

Silicon

DOI 10.1007/s12633-014-9203-5

ORIGINAL PAPER

1

2 Preliminary Evaluation of Median Lethal Concentrations 3 of Stöber Silica Particles with Various Sizes and Surface 4 Functionalities Towards Fibroblast Cells

5 Cecilia Galindo Gonzalez · Gisela Solange Álvarez · Daniela Edhit Camporotondi ·
6 Maria Lucia Foglia · Carole Aimé · Luis Eduardo Diaz · Thibaud Coradin ·
7 Martin Federico Desimone

8 Received: 6 November 2013 / Accepted: 1 May 2014
9 © Springer Science+Business Media Dordrecht 2014

10 **Abstract** The application of silica particles in the biomed-
11 ical field experienced a great development in recent years,
12 especially in the design of nanoparticles having homoge-
13 neous size, structure and amenable to specific grafting. In
14 this way, it becomes possible to control the interaction of
15 nanoparticles with cells in order to meet the requirements
16 for desired applications. This work explores the cytotoxicity
17 of silica particles of various sizes and surface function-
18 ality towards L929 fibroblast cells. In particular, the median
19 lethal concentration of the different silica particles has been
20 established. Preliminary investigations of silica nanoparti-
21 cles prepared by the Stöber method with size ranging from
22 100 nm to 500 nm showed that the largest particles are
23 less harmful for the cells. Moreover, cytotoxicity towards
24 L929 fibroblasts was mainly observed for bare particles,
25 whereas sulfonate-, amine- and thiol-grafted particles had
26 less detrimental effects. This shows the key influence of
27 particle surface curvature and chemistry on nanomaterials
28 cytotoxicity.

Q1 29 **Keywords** Median lethal concentrations · Silica
30 particles · Fibroblast cells · Cytotoxicity

G. S. Álvarez · D. E. Camporotondi · M. L. Foglia · L. E. Diaz
IQUIMEFA-CONICET. Facultad de Farmacia y Bioquímica,
Universidad de Buenos Aires, Junín 956 Piso 3°, (1113),
Ciudad Autónoma de Buenos Aires, Argentina

Q2 C. G. Gonzalez · C. Aimé · T. Coradin (✉) · M. F. Desimone (✉)
UPMC Univ Paris 06; CNRS, Laboratoire de Chimie de la Matière
Condensée de Paris, Collège de France, 11 place Marcelin
Berthelot, 75005 Paris, France
e-mail: thibaud.coradin@upmc.fr
e-mail: desimone@ffyb.uba.ar

1 Introduction 31

32 The potentialities of silica nanoparticles for biological
33 applications have now been well-identified, including
34 molecular and gene delivery [1, 2], small animals and cell
35 imaging [3], photodynamic therapy [4], biomaterials surface
36 functionalization [5] and hydrogels reinforcement [6, 7].
37 Currently, strategies for optimizing drug loading, stealthi-
38 ness, cell targeting as well as controlled release properties
39 are being developed at a fast rate [8–10]. In parallel, *in*
40 *vitro* evidences are being accumulated about silica par-
41 ticle interactions with living cells, including cytotoxicity
42 [11], internalization [12] and degradation [13]. All of these
43 have reported significant variations as a function of par-
44 ticle size [14], shape [15] and porosity [16] but also of
45 culture medium [17, 18] testing methods [19] and cell types
46 [20]. Indeed the presence of (bio)-organic molecules over or
47 within the particles, necessary to introduce biofunctionality,
48 further increases the complexity of the processes [21, 22].

49 In this perspective, we have examined here the effect of
50 silica nanoparticles on the viability of fibroblast cells. Pure
51 amorphous silica nanoparticles are usually considered as the
52 more benign nanoscale oxides [23] although some recent
53 investigations suggest possible toxicity for 20 nm colloids
54 at relatively low dose (20 mg.L⁻¹-0.3 mM) [24]. As a mat-
55 ter of fact, it is somehow difficult to establish comparison
56 between various studies, especially concerning silica parti-
57 cle cytotoxicity towards mammalian cells [25–28]. Hence,
58 we have tried to evaluate here the median lethal concen-
59 tration LC₅₀, i.e. the concentration necessary to kill half
60 the members of a cell population after a defined time. We
61 have studied the effect of particle size and functionalization,
62 including sulfonate functions whose effect on nanoparticle
63 cytotoxicity was never reported so far, on L929 fibroblast
64 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:H₂O:NH₃ 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 (-NH₂) group, thiol function (-SH) and its oxidation into sulfonate (-SO₃⁻) was performed using aminopropyltriethoxysilane and mercaptopropyltrimethoxysilane, respectively (Scheme 1), as described elsewhere [30–32].

