



# Genotoxic effects of synthetic amorphous silica nanoparticles in the mouse lymphoma assay



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## ABSTRACT

Synthetic amorphous silica nanoparticles (SAS NPs) have been used in various industries, such as plastics, glass, paints, electronics, synthetic rubber, in pharmaceutical drug tablets, and as a food additive in many processed foods. There are few studies in the literature on NPs using gene mutation approaches in mammalian cells, which represents an important gap for genotoxic risk estimations. To fill this gap, the mouse lymphoma L5178Y/*Tk*<sup>+/-</sup> assay (MLA) was used to evaluate the mutagenic effect for five different concentrations (from 0.01 to 150  $\mu\text{g/mL}$ ) of two different sizes of SAS NPs (7.172 and 7.652 nm) and a fine colloidal form of silicon dioxide ( $\text{SiO}_2$ ). This assay detects a broad spectrum of mutational events, from point mutations to chromosome alterations. The results obtained indicate that the two selected SAS NPs are mutagenic in the MLA assay, showing a concentration-dependent effect. The relative mutagenic potencies according to the induced mutant frequency (IMF) are as follows: SAS NPs (7.172 nm) ( $\text{IMF} = 705.5 \times 10^{-6}$ ), SAS NPs (7.652 nm) ( $\text{IMF} = 575.5 \times 10^{-6}$ ), and  $\text{SiO}_2$  ( $\text{IMF} = 57.5 \times 10^{-6}$ ). These *in vitro* results, obtained from mouse lymphoma cells, support the genotoxic potential of NPs as well as focus the discussion of the benefits/risks associated with their use in different areas.

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## 1. Introduction

Nanomaterials (NMs) are described as particles, which have a maximum size of 100 nm or less and exhibit novel physicochemical properties including high tensile strength, thermal and chemical stability, high hydrophobicity, heat and electrical insulation, hydrogen storage capacity, resistance to oxidation, etc. NMs are at the leading edge of the rapidly developing field of nanotechnology. The use of nanotechnology has surged in recent years, and the increasing applications of NMs in diverse fields, such as energy, electronics, food and agriculture, biomedical devices, imaging, bio-sensing and chips, environmental clean-up, household products, paints, consumer products, sports biotechnology, life sciences, medicine, defence, and engineering [1] have raised awareness of the potential toxicity of NMs. These particles, by virtue of their size could enter the body through various routes such as oral, inhalation, absorption, or injection in medical procedures. There is concern about the potential side effects of these substances on human health and various studies have been performed revealing these biolog-

ical effects. Thus, studies in nano-genotoxicology to evaluate the harmful effects of NMs are increasing [2–6].

Synthetic amorphous silica nanoparticles (SAS NPs) are used as a food additive in many processed foods, in pharmaceutical drug tablets, glass, electronics, and in hydrophobic anticancer drugs [7]. Silica ( $\text{SiO}_2$ ) NPs induce inflammatory and oxidative stress responses both *in vivo* and *in vitro* [8,9], but cytotoxicity has only been observed at high concentrations [10]. Wang et al. [11] demonstrated that ultrafine  $\text{SiO}_2$  NPs are cytotoxic and genotoxic in cultured human lymphoblastoid cells.  $\text{SiO}_2$  NPs also have been reported to impact nuclear integrity by forming intranuclear protein aggregates that can lead to inhibition of replication, transcription and cell proliferation [12].

Although several studies dealing with the genotoxic properties of SAS NPs have been carried out, only one study on the mutagenic potential of nano-silica powder (10 nm) in L5178Y cells has been reported [13]. For this reason, we used the mouse lymphoma assay (MLA) as an *in vitro* mutagenicity test system [14–20].

The MLA has been applied to the mouse lymphoblastic cell line for *in vitro* mutagenicity testing of different mutational changes, including mitotic recombination, point mutations, chromosome aberrations, and aneuploidy [21,22], including NMs [13,19]. These features make the MLA particularly useful to evaluate the ability of chemicals to induce a wide variety of mutational events [16,22].

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In this study, the MLA was used to assess the genotoxicity of two different sizes of SAS NPs in a mouse lymphoma cell line. The MLA provides both rapid and reliable data on the genotoxicity of chemicals. The thymidine kinase (*Tk*) locus has been widely used to detect the ability of chemicals to induce genetic damage in cultured mammalian cells. Taking into account that particle structure and size can be an important modulating agent, our study included two different size ranges for the SAS NPs (7.172 and 7.652 nm). In addition, we also included a fine colloidal SiO<sub>2</sub> to determine the importance of particle size in the observed effects.

