Occupational exposure to LTA Nanozeolites: strategies of exposure monitoring and toxicity evaluation

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Abstract. Nanozeolites (NZs) are increasingly used in several sectors, but very few data are available on their toxicity. Therefore in this study we aimed to apply a standardized strategy to assess the occupational exposure integrated to an in vitro model useful to evaluate potential toxic effects in the case study of LTA-NZs. Particle number concentration (PNC) values greater than the background significant level were highlighted during the production process phase of LTA NZs. The increase of PNC in the typical size range of produced NZs (around 100nm) and the presence of airborne NZs and their agglomerates in the workplace were confirmed by both real-time (CPC and FMPS) and offline (ICP-MS and SEM-EDX) data analysis. We also investigated the potential cyto-genotoxicity of NZs produced in the factory, on human alveolar cells (A549) exposed to 10-100μg/mL. We evaluated cell viability/apoptosis by cytofluorimetric assay, membrane damage by LDH release and DNA damage by Fpg-comet assay. We found a slight increase of apoptotic cell % at 50 and 100μg/mL. We found slight, statistically significant, direct DNA damage and slight oxidative DNA damage. These findings represent the first data integrating exposure characterization and potential genotoxicity of NZs and highlight the need to perform further studies to confirm such results.

1. Introduction

Nanozeolites (NZs) are used in a lot of applications [1,2] including catalysts, ion exchange materials or thermal isolators, taking advantages from the major property of NZs to absorb water residual and moisture in order to keep the insulation of devices and products [3]. However, only few studies evaluating NZs potential toxicity are available [4-6]. In particular Kihara et al. 2011 [4] demonstrated that the cubic Lined Type A (LTA) NZs display higher toxicity compared to those with spherical or quasi-cubic morphology. Several exposure measurement strategies have been developed to assess airborne nanomaterials (NMs) in the workplace, integrating different aerosol instruments [7] to take into account the most important parameters of exposure such as size, shape, surface area, aggregation and agglomeration, water solubility and surface chemistry [8-11]. Research efforts have focused on improving the release characterization towards a comprehensive exposure assessment on occupational settings [12].

In this framework the present study is aimed to apply a standardized strategy to assess the occupational exposure to LTA NZs produced in an industrial workplace, including their chemical-physical characterization and an in vitro model useful to evaluate their potential toxic effects. We evaluated cytotoxic, genotoxic and oxidative effects of LTA NZs, simultaneously to their airborne dispersion in the industrial workplace where they were produced in order to assess the occupational exposure and their potential cyto-genotoxicity. Since inhalation represents the main route of occupational exposure to NMs, we performed our study on human alveolar epithelial (A549) cells that are widely used in the study of potential toxicity of NMs on target organ.
2. Methods

2.1 Exposure assessment

In our study the measurement strategy recommended by the OECD guidelines has been used [13, 14]. It provides for a multilevel approach with three phases of analysis. The first and the second tier include a preliminary information gathering and measurements with portable instruments. The third level concerns an intensive monitoring and sampling campaign for the off-line analysis carried out in laboratory.

The real time instruments were used for airborne nano-powders measurement such the Fast mobility Particle Sizer (FMPS, Mod. 3091, TSI, USA) and the Condensation Particle Counter (CPC Mod. 3007, TSI, USA); in the case of integration time measurements we used for particle matter sampling the inertial cascade impactors such a nano Micro Orefice Uniform Deposit Impactor (nanoMOUDI-II 122R, MSP, USA) and a SIOUTAS (SKC, USA) personal cascade impactor.

The real time instruments have a time resolution of one second; these short acquisition times allow the identification of possible spills in the different phases of production. Instead the integration time instruments allow to sample powders on filters for off-line analysis such as scanning electron microscopy (FE-SEM, Zeiss) equipped with an energy dispersive spectroscopy system (EDS, Oxford Instruments INCA) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7500ce, Agilent Technologies); in details with ICP-MS 10 different metals were analyzed on the sampling stages, including the Si concentration which is the major component of LTA NZs.

Our case study involved an industrial factory housed within a building of about 5 m high and in an area of 1300 m² and organized in different laboratories and sections. Annual NZs production totaled about 10 kg/year. Primary LTA NZs produced particles have a typical cubic shape with an average size of about 100 nanometers. The production process of LTA NZs includes synthesis, surface modification, drying, activation and mixing with a few workers involved.

The measurements were performed in two separate rooms located in different locations called as Background room and Production Laboratory, they were about 48 meters apart from each other as the crow flies. Instruments were positioned firstly in the Background room and then in the Production Laboratory. Background room and Production Laboratory have been monitored for three days each, for eight hours each day. In the Production Laboratory the instruments were located close to the operator, in a radius of about 1.5 m and the personal cascade impactor was used for personal sampling, in the breathing zone of the operator.