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 subcutaneous areolar and adipose tissue, were seeded in 24-wells culture plates at a density of 2.10⁴ 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 CO₂ (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 mitochondrial dehydrogenase enzymes of living cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) into an insoluble formazan. At the above mentioned 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 CO₂ (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.

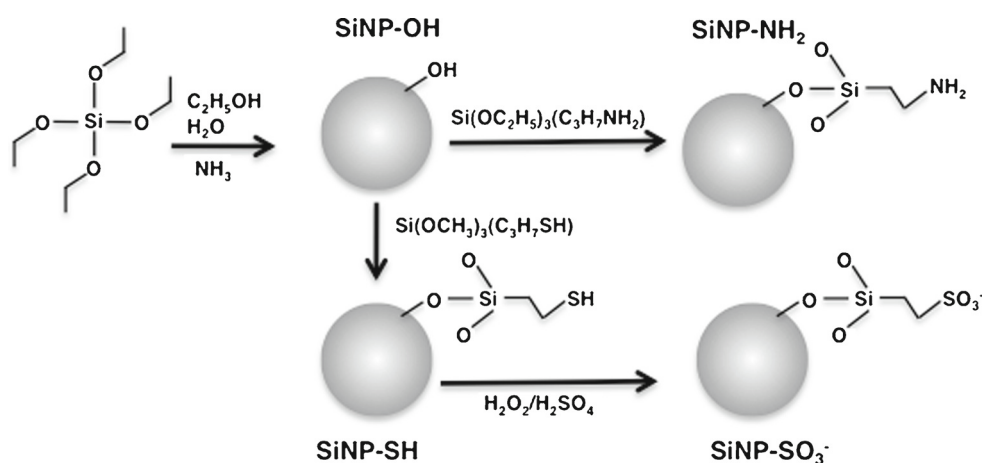
2.3 Statistical Analysis

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

Scheme 1 Preparation of silica nanoparticles (SiNP-OH) and their surface modification by amine (SiNP-NH₂), thiol (SiNP-SH) and sulfonate (SiNP-SO₃⁻) groups



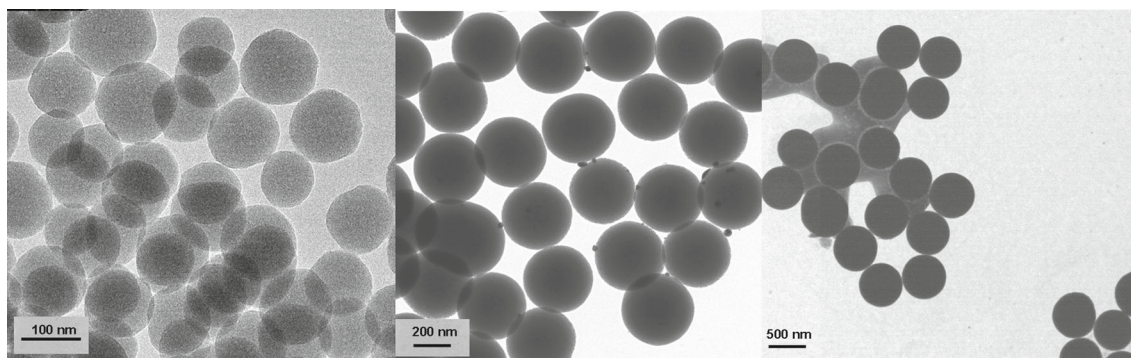


Fig. 1 TEM images of silica nanoparticles with diameters of 100 nm (SiNP-100-OH), 300 nm (SiNP-300-OH) and 500 nm (SiNP-500-OH)

functionalization, except for SiNP-100-NH₂ for which large aggregates were detected, indicating poor colloidal stability. In parallel, ζ value in water was -20 ± 5 nm for all particles except for amine-bearing particles whose ζ value was $+15 \pm 5$ nm independently of their size.