## 2. Materials and methods

### 2.1. Chemicals

SiO<sub>2</sub> NPs aqueous dispersion, amorphous, 25 wt%, 5–35 nm (99.99% purity; CAS No. 13463-67-7, US7300) and SiO<sub>2</sub> NPs aqueous dispersion, amorphous, 25 wt%, 30 nm (99.99% purity; CAS No. 13463-67-7, US7040) were from US Research Nanomaterials, Inc. Both SiO<sub>2</sub> NPs were in amorphous form. Fine size colloidal, 40 wt% suspension in H<sub>2</sub>O silicon dioxide (CAS No. 7631-86-9) was from Sigma Chemicals Co. (St. Louis, MO, USA). Methyl methanesulfonate (MMS, 99% purity; CAS No. 66-27-3), ethanol (≥99.5% purity; CAS No. 64-17-5), bovine serum albumin (BSA, ≥98% purity; CAS No. 9048-46-8), thiazolyl blue tetrazolium bromide (MTT, 99.7% purity; CAS No. 298-93-1), trifluorothymidine (≥99% purity; CAS No. 70-00-8), thymidine (CAS No. 4449-43-8), hypoxanthine (99% purity; CAS No. 68-94-0), methotrexate (CAS No. 59-05-2) and glycine (≥99% purity; CAS No. 56-40-6) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). RPMI 1640 medium, horse serum, L-glutamine solution (CAS No. 56-85-9), penicillin/streptomycin solution, sodium pyruvate solution (CAS No. 113-24-6) and amphotericin B solution (CAS No. 1397-89-3) were purchased from PAA Laboratories (Pasching, Austria).

### 2.2. Nanoparticles characterization and dispersion procedure

To characterize the selected NPs different analyses using transmission electron microscopy (TEM), dynamic light scattering (DLS), and laser Doppler velocimetry (LDV) methodologies were carried out. TEM methodologies were carried on a Tecnai G2 F30 instrument to determine size and morphology. DLS and LDV were performed on a Malvern Zetasizer Nano-ZS zen3600 instrument for the characterization of hydrodynamic size and zeta potential. For dispersion, NPs were pre-wetted in 0.5% absolute ethanol and afterwards dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water. The NPs in the dispersion medium were sonicated for 16 min to obtain a stock dispersion of 2.56 mg/mL according to the Nanogenotox protocol [23].

### 2.3. Mouse lymphoma assay (MLA)

The L5178Y/*Tk*<sup>+/-</sup>-3.7.2C mouse lymphoma cell line was used for the mutation assay. Cells were cultured in suspension in RPMI 1640 medium supplemented with 10% vol/vol DHS, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), sodium pyruvate (1 mM) and amphotericin B (2.5 μg/mL). Cultures were routinely diluted to 2 × 10<sup>5</sup> cells/mL daily to prevent overgrowth (> 10<sup>6</sup> cells/mL).

Detailed procedures and current guidance for conduct of the MLA are given elsewhere [15,24,25]. In brief, the assay was performed using liquid medium and limiting-dilution cloning in 96-well plates without S9exogenous metabolic activation according to the method previously described by us [17,18,20]. Cells were treated with different concentrations (ranging from 0.01 to 150 μg/mL) of the two different sizes of SAS NPs and the fine

sized form in RPMI 1640 medium with 10% heat-inactivated horse serum for 4 h at 37 °C. MMS was dissolved in distilled water, and 10 μg/mL was used as positive control. Cells were counted by using an automatic cell counter, and densities were adjusted using fresh medium at approximately 2 d after exposure. For mutant enumeration, trifluorothymidine (TFT) was added to the cell culture at a final concentration of 4 μg/mL, and cells were seeded onto 96-well flat-bottom microtiter plates. All plates were incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub> in air. After 12 d of incubation, colonies were counted and categorized as small or large [17,18]. A small colony was defined as a colony having a size of ≤ one-fourth of the well diameter. On the other hand, large colonies were those larger than 25% of the well diameter. Mutant frequencies, percentages of small and large mutant colonies, and cytotoxicity were determined.

Different concentrations of SAS NPs (0.01, 1, 10, 100 and 150 μg/mL) were chosen based on preliminary studies in the literature [13,26–28]. Preliminary experiments were conducted to determine the cytotoxicity of SAS NPs and their ionic forms. Cytotoxicity was determined by the relative total growth (RTG). RTG measures cytotoxicity including cell growth during treatment (4 h), expression (2 d), and cloning (12 d). RTG measurement takes into account cell loss after treatment, reduction in growth rate over the expression period, and any reduction in cloning efficiency on the day of selection for mutants [21]. The recommended highest concentration was one that corresponded with an RTG of 20% or more than 20% [29]. Therefore, the final concentrations of the test chemicals in the main experiments were 0.01–150 μg/mL.

### 2.4. Statistical analysis

The criteria of the Mouse Lymphoma Assay Workgroup of the International Workshop on Genotoxicity Tests (IWGT) were applied to determine whether a response was positive or negative [24]. IWGT considers biological relevance to be a major factor in MLA data evaluation that requires that the induced mutant frequency (IMF) exceeds some value based on the global background mutant frequency (MF). This value is the global evaluation factor (GEF), which is considered to be 126 for the micro-well version. The IMF is obtained by MF-SMF=IMF, where MF is one of the test-culture mutant frequencies, and SMF is the spontaneous mutant frequency. Positive responses are those which, for any treatment meet or exceed the GEF plus vehicle control MF, and exhibit a positive trend test as well. The statistical approach was the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the difference in MF between control and treatments. The concentration-response relationship was also evaluated by testing for a linear trend [30]. The level of statistical significance was set at 5%. Each compound was tested in two independent experiments and a good concordance was observed between both experiments.

