Preliminary Evaluation of Median Lethal Concentrations of Stöber Silica Particles with Various Sizes and Surface Functionalities Towards Fibroblast Cells

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Abstract: The application of silica particles in the biomedical field has experienced a great development in recent years, especially in the design of nanoparticles having homogeneous size, structure and amenable to specific grafting. In this way, it becomes possible to control the inter- action of nanoparticles with cells in order to meet the requirements for desired applications. This work explores the cytotoxicity of silica particles of various sizes and surface functionality towards L929 fibroblast cells. In particular, the median lethal concentration of the different silica particles has been established. Preliminary investigations of silica nanoparticles prepared by the Stöber method with sizes ranging from 100 nm to 500 nm showed that the largest particles are less harmful for the cells. Moreover, cytotoxicity towards L929 fibroblasts was mainly observed for bare particles, whereas sulfonate-, amine- and thiol-grafted particles had less detrimental effects. This shows the key influence of particle surface curvature and chemistry on nanomaterials cytotoxicity.

Keywords Median lethal concentrations; Silica particles; Fibroblast cells; Cytotoxicity

1.Introduction

The potentialities of silica nanoparticles for biological applications have now been well-identified, including molecular and gene delivery [1,2], small animals and cell imaging [3], photodynamic therapy [4], biomaterials surface functionalization [5] and hydrogels reinforcement [6,7]. Currently, strategies for optimizing drug loading, stealthiness, cell targeting as well as controlled release properties are being developed at a fast rate [8-10]. In

parallel, *in vitro* evidences are being accumulated about silica par- ticle interactions with living cells, including cytotoxicity [11], internalization [12] and degradation [13]. All of these have reported significant variations as a function of particle size [14], shape [15] and porosity [16] but also of culture medium [17,18], testing methods [19] and cell types [20]. Indeed the presence of (bio)-organic molecules over or within the particles, necessary to introduce biofunctionality, further increases the complexity of the processes [21,22].

In this perspective, we have examined here the effect of silica nanoparticles on the viability of fibroblast cells. Pure amorphous silica nanoparticles are usually considered as the more benign nanoscale oxides [23] although some recent investigations suggest possible toxicity for 20 nm colloids at relatively low dose (20 $mg.L^{-1}$-0.3 mM) [24]. As a matter of fact, it is somehow difficult to establish a comparison between various studies, especially concerning silica particle cytotoxicity towards mammalian cells [25-28]. Hence, we have tried to evaluate here the median lethal concen-tration LC_{50}, i.e. the concentration necessary to kill half the members of a cell population after a defined time. We have studied the effect of particle size and functionalization, including sulfonate functions whose effect on nanoparticle cytotoxicity was never reported so far, on L929 fibroblast cells as representatives of mammalian tissue cells.

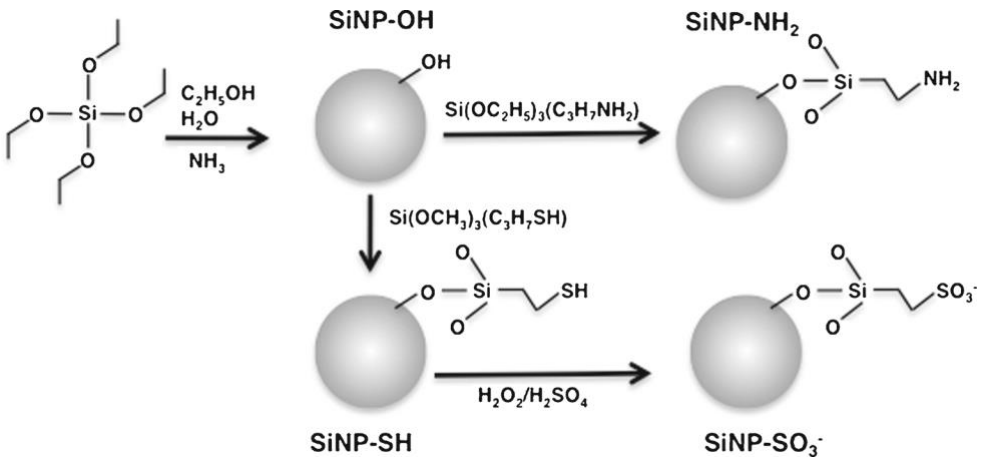
2. Materials and Methods

2.1 Silica Nanoparticles Synthesis and Characterization

All silica nanoparticles (SiNP) were synthesized using the Stöber method [29]. Basically,