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.

After this time, more pronounced cytotoxic effects allowed the estimation of the LC₅₀ values for some of the SiNPs (Fig. 3). Considering bare particles, it is possible to determine that the size has also a strong effect on the viability. LC₅₀ 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 limited impact on cells but their cytotoxicity increases with increasing diameter, whereas SO₃⁻ and NH₂-modified particles are less toxic for a 500 nm diameter. Overall, in the case of 100 nm particles all surface modifications increased the LC₅₀, as compared to unmodified SiNPs. For amine-modified particles (SiNP-100-NH₂) the LC₅₀ is between 25 and 50 mM, while for thiol-modified particles (SiNP-100-SH) and sulfonate-modified particles (SiNP-100-SO₃) the LC₅₀ 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-NH₂) and sulfonate-modified particles (SiNP-500-SO₃) are less toxic. A different situation is observed for 300 nm particles where only thiol-modified particles (SiNP-300-SH) have a higher LC₅₀ 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 particles. 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 LC₅₀ could be expected.

The observation of non-monotonous variation of LC₅₀ value with bare silica particle diameter is worth being discussed. When analyzing size effect on nanotoxicity, the systems must be considered at two scales. When considering 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 LC₅₀ 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 details. Nevertheless, one may notice that sulfonate- and amine-modified particles behave similarly to bare silica particles in terms of influence of size on LC₅₀. Hence it suggests that these functions do not induce specific interactions with L929 cells. The case of thiol groups is different as LC₅₀