## 3. Results

### 3.1. NPs characterization

This study used TEM to determine particle size of SAS NPs (7.172 nm) and SAS NPs (7.652 nm). Examples of TEM figures are shown in Figs. 1A 2A. Information on mean size and standard deviation was calculated by measuring 200 isolated NPs in random areas. As observed, the obtained measures match well with those indicated by the manufacturer: 7.08 ± 3.126 for SAS NPs (7.172 nm) and 8.04 ± 5.738 for SAS NPs (7.652 nm). The average hydrodynamic diameter and zeta potential of the SAS NPs suspensions in RPMI 1640 medium with 10% DHS were detected by DLS and LVD, respectively. Later, the sonicated NPs were dispersed in

**Table 1**Results of the toxicity and mutagenicity of fine SiO<sub>2</sub> in mouse lymphoma cells (L5178Y/Tk<sup>+</sup>/–3.7.2C) after 4 h of exposure.

Concentration (μg/mL)	Percent plating efficiency	Mutant frequency (×10 <sup>–6</sup> )	Relative total growth	MF (S/L) <sup>a</sup> (×10 <sup>–6</sup> )	IMF (MF-SMF)
Experiment 1					
0	91	70	100	58/12	–
0.01	88	79	92	79/0	9
1	82	99	82	86/13	29
10	80	95	63	70/25	25
100	77	114	53	70/44	44
150	75	134	46	80/54	64
MMS (10 μg/mL)	66	670 <sup>***</sup>	51	542/128	600
Experiment 2					
0	91	60	100	60/0	–
0.01	85	68	85	65/3	8
1	80	88	76	73/15	28
10	78	89	69	68/21	29
100	75	109	59	67/42	49
150	74	111	49	73/38	51
MMS (10 μg/mL)	80	607 <sup>***</sup>	50	470/137	547

\*\*\* P ≤ 0.001 (Significantly different from negative control).

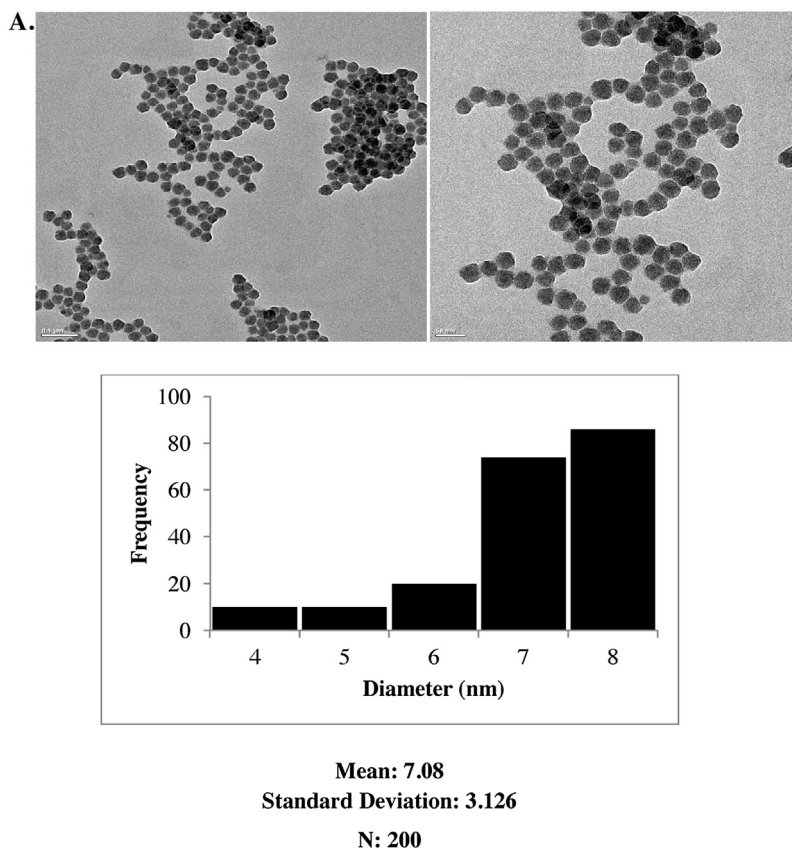
<sup>a</sup> Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

RPMI 1640 medium with 10% DHS for the genotoxic measurements. The average diameters obtained using DLS were 7.172 ± 1.774 and 7.652 ± 1.906 nm for SAS NPs (7.172 and 7.652 nm, respectively) (Figs. 1B 2B). The hydrodynamic diameter averages were similar to TEM analyses, indicating good dispersion. The zeta potential average obtained from the LDV technique was –45.9 and –58.4 mV for SAS NPs (7.172 and 7.652 nm, respectively), (Figs. 1C 2C). This highly negative surface charge results in good stability and dispersion of these nano-compounds in a medium solution. Energy

dispersive X-rays microanalysis (EDX) shows the expected Si and O peaks for the SAS NPs in the dispersion solution (Figs. 1D 2D).

