W/O microemulsions consist of nanodroplets of

complex water phase dispersed in an oil phase and stabilized in spherical reverse micelles created by the surfactant molecules. Those hydrophilic droplets can then be considered as nanoreactors and by controlling the molar ratio of the mixture oil/water/surfactant, it is possible to predetermine the size and shape of those droplets and, as a consequence, to tailor the size and shape of the final silica NPs. In this work, the complex water phase was prepared by dissolving the $Cs_2[Mo_6Br_{14}]$ cluster compound in a mixture of ethanol and distilled water (1:1 volume ratio). The concentration of the cluster sol ranges from 0.01 to 0.02 M. Typically, 47 mL of heptane (oil-phase) was mixed with Brij30 (15 mL, surfactant) followed by the dropwise addition of 1.6 mL of the cluster sol and 1.3 mL of an ammonia solution. After 1 h of magnetic stirring, 2 mL of TEOS were added. The reaction was left under magnetic stirring for 3 days. Thanks to this W/O microemulsion process, the Mo_6 cluster units were efficiently encapsulated in the silica with a good stability and reproducibility. For pure SiO_2 NPs, the complex water phase was free of cluster. The Au-CMB@SiO2 NPs were prepared through a similar microemulsion process with of course some modifications allowing the formation of gold nanocrystals inside the SiO_2NPs. Same ratio of heptane and Brij30 were used as for SiO_2 or CMB@SiO_2. Then is added successively an aqueous solution of gold (III) chloride $HAuCl_4.3H_2O$ (40 mg in 200µL of water), an aqueous ammonia solution (28%, 400µL), a solution of 0.1M $NaBH_4$ dropwise (500 µL) and finally a complex aqueous cluster sol. The precursor of silica (TEOS, 2mL) was added one hour later. After the addition of TEOS, the reaction is stirred for 3 days.

Finally, in all cases, the microemulsion is destabilized by adding ethanol and the nanoparticles were collected and washed once with ethanol by centrifuging at 20000g during 20 minutes and then five times with water in order to remove the surfactant molecules (40000g during at least 30 minutes) before to be dispersed in purified water at concentration around 15mg/ml. The average hydrodynamic size of the SiO_2 NPs in water solution was estimated by dynamic

light scattering using a Malvern Zetasizer Nano ZS apparatus. The external morphology of the nanoparticles was observed by scanning electron microscopy (SEM) using a JEOL (JSM 6301F) microscope. Samples for SEM were simply prepared by depositing precipitated and dried powders directly on aluminium metal sample holder. All the samples and especially the gold nanocrystals inside the SiO_2 NPs were studied by transmission electron microscopy (TEM) using a microscope JEOL 2100 LaB6 at 200 kV or JEOL JEM-1400 microscope operating at 120 kV. Samples for TEM analysis were prepared by placing a drop of the diluted solution in mesh copper grids, allowing the solvent in the grid to evaporate at room temperature.

Cells culture

The A549 cell line (ATCC® CCL-185™), an human lung carcinoma, the KB cell line (ATCC® CCL-17™) derived from an human epidermal mouth carcinoma and the fibroblast murine L929 cell line (ATCC® CCL-1™) were used in this study. For the analysis, cells were grown to subconfluency in RPMI-1640 medium (Lonza) for the first and DMEM (Lonza) for the other, supplemented with 2mM L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 25 mM HEPES with the addition of 10% (v/v) fetal calf serum (FCS). These cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 with exponential proliferation. Confluent cells were detached with trypsin/EDTA for 5 min.

In the 2D model, cells were seeded into 96-well plates at a concentration in each well of 110^4 cells/mL, 9.10^3 cells/mL and 75.10^2 cells/mL for A549, KB and L929 respectively. The plating number is chosen in order to have sufficient cells to obtain a significant OD at each time (D1 or D4) avoiding the confluence of the cultures.

For the 3D model, flat 96-well plates were treated with 50 µL of 1% agarose prepared in phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+} to form a thin film of a no adhesive

substrate. Single-cell suspension (700 cells / 200 µL for A549, 100 cells / 200 µL for KB and L529) was seeded into individual wells to initiate spheroid formation. The 3D cultures were used after 4 days of incubation, without moving the microplates. The initial number of cells in 3D culture is determined to obtain sufficiently large spheroids (> 200 mm) to be able to manipulate them and to avoid necrotic centers at the end of the experiment (D4).