2.2 In vitro study

A stock solution was prepared dissolving LTA NZs in H2O (2 mg/ml). At the moment of exposure the LTA NZs stock solution was sonicated for 10 min and Bovine Serum Albumin (BSA, Sigma) 15 mg/mL was added then this solution was used to perform the exposures for 24 h in cell medium (RPMI) at the used concentrations. The hydrodynamic diameter of LTA NZs suspensions were measured by dynamic light scattering (DLS) analysis using a Zetasizer nano ZS (Malvern, UK). The hydrodynamic size distribution of LTA NZs in the stock solution was analyzed to characterize the used NZs dispersion before diluting it in culture medium. The hydrodynamic diameter of LTA NZs suspensions in RPMI at the concentrations used to expose the cells was measured at the beginning of exposure (t=0).

An in vitro model already applied in the study of cyto-genotoxicity of other nanomaterials such as Multiwalled Carbon Nanotubes, Titanium Dioxide and Cobalt Oxide [15-17] was used with small modifications. In particular, Human lung epithelial (A549) cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured in RPMI 1640 (EuroClone, United Kingdom) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO2. Cells (8x10^4 cells/well) were seeded into 24 multi-well culture plate (15.6 mm well diameter) and cultured for 24 h before the exposure. After 24h exposure to 10, 25, 50 and 100 µg/mL of LTA NZs, we evaluated viability reduction and apoptosis induction by the Guava ViaCount cytofluorimetric assay (using easyCyte™ Flow Cytometer, Merk Millipore, Germany), following the manufacturer’s instructions. Moreover, we evaluated membrane damage detecting Lactate Dehydrogenase (LDH) release by Cytotoxicity Detection Kit, (Roche Diagnostics, Milan, Italy) on the culture medium of cells exposed to LTA NZs.

Direct and oxidative DNA damage were evaluated by the Formamido-pyrimidine glycosylase (Fpg) comet assay which allows to specifically evaluate simultaneously direct and oxidative DNA damage [18]. It represents a very sensitive biomarker of early genotoxic effects widely used in nanotoxicology. Unexposed
cells were used as negative control and cells exposed for 30 min to 100 µM H$_2$O$_2$ were used as positive control. The previously described protocol [17] was used. Images of 100 randomly selected comets from either the Fpg enzyme-treated or untreated slides, stained with ethidium bromide, were acquired and analyzed from each sample, with specific image analyzer software (Delta Sistemi, Rome, Italy). The percentage of DNA in the tail (% tail DNA) was obtained from the analysis. For each experimental point, we calculated the mean % tail DNA of 100 comets from enzyme untreated cells (% tail DNA) which indicates the direct DNA damage, and the mean % tail DNA of 100 comets from Fpg-enzyme treated cells (% tail DNA enz), evaluating direct and oxidative DNA damage. Direct DNA damage was assessed by calculating for each experimental point the % tail DNA values of exposed cells normalized in respect to unexposed cells. Oxidative DNA damage was evaluated in terms of oxidized DNA bases (sites recognized and cut by Fpg) and calculated by deducting % tail DNA from the % tail DNA enz, both in exposed and unexposed cells. The results were expressed as means of three independent experiments.

3. Results and discussion
3.1 Exposure assessment
The mean concentration value of the Far Field (FF) background calculated in a time range of four hours (13:00-17:00), resulted lower than the mean concentration value of Near Field (NF) background calculated in a time range of 18 minutes. Furthermore, the mean particle number concentration (in a typical working day of about 8 hours) during the Production Phases (PP) resulted slightly higher than NF, but with a much higher variability (table 1). Moreover, the Significant Level (SL), above which the signal recorded by the CPC could be attributed to the LTA NZs production process, has been calculated as the NF background value plus three times the corresponding standard deviation [19, 20]. The SL value, according to the procedure described above, is 5152 #/cm$^3$. Figure 1 shows particle number concentration measured during a working day by CPC in the Production Laboratory compared to the calculated background SL.

| Parameter   | Particle Number Concentration (#/cm$^3$) | Standard deviation (#/cm$^3$) |
|-------------|------------------------------------------|-------------------------------|
| FF background | 956                                      | 155                           |
| NF background | 4423                                     | 243                           |
| PP           | 5753                                     | 1523                          |

Table 1. Particle number concentration and standard deviation of FF background, NF background and PP

![Figure 1](image1.png)
The particle size distribution obtained by FMPS in the Production Laboratory was compared to that in the Background room and the results showed a three modal trend for both cases. In particular, the particle size distribution detected in the Production Laboratory showed a shift towards the dimensions close to the produced NZs typical size about 100 nm (Figure 2). In addition the gravimetric analysis, performed on the filters sampled with the nanoMOUDI, showed an increase, corresponding to the stages with nominal cut-off sizes in a range that comes from 56 to 100 nm, when compared to the total mass sampled.