### 3.2. Mutagenicity as detected by the MLA

The genotoxic potentials of SAS NPs and fine colloidal SiO<sub>2</sub> were assessed at various concentrations in the MLA. The results obtained after treatment of the cells with SAS NPs and SiO<sub>2</sub> are indicated in Tables 1–3 and Figs. 3–5, respectively. Each compound was tested in two experiments with two replicates. Prior to use, SAS



**Fig. 1.** Characterization of SAS NPs (7.172 nm). (A) Typical TEM image and size distribution histogram using such images. (B) and (C) represents size distribution and zeta potential, by DLS and LDV characterization. (D) Energy dispersive X-rays microanalysis (EDX) spectrum shows the chemical composition of SAS NPs (7.172 nm) in the dispersion solution.

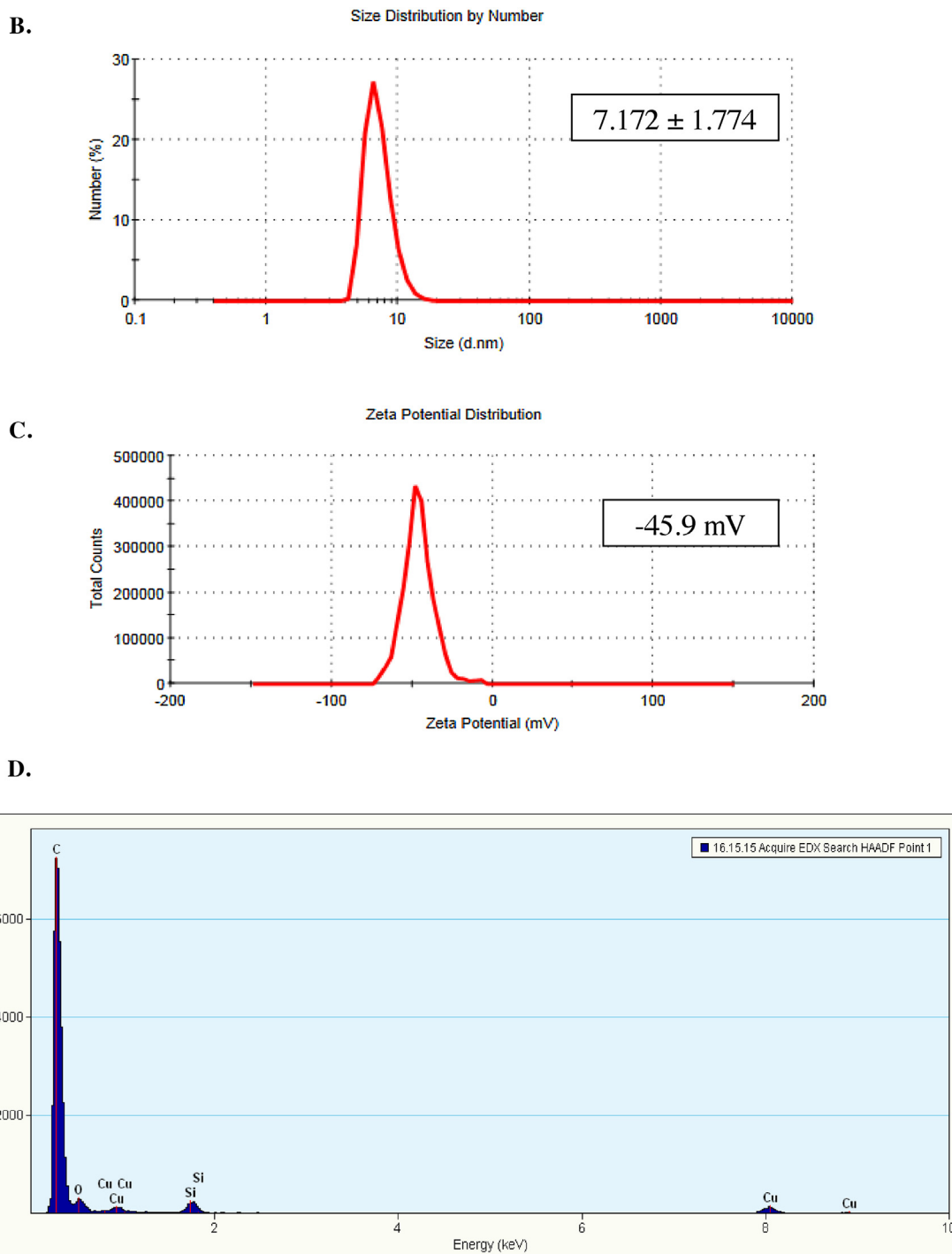


Fig. 1. (Continued)

NPs and SiO<sub>2</sub> were dispersed in distilled water containing 0.05% BSA. This suspension solution was used as the negative control. MMS (10 µg/mL), which was used as a positive control, showed a strong mutagenic effect. The negative control mutant frequency was in good agreement with the normal background range previously reported [18,20]. Significant increases were observed for the positive controls carried out using 10 µg/mL of MMS, which would support the validity of both the results observed and the protocols used.