Material exposure

The stock suspensions of each silica NPs were sterilized by 0.22 µm filter and stored at 4°C. For each analysis, the stock suspensions were freshly diluted in the cell culture medium at concentrations 1 µg/mL, 10 µg/mL and 100 µg/mL each. Serial dilution of NP were carried out in culture media (DMEM or RPMI) with low serum (1% FBS) to prevent particle agglomeration. The final dilution is realized in medium with a usual serum concentration. After initial cell culture, supernatant was replaced with freshly dispersed nanoparticles suspensions.

Cells free of nanoparticles were used as control cells throughout each assay. After 30 hours, allowing the cell adhesion, the conventional cultures were exposed to the nanoparticles for 4 days at the range of concentrations. Cytotoxicity was evaluated at 2 times: day 1 and day 4. 3D culture were exposed at the same range of NP concentrations for 96h.

Cytotoxicity in 2D model

Cell viability was measured by the MTT assay. Following the exposure to nanoparticles, the cells were incubated with MTT (1 mg/mL) for 4 hours. The supernatant was then removed and 100 µL of DMOS were added into each well to dissolve formazan crystals. After thoroughly mixing, Optical Density was measured at 570 nm with ELISA reader. Survival

rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of control (cells alone =100%).

Cell viability in 3D model

Time dependent spheroids growth

Spheroids integrity can easily be visualized by phase-contrast imaging. The spheroids were photographed from 24 to 96 hours. Diameters were measured on phase-contrast images with Photoshop data software. Mean diameters were calculated for each time-point (24, 48, 72 and 96 h).

Cell viability measure

Quantification of cytosolic acid phosphatase activity [22] was used for the 3D model. Intracellular acid phosphatase hydrolyzes p-nitrophenyl phosphate to p-nitrophenol, in viable cells. Its absorption at 405 nm is directly proportional to the cell number in the range of 10^3 to 10^5 monolayer cells. Spheroids were washed twice with PBS. Centrifugation was repeated, and the supernatant was discarded to a final volume of 100 µL. Then, 100 µL of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, supplemented with ImmunoPure p-nitrophenyl phosphate) was added per well and incubated for 90 min at 37 °C. Following incubation, 10 µL of 1 N NaOH was added to each well, and absorption at 405 nm was measured within 10 min on a micro plate reader. For each experiment, a standard curve was used to determine the linear correlation between APH activity and the number of cells within spheroids. Cell viability was expressed as a proportion of the 10^4 cells initially seeded forward.

Oxidative stress

An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. The OxiSelect™ Total Glutathione Assay Kit (Cell Biolabs, Inc) measures the total glutathione content within a sample in comparison with a predetermined glutathione standard curve. The rate of chromophore production is proportional to the concentration of glutathione. After 4 days' exposure to the nanoparticles, spheroids were washed twice with PBS. Centrifugation was repeated and the assay was performed on 100 µL of supernatants according to manufacturer' protocol. In a 96-well plate, 25 µL of Glutathione Reductase solution and 25 µL of NADPH solution were added to each well. The samples were mixed thoroughly and 50 µL of the chromogen was added per well and mixed briefly. A kinetic reading of the absorbance was recorded at 405 nm. The glutathione level was expressed as the percentage of control.

Statistical analysis

For all the tests, data are expressed as the mean ± SD of three independent experiments. The one-way ANOVA followed by Fisher's PLSD was used to analyze differences in treatment means and $p < 0.05$ was considered statistically significant. All statistical analyzes were performed using commercially available statistical software StatView® v5.0.

RESULTS

Particles characterization

The hydrodynamic diameter of all the types of silica NPs was found to be centred around 60 nm from the dynamic light scattering data in aqueous dispersion at pH = 7.4. The result obtained for CMB@SiO_2 is represented in figure 1 as example. This result is in the same range as the size observed by SEM (not shown) and HRTEM for the three types of sample.