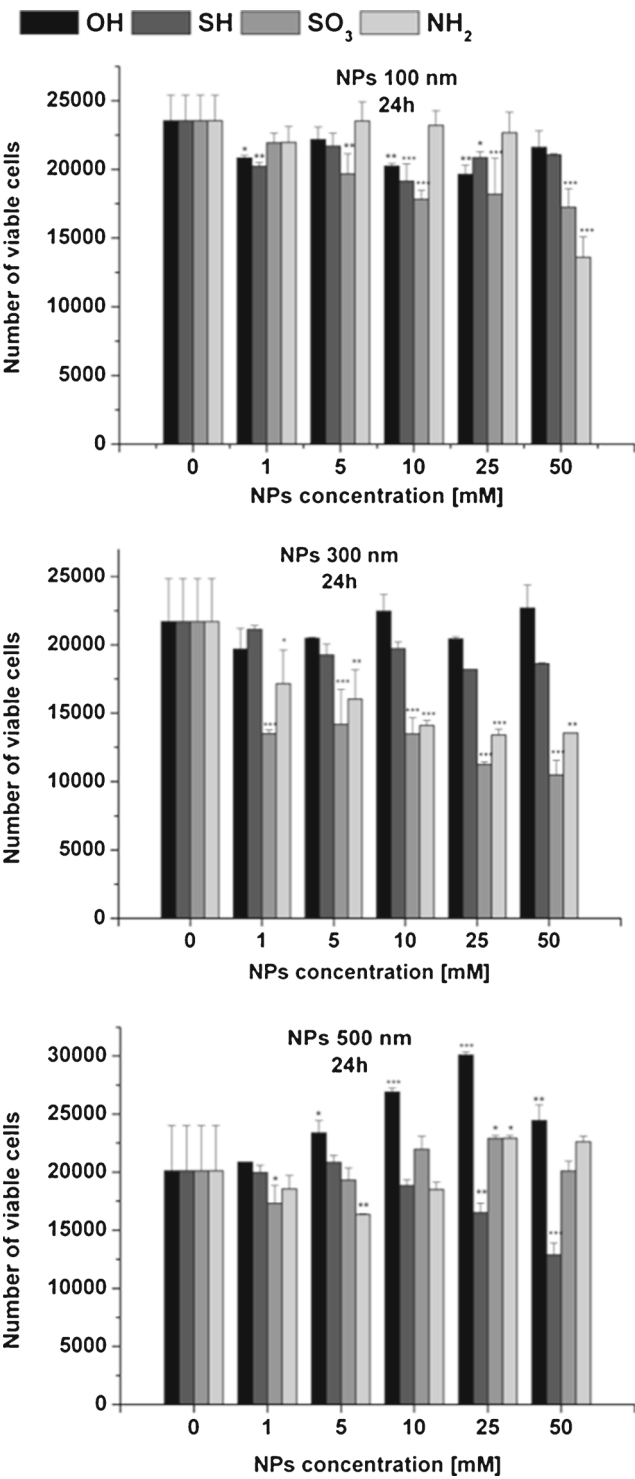


Fig. 2 Effect of nanoparticle concentration, size, functionalization after a 24h exposure time on L929 cells. Two-way ANOVA, double interaction. * Significantly different from: $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$

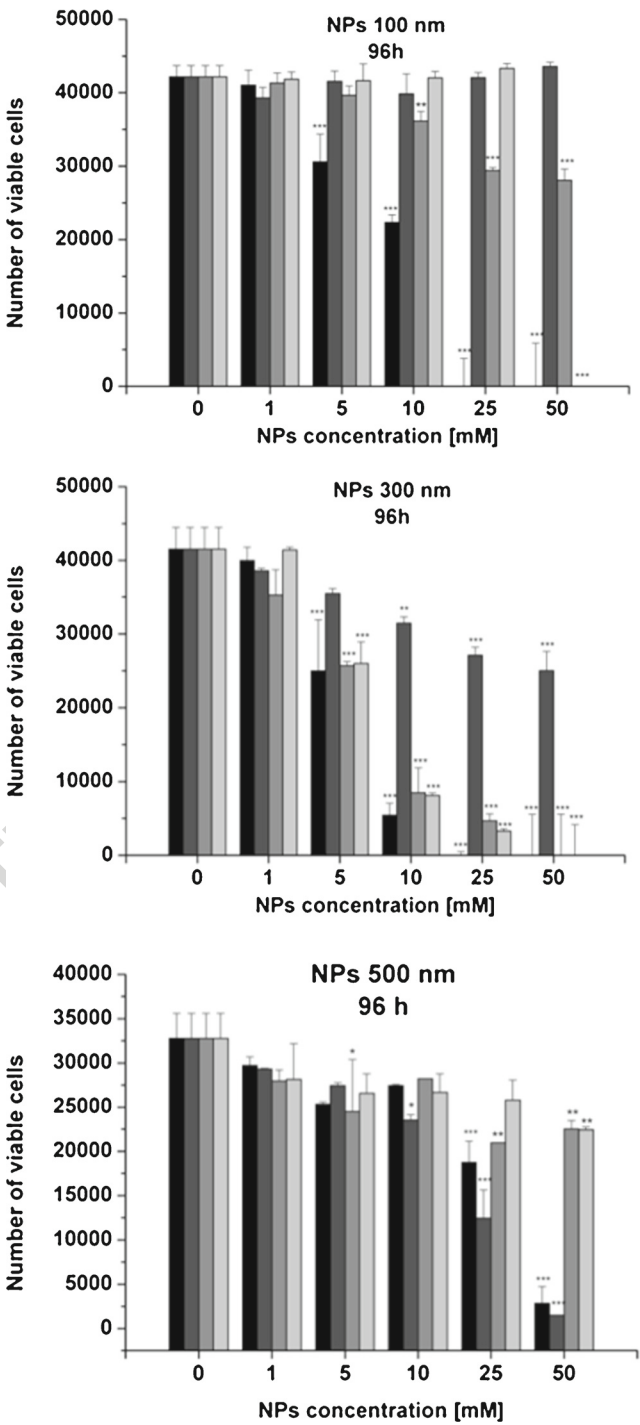


Fig. 3 Effect of nanoparticle concentration, size, functionalization after a 96h exposure time on L929 cells. Two-way ANOVA, double interaction. * Significantly different from: $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$

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].

Table 1 Median lethal concentration LC₅₀ for 2.10⁴ fibroblast cells exposed to various silica particles after 96 h exposure

Size (nm)	Surface functionality			
	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

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 cellular response to SiNPs is exposure time and concentration dependent. Indeed, increasing silica particles concentration will also increase the effect produced and the extent of cytotoxicity. 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.