Figure 2. Percentage contribution for each size compared to the total concentration measured for both the Background days and production days

Figure 3 shows the percentage contribution to the total particle number concentration obtained by FMPS, in the size range 52-100 nm, both for the Production Laboratory (33 %) and for the Background room (16 %). For the same size range it is also reported the silicon (Si) concentration percentage obtained by ICP-MS on the sampled filters with the nanoMOUDI is 36 % and 18 % for Production Laboratory and for Background room, respectively. The results highlight that, in the stages with the nominal cut-off sizes close to the produced NZs, Si concentration in the Production Laboratory is more than twice as much background values. On the contrary, the concentration values of different metals, including Si, obtained on the stages with the nominal cut-off sizes far from those of produced LTA NZs (> 100nm) are similar in the Background room and in the Production Laboratory.

Figure 3. Percentage of Si concentration (nominal cut-off sizes from 56 to 100 nm), obtained by ICP-MS and percentage of the total particle number concentration (size range from 52 to 100 nm) obtained by FMPS in the Background room (blue) and in the Production Laboratory (orange).
Figure 4. SEM images of LTA NZs: a) trial sample, b) sample collected by the inertial impactor (Sioutas).

Figure 4 shows SEM images of LTA NZs deposited (trial sample, panel a) and collected by Sioutas (panel b) on aluminum substrates; the SEM images were taken using the INLENS detector, without sample coating. At high resolution (fig.4 b) is well highlighted the characteristic cubic shape and the size of about 100 nm, both corresponding to those of the produced LTA NZs.

3.2 In vitro study

Figure 5 shows the results of DLS analysis with the Z-AVERAGE (ZAV) diameters of LTA NZs suspensions in cell medium at each used concentration and demonstrates a dose-dependent increase of the agglomerate sizes. DLS analysis shows a better dispersion in cell medium (with 10% fetal bovine serum) at the lowest concentration of the tested nanomaterials, ZAV diameter ranged from 100.8 to 299.4 nm and Polydispersivity Index (PDI) from 0.455 to 0.812.

Figure 5. DLS characterization of tested LTA NZs concentrations in culture medium (RPMI).
Figure 6 shows lack of statistically significant viability reduction after 24h exposure to LTA NZs. However at 100 μg/mL we found a slight although statistically significant increase of apoptotic cell percentage and a slight increase of LDH release.

Figure 6. Percentages of viable A549 cells (upper panel) and of apoptotic cells (lower panel) evaluated by cytotoxic-fluorimetric Guava assay after 24h exposure to LT NZs.

Figure 7. LDH release after 24h exposure to LTA NZs.

The results of the fpg-comet assay after 24h exposure are reported in figure 8 which shows both direct and oxidative DNA damage. We found a dose-dependent direct DNA damage expressed as percentage of DNA in
the tail (statistically significant beginning from 25 µg/mL). We also found a slight induction of oxidative DNA damage.

![Direct DNA damage graph](image1)

**Figure 8.** Direct (upper panel) and oxidative (lower panel) DNA damage in A549 cells evaluated by Fpg comet assay after 24h exposure to LTA NZs.

The application of our in vitro model on human respiratory cells to evaluate the cyto-genotoxicity of LTA NZs produced in the studied industrial workplace, allow us to obtain (at the best of our knowledge) the first results on the genotoxic effects of such nanomaterial. In fact the only available data on potential LTA NZs toxicity are related to cytotoxic effects in terms of viability reduction, apoptosis and membrane damage. In particular, Khiara et al. 2011 [4] evaluated the effect of composition, morphology and size of NZs on their in vitro cytotoxicity and found on Hela cells that pure-silica NZs were non cytotoxic while aluminum-containing NZs such as NZ LTA, induced a dose-dependent cytotoxicity influenced by both alumina component and NZ crystal shape. Otherwise, the study of Meczynska-Wielgosz [6] evaluating on the same Hela cells the effects of pristine and differently modified NZ A did not find significant cytotoxicity induction. Thomassen et al. 2012 [5] evaluated cytotoxic effects of NZ A and Y, in terms of viability reduction and LDH release on human alveolar epithelial cell line (A549), human endothelial cell line (EA.hy926) and human monocyteic leukemia cell line (THP-1). They tested a big range of concentrations (25-2000 µg/ml) of NZs and they did not find any significant effect below 250 µg/ml. Our results seem to confirm the low cytotoxicity found by Thomassen (2012) [5] for NZs A on A549 cells and they also demonstrated that on this kind of transformed cells (particularly sensitive to genotoxic effects) such nanomaterials could induce DNA damage.
Conclusion

The present study characterizes the occupational exposure to LTA NZs in the production process and evaluates simultaneously their potential cyto-genotoxicity on human alveolar epithelial cells, which represent the main target of NM occupational exposure by inhalation. The multi-parametric approach allowed us to have a comprehensive assessment of the exposure to airborne LTA NZs produced in the workplace. Moreover, since we found detectable levels of such NMs, we highlight the need to perform further studies to quantify NZs used in such industrial applications and to evaluate their toxicity also in other kind of cell lines.

In conclusion, these findings provide the first data integrating the exposure characterization and the potential toxicity of LTA NZs and suggest to perform other studies to better quantify the real exposure condition to be correlated to possible induced toxicity.

4. References

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