The TEM images of the SiO_2, CMB@SiO_2 and Au-CMB@SiO_2 are as shown (Fig. 2). The dark point in the center of Au-CMB@SiO_2 is the gold nanoparticle. It should be mentioned that due to resolution limit of the TEM available for this work, it is not possible to see the nanosized metal cluster inside the SiO_2 NPs. For this particular point, the reader should see these references [11-13,23]. The histograms obtained from the TEM image using an ImageJ show that the size of the nanoparticles is in the range of 30-50 nm (Fig. 3). The specific surface areas of the 3 types of silica Nps were estimated to be around 335 m^2/g (±10 m^2/g). [23].

Time course and dose-dependent cytotoxicity in 2D cell culture model

After A549 cells were exposed to CMB@SiO_2, Au-CMB@SiO_2 and pure SiO_2 NPs at 1, 10 and 100 µg/mL for 24h and 96h, cell viability decreased as a function of dosage levels in the conventional cell culture model. Interestingly, the cytotoxicity of Au-CMB@SiO_2 NPs, which caused decrease of cell viability with percentages of 0%, 10% and 35% for each concentration respectively, was the lowest compared to CMB@SiO_2 and pure SiO_2 NPs. On the other hand, there was no time course-dependent cytotoxicity (Fig. 4a).

For the L929 cells, the dose-dependent cell viabilities at 24h were more than 100%, 95% and 88% after exposure to each concentration of Au-CMB@SiO_2 NPs. Similar results were observed at 96h (Fig. 4b). There is not increased cytotoxicity with dose increased for SiO_2 and CMB@SiO_2.

Inversely, the KB cells viability decreased significantly in a dose- and time-dependent manner for each nanomaterial tested. However, once more, the Au-CMB@SiO_2 NPs induced the lower cytotoxicity compared to other nanoparticles with 100%, 96% and 68% of cell viability at 96h (Fig. 4c).

Cytotoxicity in 3D model

Time dependent spheroid growth

Increasing the diameter of the spheroids (fig.5) confirmed cell proliferation in the absence of nanoparticles. The number of cell plating allowed sufficient large spheroids at the start and to avoid excessive dimensions after four days of culture and the absence of a necrotic center.

Both CMB@SiO_2 and Au-CMB@SiO_2 NPs did not exhibit any inhibition of the proliferation with A549 cells even at the higher dosage level. These results were confirmed by the observation of phase-contrast images; the A549 spheroids showed the same integrity and the same size compared to the control. Similar results were found for the 3D L929 cells. No alteration in the growth curves was observed whatever the nanomaterial and whatever the dosage level. The opposite effect was observed for the KB cells. Both CMB@SiO_2, Au-CMB@SiO_2 and pure SiO_2 NPs were cytotoxic as reflected by a dose-dependent inhibition of the growth of spheroids even at the lowest concentration (Fig. 6).

Nanoparticles effect on spheroid viability

Addition of Au-CMB@SiO_2 and CMB@SiO_2 NPs to the cell culture medium did not affect the A549 cells viability after exposure for 96h, independently of the dosage level. However, pure SiO_2NPs caused a decrease of cell number of 78% related to the control (untreated cells) at the highest concentration (100 µg/mL).

For the L929 three-dimensional cultured cells, a non-significant proliferative effect was even observed with Au-CMB@SiO_2 and CMB@SiO_2 NPs whatever the dosage level. Only the pure SiO_2 NPs induced a decrease of cell viability (70%) at the 100 µg/mL dose. However, we note a moderate proliferation (+10/20%) but significant in the presence of the Mo_6 clusters.

The cytotoxic effect of all nanomaterials tested on the KB cells was confirmed with a significant decrease of the cell viability. However, the Au-CMB@SiO_2 NPs induced the

lowest decrease of the cell viability of 12%, 21% and 80% at 1 µg/mL, 10 µg/mL and 100 µg/mL respectively, related to untreated cells as controls (Fig. 7)

Total glutathione levels

To assess the involvement of oxidative stress in particle-mediated cytotoxicity, the amount of total intracellular glutathione was measured (both oxidised (GSSG) and reduced GSH, with the GSSG converted to GSH) after 96 h treatment with the different nanoparticles.

In 549 and L929 cells, cellular glutathione level is almost identical to that of the control except for the SiO_2 NPs at 100 µg/mL where GSH quantity is both significantly greater.(fig 8 a and b). For L929 in contact with CMB@SiO_2 NPs, 10 µg/mL and 100 µg/mL, the GSH level is lower compared to the control but it is not statistically significant.