tetraethoxysilane (TEOS) and deionized water were added under stirring to 100 mL of ethanol. Ammonia (30 wt% in water) was then slowly added and the mixture was left under stirring during 48 h. The TEOS:H_2O:NH_3 volume ratios (in mL) of (3.5 : 5.4 : 8), (3.5 : 3.5 : 6.7) and (3.7 : 3.4 : 3.1) were used to obtain silica nanoparticles with diameters of 500 nm (SiNP-500- OH), 300 nm (SiNP-300-OH) and 100 nm (SiNP-100-OH), respectively.

Surface grafting of amine (-NH_2) group, thiol function (-SH) and its oxidation into sulfonate (-SO_3-) was performed using aminopropyltriethoxysilane and mer-captopropyltrimethoxysilane, respectively (**Scheme 1**) , as described elsewhere [30-32].



Scheme 1 Preparation of silica nanoparticles (SiNP-OH) and their surface modification by amine (SiNP-NH_2), thiol (SiNP-SH) and sulfonate (SiNP-SO_3-) groups

The mean diameter and hydrodynamic radius of the nanoparticles were determined by Transmission Electron Microscopy (TEM, JEOL 1011 electron microscope operating at 100 kV) and Dynamic Light Scattering (DLS, BI ZetaPlus Brookhaven instrument). The zeta potential of nanoparticles was measured in deionized water using the same equipment.

2.2 Cytotoxicity Assays on Fibroblast Cells

L-929 mouse fibroblast cells, derived from normal sub- cutaneous areolar and adipose tissue, were seeded in 24- wells culture plates at a density of 2.10^4 cells per well in 1 mL of Minimum Essential Medium Non-essential Amino Acid (MEM-NEAA) supplemented with 10 % fetal bovine and 1 % (v/v) Penicillin/Streptomycin mixture under a CO_2 (5 %) atmosphere and at 37 °C for different times. After overnight incubation, the medium was replaced with MEM-NEAA without fetal bovine serum and incubated at 37 °C in order to induce cell cycle arrest. After 4 h, cells were treated with 1, 5, 10, 25 and 50 mM silica particle suspensions and incubated at 37 °C for 24 h and 96 h.

As an indicator of cell viability, mitochondrial redox activity was assessed *via* reduction of the MTT reagent. This colorimetric assay is based on the ability of the mito- chondrial dehydrogenase enzymes of living cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bro- mide (MTT) into an insoluble formazan. At the above men- tioned culture times, 900 μl of MEM-NEAA and 100 μl of MTT solution (5 $mg.mL^{-1}$ in PBS) were added to each sample and incubated for 4 h at 37 °C, under a CO_2 (5 %) atmosphere. Medium was withdrawn through aspiration and cultured fibroblast cells were treated with 1 mL of ethanol 99.5 % for 15 minutes. The absorbance at 570 nm was measured using a UV-Visible spectrophotometer. Readings were converted to cell number using a calibration standard curve. In all cases results are expressed as mean SD from triplicates experiments.

2.3 Statistical Analysis

The data are means SE. The differences were analyzed using two-way ANOVA, followed by

Bonferroni multiple comparisons test. $p < 0.05$ was considered significant.