Tables 1–3 summarizes the results of the MLA after exposure of L5178Y cells to SAS NPs and fine SiO<sub>2</sub> at five concentrations for 4 h.

In this study, the fine form of SiO<sub>2</sub> (Table 1 and Fig. 3) was tested from 0.01 to 150 µg/mL. Although slight increases were observed after fine colloidal SiO<sub>2</sub> exposure, it did not cause a significant mutagenic response in the MLA assay, and the cytotoxicity test did not show any viability reduction (taking into account the RTG values) after exposure to up to 150 µg/mL fine SiO<sub>2</sub> (Table 1). In other words, the results demonstrated that fine SiO<sub>2</sub> at the concentrations tested was not genotoxic in this assay.

When SAS NPs (7.172 and 7.652 nm) were tested for the induction of mutagenicity in the MLA, significant mutagenic effects were observed (Tables 2 and 3 and Figs. 4 and 5). RTG values were used

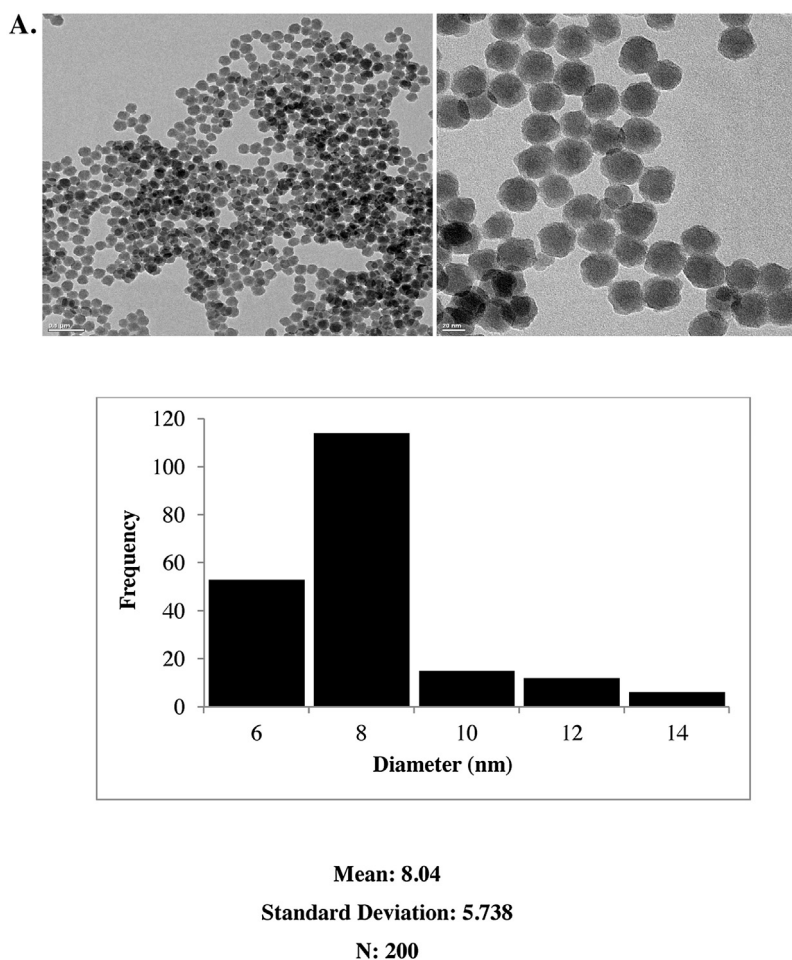
**Table 2**Results of the toxicity and mutagenicity of SAS NPs (7.172 nm) in mouse lymphoma cells (L5178Y/Tk<sup>+/−</sup>-3.7.2C) after 4 h of exposure.

Concentration (μg/mL)	Percent plating efficiency	Mutant frequency ( $\times 10^{-6}$ )	Relative total growth	MF (S/L) <sup>a</sup> ( $\times 10^{-6}$ )	IMF (MF-SMF)
Experiment 1					
0	95	64	100	64/0	–
0.01	88	76	87	60/16	12
1	76	104	66	74/30	40
10	74	115	58	73/42	51
100	67	449 <sup>***</sup>	45	309/140	385
150	62	785 <sup>***</sup>	36	588/197	721
MMS (10 μg/mL)	61	879 <sup>***</sup>	55	698/181	815
Experiment 2					
0	98	62	100	48/14	–
0.01	93	72	86	54/18	10
1	81	97	69	66/31	35
10	75	122	53	76/46	60
100	70	500 <sup>***</sup>	44	395/105	438
150	63	752 <sup>***</sup>	35	535/217	690
MMS (10 μg/mL)	61	808 <sup>***</sup>	49	620/188	746