In KB cell, cellular glutathione level exhibited, with all of the nanoparticles, an important and dose-dependent increase. For example, the level was twice more elevated after exposure to both CMB@SiO_2 and SiO_2 NPs compared to the control (Fig. 8 c).

Discussion

With the rapid developments in the fields of nanoscience and nanotechnology, more and more nanomaterials and their based consumer products have been used into our daily life. The safety concerns of nanomaterials have been well recognized by the scientific community and the public. Developing in vitro models for studying cell biology and cell physiology is of great importance to the fields toxicity testing, as well as the emerging fields of tissue engineering and regenerative medicine. Traditional two-dimensional (2D) methods of mammalian cell culture have several limitations and it is increasingly recognized that cells grown in a three-dimensional (3D) environment more closely represent normal cellular function due to the increased cell-to-cell interactions, and by mimicking the in vivo

architecture of natural organs and tissues. Three-dimensional (3D) tissue constructs consisting of human cells have opened a new avenue for tissue engineering, pharmaceutical applications, and have great potential to estimate toxicity expression of nano-materials. To date, few studies demonstrated the importance of 3D screening for nanoparticle as important toxicological data could be missed with only monolayer screening [24-26].

Recently, a number of studies have focused on the interaction between SiO_2 NPs and biological systems to explore their biocompatibility at the systemic and cellular level. *In vitro* experiments have revealed cytotoxic effects of nanoparticles for human cell lines. SiO_2 NPs induced gene expression related to surface area. Moreover, SiO_2 NPs caused a proinflammatory reaction. On the other hand, an *in vivo* study demonstrated that SiO_2 NPs were not toxic and, therefore, could be used *in vivo* or for other biomedical applications. As previously well documented, nanoparticles could be transported into cells through endocytosis in addition, the interaction between nanoparticles and cell membranes could proceed by nonspecific cellular uptake [27]. As the potential applications of silica have encompassed areas such as bioanalysis and imaging or diagnoses, the particles may be directly injected into the human body. Once systematically available, the nanomaterials appear capable of distributing to most organ systems and even may cross biological barriers [28,29].

In this study, the cytotoxicity of two kinds of functional @SiO_2 NPs was investigated in three cell lines in comparison to pure SiO_2 NPs with 2 and 3D cell culture. Generally, the biological activity increases as the particle size decreases [30] and/or specific surface increase [31], moreover a study demonstrated that particle composition probably played a primary role in the cytotoxic effects of different nanoparticles [32]. For these three cell lines, when investigation was conducted in a conventional 2D culture model, the same tendency was observed with, therefore, a more important cytotoxicity in a dose-dependent manner even if there are

variations in the survival rate between each line. These results were in agreement with those of a previous study which found that 15 nm SiO_2 NPs produced a cell viability decreased as a function of both concentration and time for A549 cells exposed to 10, 50 and 100µg/mL dosage levels for 24h, 48h and 72h [21]. In an *in vivo* study, other authors also found that 10 nm SiO_2 NPs induced a pulmonary inflammatory response after instillation of particles into the lung of rats at doses of 1 or 5 mg/Kg [33]. On the other hand, Chang et al. [34] demonstrated that the decrease of cell viability was negligible (mostly > 85% of control at 667µg/mL exposure 48h) for epithelial and fibroblast pulmonary cells treated with silica-chitosan composite nanoparticles.

Interestingly, both @SiO_2 NPs containing CMB induced no toxicity in A549 and L929 cell spheroids whatever the dosage level. These results support those concerning drug sensitivity. Comparison of gene and protein expression reveals that metabolic, cell stress-response, structural, signal transduction, and cellular transport proteins are expressed at elevated levels in spheroids compared to 2D-cultured cells. Moreover, cell adhesion and junction proteins that influence cell aggregation and compaction can be upregulated in spheroids compared to cells in monolayer [35]. Differences in drug distribution and penetration, generation of hypoxia and ROS, enhanced expression of multidrug resistant genes, activation of survival pathways and increased cell–cell and cell–matrix adhesions, may explain the differences in drug activity between 3D-cultured and 2D-cultured cells [36]. Several studies have shown that increased cell–cell and cell–matrix adhesions may activate downstream signaling pathways leading to changes in gene expression, influencing cell sensitivity to drugs. These explanations can be applied to nanotoxicology.