3 Results and Discussion

Silica particles of various sizes and surface chemistry were obtained using the Stöber process followed by surface grafting via silanization. Mean particle diameter were 540 ± 30 nm (SiNP-500), 290 ± 20 nm (SiNP-300) and 95 ± 15 nm (SiNP-100) as determined by TEM (**Fig. 1**). DLS studies performed in deionized water indicated good dispersion of all particles independently of their size and surface functionalization, except for SiNP-100-NH_2 for which large aggregates were detected, indicating poor colloidal stability. In parallel, the ζ value in water was - 20 ± 5 nm for all particles except for amine-bearing particles whose ζ value was 15 ± 5 nm independently of their size.

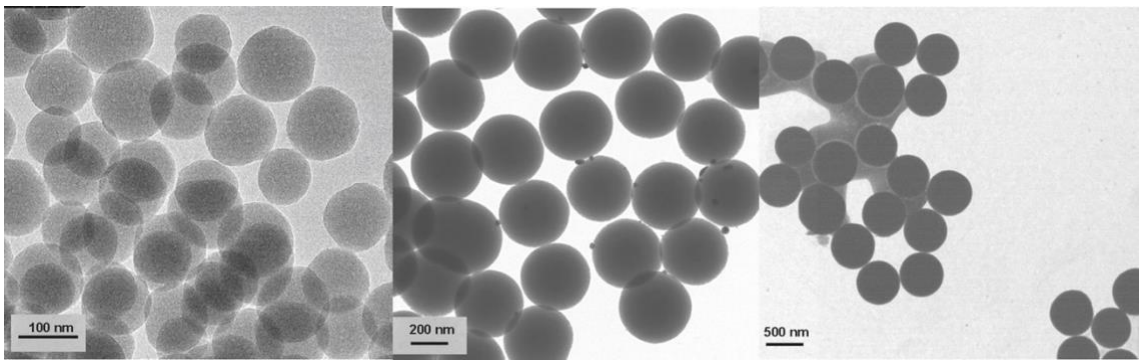


Fig. 1 TEM images of silica nanoparticles with diameters of 100 nm (SiNP-100-OH), 300 nm (SiNP-300-OH) and 500 nm (SiNP-500-OH)

We investigated the effects of size, surface chemistry, concentration and exposure time. First, it was observed that the toxicological effects of the SiNPs strongly depend on the

exposure time. After 24h, in most conditions, the cells exposed to bare silica particles grow at a similar or even higher (see SiNP-500-OH) level than the control cells that were not exposed to particles **(Fig. 2**). In contrast, after this delay, the effect of surface functionalization was apparent, especially for the amine- and sulfonate-modified 300 nm silica particles at all concentrations. However, none of these situations led to the death of half the cell population so that the experiments were continue up to 96 h.