\*\*\*  $P \leq 0.001$  (Significantly different from negative control).<sup>a</sup> Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

to determine the toxicity at each concentration level. Thereby, the selected ranges for SAS NPs, taking into account the obtained RTG values, were 0–150 μg/mL (Tables 2–3). At concentrations higher than 10 μg/mL, distinct decreases in the relative total growth (RTG) were found and the two highest tested doses (100 and 150 μg/mL) were clearly mutagenic. The data suggest that SAS NPs may cause

mutagenicity and damage at the chromosomal level in the MLA. The relative mutagenic potency according to the induced mutant frequency (IMF) was  $705.5 \times 10^{-6}$  at the highest concentration of SAS NPs (7.172 nm) and  $575.5 \times 10^{-6}$  at the highest concentration of SAS NPs (7.652 nm). According to these results, we selected 150 μg/mL for the final concentration of SAS NPs in the MLA exper-



**Fig. 2.** Characterization of SAS NPs (7.652 nm). (A) Typical TEM image and size distribution histogram using such images. (B) and (C) represents size distribution and zeta potential, by DLS and LDV characterization. (D) Energy dispersive X-rays microanalysis (EDX) spectrum shows the chemical composition of SAS NPs (7.652 nm) in the dispersion solution.

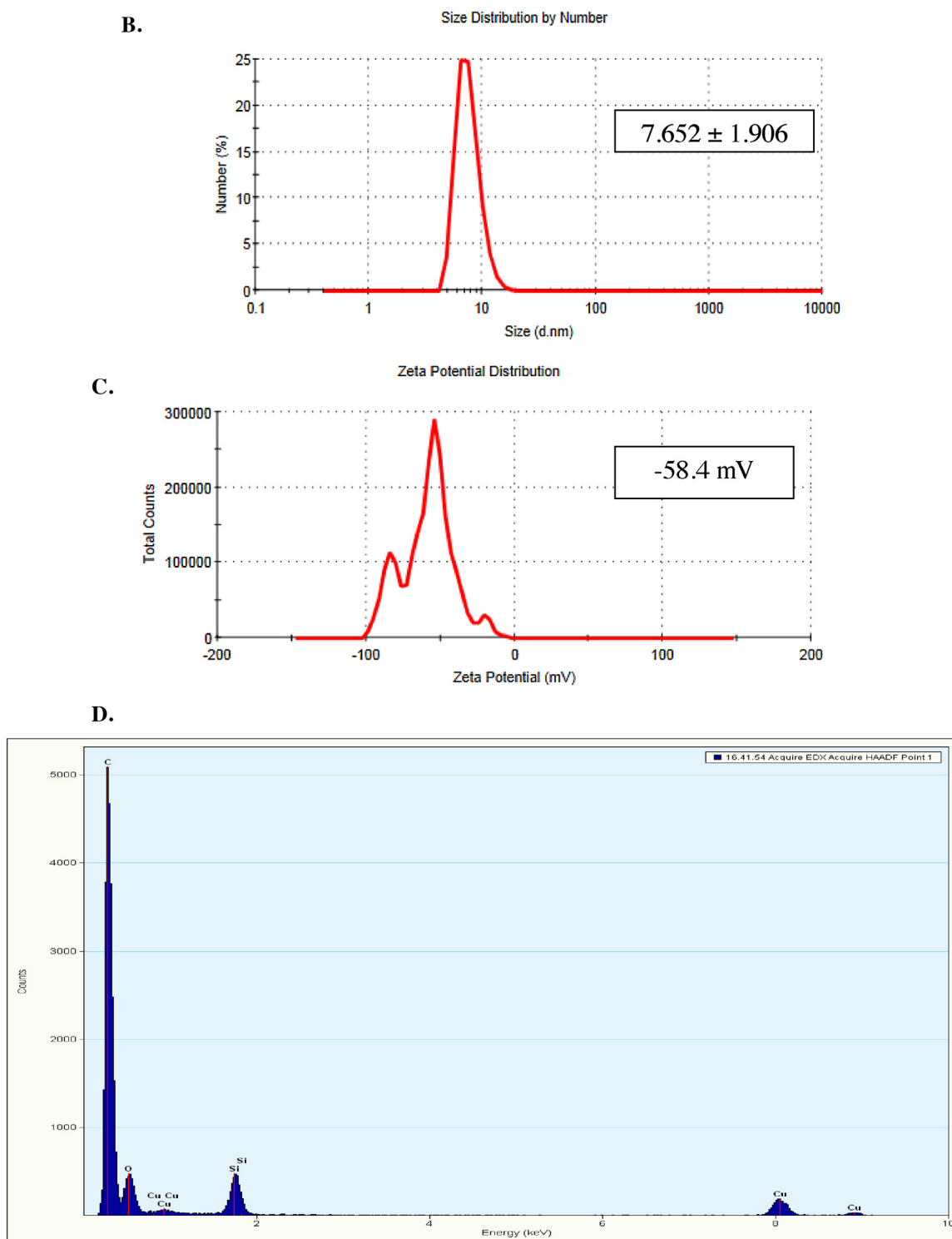


Fig. 2. (Continued)

iments. Above this concentration, the RTG was lower than 20%, which is usually accepted as the maximum level of toxicity for MLA.