Only the exposure to pure SiO_2 NPs at the highest concentration resulted in significant reduced cell viability. Precisely for these concentrations, the strongest level of glutathione is

observed, confirming that the ROS induction is the cause of the significant decrease in cell viability.

In the presence of silica, CMB@SiO_2 and Au-CMB@SiO_2 , (@Si10 and @Si100), murine L929 fibroblasts in 3D culture presented moderate proliferation relative to spheroid control. For these concentrations, these mouse connective tissue cells had a viability decrease of about 20% in the 2D model culture. This cell proliferation increase concerned only the mouse line and not the human ones.

Silica and silica complexes enhance the synthesis of type I collagen [37] which is part of the extracellular matrix synthesized in the spheroid. Our hypothesis is that this larger amount of collagen is a cell proliferation stimulating factor. A mixture of silicon and calcium, or mixtures with various other materials, including zinc and magnesium, improve both gene expression and biological performance. Many studies have shown that silica-based scaffolds and silica-coated plates induce cell proliferation, attachment, and biocompatibility improves both gene expression and biological performance. Interestingly, SiO_2 NPs have effects on MAPK signaling and cell proliferation. Exposure of cells to SiO_2 NPs medium initially resulted in potent activation of ERK1/2 signaling [38].

Inversely, the two kinds of functional @SiO_2 NPs as much as pure SiO_2 NPs caused cell injury in the epithelial cell line KB spheroid and that even at lower concentration (1µg/mL). Although several findings suggest that spheroid culture reduce their sensitivity to drugs compared to monolayer of cells, it does not necessarily hold true under all circumstances. Some recent study recently demonstrated higher sensitivity of the tumor spheroids to treatment compared to 2D-cultured cells, as the cells of the spheroid displayed increased activation and dependence to some signal transduction [39]. In the same way, Yang et al. [40] demonstrated that exposure of human epidermal keratinocyte cell line HaCaT to SiO_2 NPs

resulted in significantly decreased cell viability in a dose-dependent manner (80% of cell viability at the concentration of 10µg/mL to 5% of cell viability at the concentration of 80µg/mL). Cytotoxicity induced by SiO_2 and @SiO_2 NPs seems to be highly cell-line-dependent due to comparable sizes or specific surface area.

Literature showed that SiO_2 NPs could react with oxygen molecules and produce superoxide and other ROS through a disproportionate reaction. The *in vitro* studies commonly report intracellular oxidative stress as a primary mechanism of cellular degradation to explain the toxicology of SiO_2 NPs [41,42].

Oxidative stress occurs when there is an excess of free radicals or reactive oxygen species in the cell [43]. Cellular antioxidant response involves the action of intracellular molecular antioxidants such as glutathione. An increased oxidized to reduced glutathione ratio is considered indicative of oxidative stress. In contrast, our results showed no modification of intracellular glutathione homeostasis after exposure to the functional @SiO_2 NPs in 2 cell lines. This point could be explained like this; the $Cs_2Mo_6Br_{14}$@SiO_2 NPs reported here are particularly stable. As already demonstrated, the cluster units do not leak from the nanoparticles, even after repeated centrifuging cycles at relatively high relative centrifugal force (up to 40000g) in ethanol or in water [23]. Moreover, it was also demonstrated that a part of the Mo_6 clusters is located at the surface and this changes probably the silica surface comparing with pure SiO_2 NPs. For A549 and L929 cell lines, the only conditions where oxidised glutathione was significantly increased compared to the control was exposure to pure SiO_2 NPs and at the highest concentration. A previous study suggested that SiO_2 NPs can generate $HO^{\bullet}$ radical in the absence of trace metal [21]. So, the metal contaminants cannot account for the oxidative effects exerted by various nanoparticles, more the impact of free metal ions released on oxidative stress could be minimal [44].

Inversely, a significant and important increase of oxidised intracellular glutathione was observed in KB cell lines whatever the nanoparticle type in a dose-dependent manner. These results suggest that the diminution of cell viability is due to increased cellular stress that leads to increased mortality as indicated by APH assay. The accumulation of ROS could deplete the defensive effect of cellular antioxidant enzymes and consequently, redundant ROS would interact with biomolecules, including proteins, enzymes, membrane and DNA that decrease the cell viability.