Acknowledgments María Lucia Foglia is grateful for their doctoral fellowship granted by the National Research Council (CONICET). C. Galindo-Gonzalez thanks the TECSAN program of the French ANR for funding her post-doc. The authors would like to acknowledge the support of grants from the University of Buenos Aires UBA-CYT 20020110100081 and from Agencia Nacional de Investigaciones Científicas y Técnicas PICT 2012-1441 (to M.F.D.). M.F.D. and T.C. thank the Argentina-France MINCYT-ECOS-Sud (project A12S01) and CONICET-CNRS programs for financial support.

References

1. Slowing II, Vivero-Escoto JL, Wu CW, Lin VSY (2008) Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. *Adv Drug Deliv Rev* 60(11):1278–1288
2. Foglia ML, Alvarez GS, Catalano PN, Mebert AM, Diaz LE, Coradin T et al (2011) Recent patents on the synthesis and application of silica nanoparticles for drug delivery. *Recent Patents Biotechnol* 5(1):54–61
3. Wang K, He X, Yang X, Shi H Functionalized silica nanoparticles: a platform for fluorescence imaging at the cell and small animal levels. *Acc Chem Res* (in press)
4. Ohulchanskyy TY, Roy I, Goswami LN, Chen Y, Bergey EJ, Pandey RK et al (2007) Organically modified silica nanoparticles with covalently incorporated photosensitizer for photodynamic therapy of cancer. *Nano Lett* 7(9):2835–2842
5. Hu Y, Cai K, Luo Z, Jandt KD (2010) Layer-by-layer assembly of 1²-estradiol loaded mesoporous silica nanoparticles on titanium substrates and its implication for bone homeostasis. *Adv Mater* 22(37):4146–4150
6. Desimone MF, Helary C, Rietveld IB, Bataille I, Mosser G, Giraud-Guille MM et al (2010) Silica-collagen bionanocomposites as three-dimensional scaffolds for fibroblast immobilization. *Acta Biomater* 6(10):3998–4004
7. Heinemann S, Coradin T, Desimone MF (2013) Bio-inspired silica-collagen materials: applications and perspectives in the medical field. *Biomater Sci* 1:688–702
8. Rosenholm JM, Sahlgren C, Linden M (2010) Towards multifunctional, targeted drug delivery systems using mesoporous silica nanoparticles - opportunities & challenges. *Nanoscale* 2(10):1870–1883
9. Vallet-Regi M, Colilla M, Gonzalez B (2011) Medical applications of organic-inorganic hybrid materials within the field of silica-based bioceramics. *Chem Soc Rev* 40(2):596–607
10. Yang P, Gai S, Lin J (2012) Functionalized mesoporous silica materials for controlled drug delivery. *Chem Soc Rev* 41(9):3679–3698
11. Ahamed M (2013) Silica nanoparticles-induced cytotoxicity, oxidative stress and apoptosis in cultured A431 and A549 cells. *Human Exp Toxicol* 32(2):186–195
12. Shapero K, Fenaroli F, Lynch I, Cottell DC, Salvati A, Dawson KA (2011) Time and space resolved uptake study of silica nanoparticles by human cells. *Mol BioSyst* 7(2):371–378
13. Quignard S, Mosser G, Boissiere M, Coradin T (2012) Long-term fate of silica nanoparticles interacting with human dermal fibroblasts. *Biomaterials* 33(17):4431–4442
14. Rabolli V, Thomassen LCJ, Princen C, Napierska D, Gonzalez L, Kirsch-Volders M et al (2010) Influence of size, surface area and microporosity on the in vitro cytotoxic activity of amorphous silica nanoparticles in different cell types. *Nanotoxicology* 4(3):307–318
15. Huang X, Teng X, Chen D, Tang F, He J (2010) The effect of the shape of mesoporous silica nanoparticles on cellular uptake and cell function. *Biomaterials* 31(3):438–448
16. Lin YS, Haynes CL (2010) Impacts of mesoporous silica nanoparticle size, pore ordering, and pore integrity on hemolytic activity. *J Amer Chem Soc* 132(13):4834–4842
17. Lin YS, Abadeer N, Haynes CL (2011) Stability of small mesoporous silica nanoparticles in biological media. *Chem Commun* 47(1):532–534
18. Drescher D, Orts-Gil G, Laube G, Natte K, Veh RW, Osterle W et al (2011) Toxicity of amorphous silica nanoparticles on eukaryotic cell model is determined by particle agglomeration and serum protein adsorption effects. *Anal Bioanal Chem* 400(5):1367–1373
19. Fede C, Selvestrel F, Compagnin C, Mognato M, Mancin F, Reddi E et al (2012) The toxicity outcome of silica nanoparticles (Ludox®) is influenced by testing techniques and treatment modalities. *Anal Bioanal Chem* 404(6–7):1789–1802