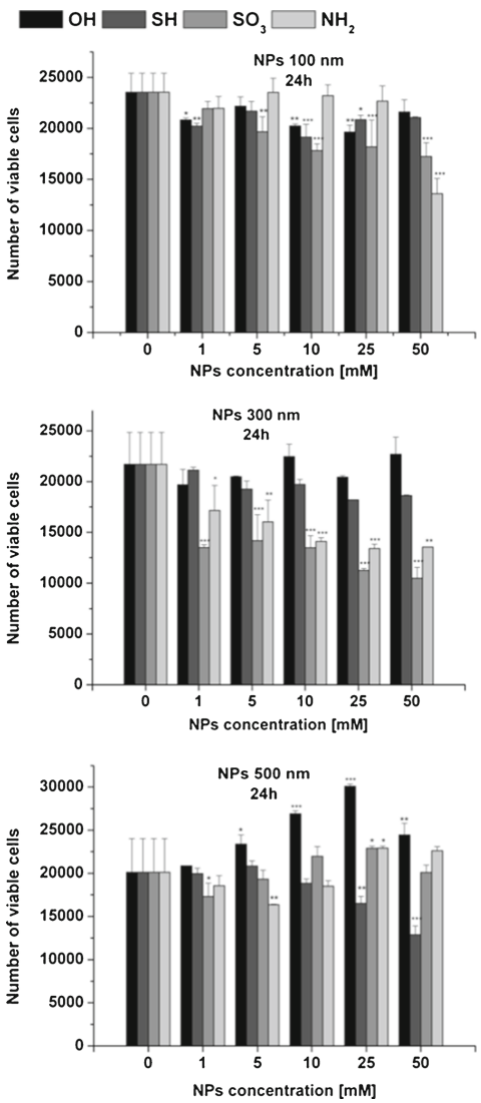


Fig. 2 Effect of nanoparticle concentration, size, functionalization after a 24h exposure time on L929 cells. Two-way ANOVA, double interaction. * Significantly different from: $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$

After this time, more pronounced cytotoxic effects allowed the estimation of the LC_{50} values for some of the SiNPs (**Fig. 3**).

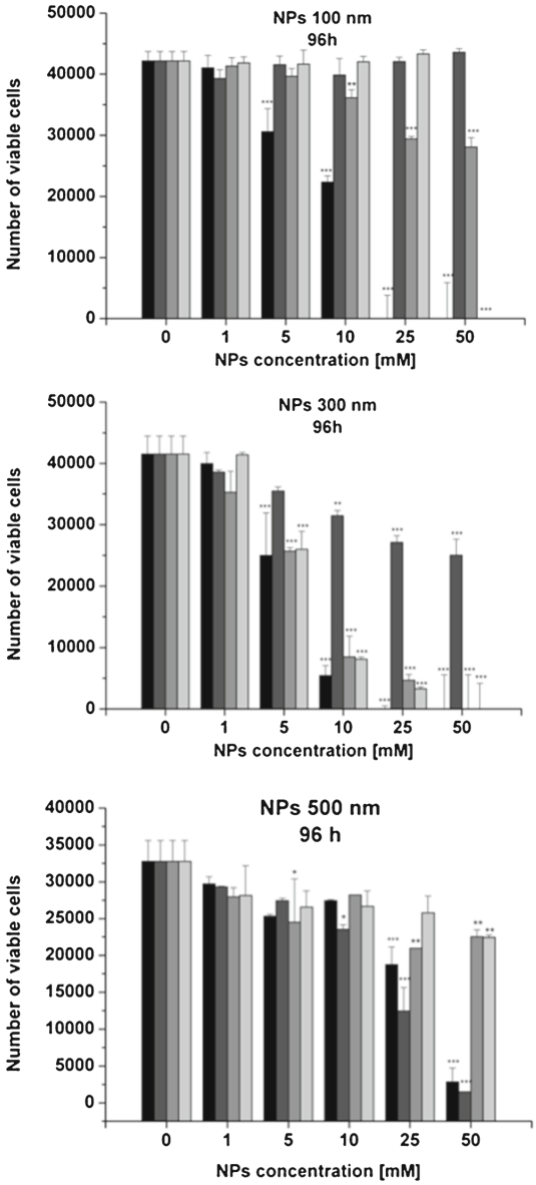


Fig. 3 Effect of nanoparticle concentration, size, functionalization after a 96h exposure time on L929 cells. Two-way ANOVA, double interaction. * Significantly different from: $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$

Considering bare particles, it is possible to determine that the size has also a strong effect on the viability. LC_{50} value ranges between 10 mM and 25 mM for SiNP-100-OH particles, between 5 and 10 mM for SiNP-300-OH and between 25 and 50 mM for SiNP-500-OH (**Table 1**). Interestingly, the evolution of toxicity with particle diameter is different depending on the surface chemistry. Hence 100 nm thiol-modified particles have lim- ited impact on cells but their cytotoxicity increases with increasing diameter, whereas SO_{3-} and NH2 -modified par- ticles are less toxic for a 500 nm diameter. Overall, in the case of 100 nm particles all surface modifications increased the LC_{50}, as compared to unmodified SiNPs. For amine- modified particles (SiNP-100-NH_2*)* the LC_{50} is between 25 and 50 mM, while for thiol-modified particles (SiNP-100-SH) and sulfonate-modified particles (SiNP-100-SO_3) the LC_{50} is higher than 50 mM. These differences between bare and functionalized particles are also observed for 500 nm particles although the difference is not significant for thiol-modified particles (SiNP-500-SH), while amine-modified particles (SiNP-100-NH_2) and sulfonate-modified particles (SiNP-500-SO_3) are less toxic. A different situation is observed for 300 nm particles where only thiol-modified particles (SiNP-300-SH) have a higher LC_{50} than SiNP-300-OH.