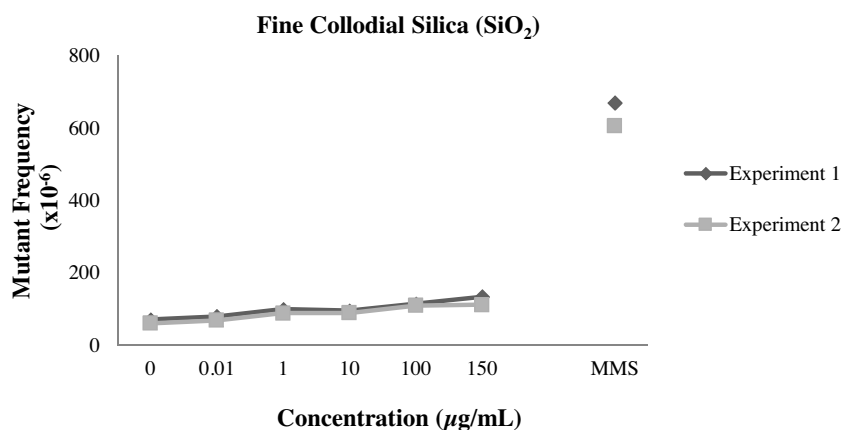
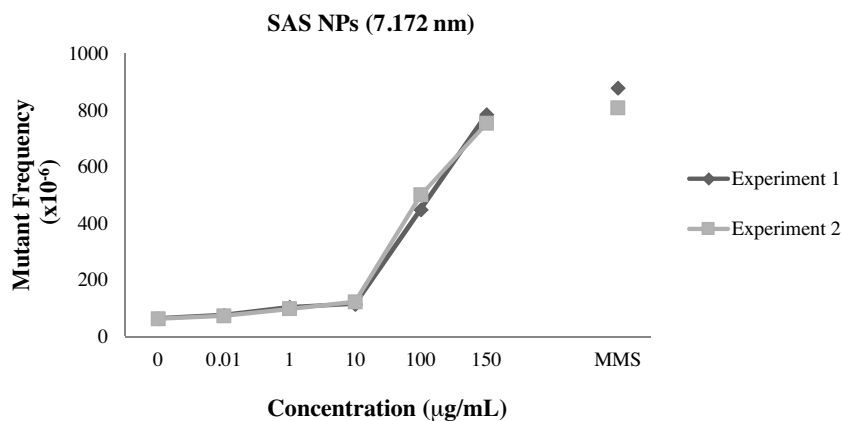
#### 4. Discussion

The use of MLA in the evaluation of mutagenicity has been well established as a genotoxicity test-system. In this context, we present studies using the MLA assay to extend our knowledge of the potential genotoxic risk of SAS NPs.

Several studies in the literature have demonstrated that some NMs are toxic and cytotoxic, with some NPs inducing allergic or inflammatory responses [6]. It is assumed that some NMs can generate reactive oxygen species (ROS), inducing oxidative stress, DNA damage, micronucleus formation, inflammatory events and fibrosis in different target organs [31]. Nevertheless, NMs' toxic effects depend on their unique physicochemical properties, such as form, size, chemical stability, dissolution rate, coating, surface charge, agglomerations/aggregations, crystal structure, and size [32,33].

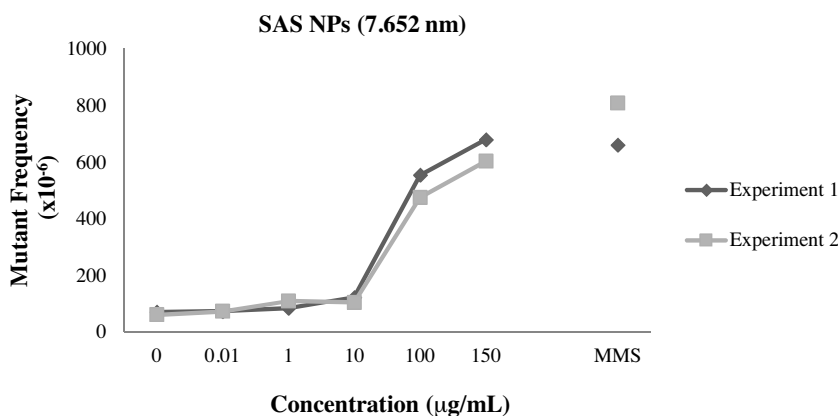
**Table 3**Results of the toxicity and mutagenicity of SAS NPs (7.652 nm) in mouse lymphoma cells (L5178Y/*Tk*<sup>+/-</sup>-3.7.2C) after 4 h of exposure.