20. Chang JS, Chang KLB, Hwang DF, Kong ZL (2007) In vitro cytotoxicity of silica nanoparticles at high concentrations strongly depends on the metabolic activity type of the cell line. *Environ Sci Technol* 41(6):2064–2068
21. Yu T, Malugin A, Ghandehari H (2011) Impact of silica nanoparticle design on cellular toxicity and hemolytic activity. *ACS Nano* 5(7):5717–5728
22. Lin IC, Liang M, Liu TY, Jia Z, Monteiro MJ, Toth I (2012) Effect of polymer grafting density on silica nanoparticle toxicity. *Bioorg Med Chem* 20(23):6862–6869
23. Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, Limbach LK et al (2006) In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. *Environ Sci Technol* 40(14):4374–4381
24. Jiang W, Mashayekhi H, Xing B (2009) Bacterial toxicity comparison between nano- and micro-scaled oxide particles. *Environ Pollut* 157(5):1619–1625
25. Eom HJ, Choi J (2009) Oxidative stress of silica nanoparticles in human bronchial epithelial cell, Beas-2B. *Toxicol Vitro* 23(7):1326–1332
26. Shi Y, Yadav S, Wang F, Wang H (2010) Endotoxin promotes adverse effects of amorphous silica nanoparticles on lung epithelial cells in vitro. *J Toxicol Environ Health - Part A: Curr Issues* 73(11):748–756
27. Stayton I, Winiarz J, Shannon K, Ma Y (2009) Study of uptake and loss of silica nanoparticles in living human lung epithelial cells at single cell level. *Anal Bioanal Chem* 394(6):1595–1608
28. Akhtar MJ, Ahamed M, Kumar S, Siddiqui H, Patil G, Ashquin M et al (2010) Nanotoxicity of pure silica mediated through oxidant generation rather than glutathione depletion in human lung epithelial cells. *Toxicology* 276(2):95–102
29. Stöber W, Fink A, Bohn E (1968) Controlled growth of monodisperse silica spheres in the micron size range. *J Colloid Interface Sci* 26(1):62–69
30. Badley RD, Ford WT, McEnroe FJ, Assink RA (1990) Surface modification of colloidal silica. *Langmuir* 6(4):792–801
31. Wu J, Silvent J, Coradin T, Aimé C (2012) Biochemical investigation of the formation of three-dimensional networks from DNA-grafted large silica particles. *Langmuir* 28(4):2156–2165
32. Aime C, Mosser G, Pembouong G, Bouteiller L, Coradin T (2012) Controlling the nano-bio interface to build collagen-silica self-assembled networks. *Nanoscale* 4(22):7127–7134
33. Zhang H, Dunphy DR, Jiang X, Meng H, Sun B, Tarn D et al (2012) Processing pathway dependence of amorphous silica nanoparticle toxicity: colloidal vs pyrolytic. *J Amer Chem Soc* 134:15790–15804
34. Kamiya H, Mitsui M, Takano H, Miyazawa S (2000) Influence of particle diameter on surface silanol structure, hydration forces, and aggregation behavior of alkoxide-derived silica particles. *J Amer Ceram Soc* 83(2):287–293
35. Park YH, Kim JN, Jeong SH, Choi JE, Lee SH, Choi BH, et al. (2010) Assessment of dermal toxicity of nanosilica using cultured keratinocytes, a human skin equivalent model and an in vivo model. *Toxicology* 267(1–3):178–181