As a general trend, and after 96 h exposure, bare particles showed the highest toxicity towards L929 compared to surface-modified ones, especially for 100 and 500 nm par- ticles. This observation is in good agreement with a recent report suggesting that isolated silanol groups are mainly responsible for silica particle toxicity [33]. As surface modification through silanization decreases the amount of free silanols, an increase in LC_{50} could be expected.

Table 1 Median lethal concentration LC_{50} for 2.10^4 fibroblast cells exposed to various silica particles after 96 h exposure

	Surface functionality			
Size (nm)	OH	SH	SO_3	NH_2
100	10–25mM	>50mM	>50mM	25–50mM
300	5–10mM	>50mM	5–10mM	5–10mM
500	25–50mM	10–25mM	>50mM	>50mM

The observation of non-monotonous variation of LC_{50} value with bare silica particle diameter is worth being dis- cussed. When analyzing size effect on nanotoxicity, the systems must be considered at two scales. When consider- ing the whole particle population (per g or mole of silica), larger particles have lower specific surface area, i.e. lower total surface contact with cells. Moreover, the density of the free silanol groups is related to the surface curvature of the particle, i.e. their density decreases with increasing diameter [34]. Hence, cytotoxicity is expected to decrease with increasing diameter. However, when considering each particle individually, their surface increases with particle diameter. An important consequence is that the amount of molecules (medium proteins, membrane components) per particle that can be adsorbed increases with its size. Therefore, larger particles may interact more strongly with cells. Thus it can be suggested that the minimum LC_{50} value obtained for 300 nm silica particles results from the balance of the two effects.

Coming to the comparison of the various surface functionalization, it is difficult to

interpret all data in detail. Nevertheless, one may notice that sulfonate- and amine- modified particles behave similarly to bare silica particles in terms of influence of size on LC_{50}. Hence it suggests that these functions do not induce specific interactions with L929 cells. The case of thiol groups is different as LC_{50} decreases with increasing particle size. As the number of thiol groups per particles increase with particle size, it is possible to propose that this function has a detrimental effect on cell viability, although lower than that of silanols. Indeed other parameters such as aggregation state and possible protein adsorption from the culture medium can contribute to the complexity of the observed effects [18].

4 Conclusions

This work has explored the cytotoxicity of silica particles towards L929 fibroblast cells. In particular, it was possible to evaluate the median lethal concentrations for different particle size and surface chemistry. This parameter is important for the development of nanomaterials for biomedical applications but is scarcely found in the literature.

From the analysis of the results, it was found that cel- lular response to SiNPs is exposure time and concentration dependent. Indeed, increasing silica particles concentration will also increase the effect produced and the extent of cytoxicity. In addition, surface modification can drastically change cellular response to nanoparticles. The mammalian cells show high sensitivity to surface modification of the particles but our results suggest that organic groups efficiently screen the detrimental interaction of cell membrane with silanol groups. Based on a compromise in terms of particle size and concentration, it was possible to

obtain nanomaterials with low impact on considered mammalian cells. Indeed, these studies must now be extended to *ex vivo* and *in vivo* environments where the living tissues can have a significant impact on the cytotoxicological effects [35]. In parallel further formulation/processing steps are now necessary to favor particles permeation or integration on/within relevant biomaterials.

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