Concentration ( $\mu\text{g/mL}$ )	Percent plating efficiency	Mutant frequency ( $\times 10^{-6}$ )	Relative total growth	MF (S/L) <sup>a</sup> ( $\times 10^{-6}$ )	IMF (MF-SMF)
Experiment 1					
0	95	71	100	71/0	–
0.01	87	74	85	74/0	3
1	78	85	69	82/3	14
10	69	123	53	86/37	52
100	62	554 <sup>**</sup>	45	448/106	483
150	56	680 <sup>***</sup>	37	548/132	609
MMS (10 $\mu\text{g/mL}$ )	60	661 <sup>***</sup>	47	492/169	590
Experiment 2					
0	95	61	100	61/0	–
0.01	80	73	75	73/0	12
1	71	110	54	95/15	49
10	69	105	43	80/25	44
100	62	475 <sup>***</sup>	34	364/111	414
150	55	603 <sup>***</sup>	26	454/149	542
MMS (10 $\mu\text{g/mL}$ )	50	808 <sup>***</sup>	33	652/156	747

<sup>\*\*</sup>  $P \leq 0.01$ .<sup>\*\*\*</sup>  $P \leq 0.001$  (Significantly different from negative control).<sup>a</sup> Total mutant frequency divided into small/large (S/L) colony mutant frequencies.**Fig. 3.** Mutation frequencies for fine colloidal silica ( $\text{SiO}_2$ ), on L5178Y/*Tk*<sup>+/-</sup> mouse lymphoma cells. Positive control (MMS, 10  $\mu\text{g/mL}$ ). Results are from two different experiments with two replicates each.**Fig. 4.** Mutation frequencies for SAS NPs (7.172 nm), on L5178Y/*Tk*<sup>+/-</sup> mouse lymphoma cells. Positive control (MMS, 10  $\mu\text{g/mL}$ ). Results are from two different experiments with two replicates each.

NMs can easily enter the cell membrane and accumulate in the cytoplasm, disrupt metabolism, and induce cell death [34]. SAS NPs may play a role in a variety of effects, such as mitochondrial dysfunction, oxidative stress, lipid peroxidation, and cell death by direct or indirect mechanisms [35–37].

At present there is limited evidence concerning whether or not SAS are genotoxic, with contradictory results being reported [38,39]. Choi et al. [13] demonstrated no mutagenicity of nano-silica (10 nm) using the MLA in L5178Y/*Tk*<sup>+/-</sup> mouse lymphoma cells in the absence or presence of S-9 activation for 3 h. In contrast, SAS NPs did show mutagenicity at the highest concentrations



**Fig. 5.** Mutation frequencies for SAS NPs (7.652 nm), on L5178Y/*Tk*<sup>+</sup> mouse lymphoma cells. Positive control (MMS, 10 µg/mL). Results are from two different experiments with two replicates each.

(100 and 150 µg/mL) for 4 h in the same cell line according to our findings. It is not clear why results of these studies differ. However, it should be noted that a high degree of dispersion was achieved in the present study without use of chemical dispersants.

*In vitro* studies conducted to detect chromosome damage induction have reported positive effects for 80 nm SAS NPs in 3T3-L1 mouse fibroblasts in the micronucleus assay [40], but no effects were observed in the same assay using Balb/3T3 mouse fibroblasts with SAS NPs having diameters ranging from 15 to 300 nm [41] and with human lymphocytes for 15 and 55 nm SAS NPs [42]. When the induction of primary DNA damage was assayed using the Comet assay negative results were reported in 3T3-L1 mouse fibroblasts [43] and in A549 human lung carcinoma cells [44]. In contrast, positive effects have been obtained in human lung alveolar (A549) epithelial cells with 20 and 100 nm SAS NPs [45], as well as in human umbilical vein endothelial cells [46], in human peripheral blood lymphocytes and cultured human embryonic kidney (HEK293) cells using 6, 15, 30, and 55 nm SAS NPs with and without formamidopyrimidine DNA *N*-glycosylase (Fpg) and endonuclease III (Endo III) enzymes [26], and in L5178Y and BEAS-2B cells for 10 nm SAS NPs [13]. In another study, Gerloff et al. [47] showed that amorphous fumed nano-silica (14 nm) administration for 24 h caused DNA damage and cytotoxicity in the human colon epithelial cell-line, Caco-2. With respect to SAS NPs toxicity it is assumed that their toxicity is mediated by oxidative stress, inflammatory, and apoptotic mechanisms, as these responses have been shown in both *in vivo* and *in vitro* [8,9,28,48–50]. However, from the genotoxic point of view the obtained results are contradictory. Our positive results suggest that further genotoxicity studies with SAS NPs using different cell lines and different *in vitro* assays are warranted.

In the present study, the genotoxic potential of two different NP sizes of SAS NPs using the MLA was much greater than an equal mass concentration of fine colloidal silica. Since the fine silica sample was in the micrometer size range (average particle size (diameter): 12 nm, specific surface area: 220 m<sup>2</sup>/g SiO<sub>2</sub>), on a mass basis, cells were exposed to a far greater particle number and total particle surface with SAS NPs than with the fine silica sample. The greater toxicity of NPs on a mass basis vs. fine particles of the same composition is well documented [51,52].

In conclusion, results from the present study confirm the usefulness of the MLA in evaluation of the genotoxicity of NPs. The fact the SAS NPs exhibit a significant concentration-dependent mutagenicity indicate that this response should be considered in the evaluation of the risk/benefits associated use of SAS NPs.

### Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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