The results described above seem to be more dependent on cell lines and on the model of cell cultures employed than the surface state of the nanoparticles. It is possible that the differences in cellular susceptibility to damage by nanoparticles are driven by not only the metabolic activity of the chosen cell line as demonstrated by Chang et al. [34] but by the antioxidant ability of the cell line as well. So, our results are corroborated with those of a previous study in which MTT analysis of A549 cells indicated that only a slight and non-significant decrease in cellular proliferation occurred at the higher SiO_2 NPs tested (minimum 91,4% of control; 75 µg/mL). Alternatively, MeT-5A pleural mesothelial cells yielded significant decreases in cellular proliferation at exposure above 75 µg/mL (53.3% lower than control) [45].

The use of inorganic nanoparticles such as gold for diagnostic and therapeutic purposes has gained an increasing interest in recent years [46,47]. Thus, gold nanoparticles have been explored as nanovectors in cell imaging, biosensors, drug delivery, cancer diagnoses and therapeutic applications. A recent study used an original experimental setup in which the gold nanoparticles were administrated under laminar unidirectional flow to human endothelial cells (HUVEC) grown in micro fluidic devices to mimic the physiological situation. The results of cytotoxicity tests showed no toxicity of Au NPs even after a long incubation time (48h). Inversely, exposure of HUVEC to Au NPs in a conventional cell culture model caused a significant decrease of cell viability in a dose- and time-dependent manner. The authors had

shown that the number of nanoparticles aggregates deposited above cells was clearly larger for cells cultured under static conditions [48]. The toxicity of nanoparticles could be explained by the ability of nanoparticles to aggregate [49]. So, the lower toxicity found in this study, when the cells were exposed to nanoparticles in the spheroid cell culture device, could be due to aggregation properties of both nanoparticles under these conditions compared to a more conventional cell culture model.

Conclusion

In conclusion, this *in vitro* study revealed that functional silica NPs containing CMB may become toxic to cultured cells but only at a very high dosage level. Therefore, this toxicity depends on cell lines and more, on the model of cell cultures employed. The selection of appropriate cell line for use in mechanistic-based studies remains a critical component in nanotoxicology. These results are relevant to future applications of Au-CMB@SiO_2 NPs in controlled release applications.

Additionally, the use of multicellular spheroids in an *in vitro* toxicity study could further strengthen nanoparticles hazard identification, thereby contribute to future nanomaterials risk assessments.

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Declaration of interest statement

The authors report no declarations of interest.

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Figure legends

Figure 1: DLS measurement of CMB@SiO_2 NPs in aqueous solution (pH = 7,4).

Figure 2: TEM image of SiO_2 NPs (a), CMB@SiO_2 NPs (b) and CMB-Au@SiO_2 NPs (c).

Figure 3: Size-distribution of SiO_2, CMB@SiO_2 and CMB-Au@SiO_2 NPs (from left to right). Nanoparticles have a mean diameter (from left to right) of: 34, 42 and 39 nm ± 3 nm.

Figure 4: Time-dependent (24 and 96 h) toxicity after exposure to increasing doses (1, 10 and 100 µg/mL) of each nanoparticles tested cell lines 2D models. Values are mean ± SD from three independently reproduced experiments. Significance indicated by: * $p < 0.005$ versus control cells.

Figure 5: Evolution of spheroid diameter

Figure 6: Morphological analysis (x10) of the three cell spheroids at the highest concentration of nanoparticles (100µg/mL): negative control (a), SiO_2 NPs (b), CMB@SiO_2 NPs (c) and CMB-Au@SiO_2 NPs (d).

Figure 7: APH assay results for the 3 cell lines spheroids (3D) at 96 h, after exposure to each nanoparticles. Values are expressed as proportions of the 104 cells initially seeded ($p < 0.005$). Each value represents mean ± SD of three independent experiments. Significance indicated by: * $p < 0.005$ versus control cells.

Figure 8: Total intracellular glutathione (GSH + GSSG) contents were measured in A549 (a), L929 (b) and KB (c) 3D cells culture after 4 days of exposure. Data are presented as mean percentage difference from control ±SD of three independent experiments. Significance indicated by: * $p < 0.05$ versus control (unexposed) cells.