Development of a novel method to detect a potential inhalation of nanoaerosols

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Abstract. In the context of the precautionary principle for engineered nanoparticles, a test able to verify the non exposure of individuals to nanoaerosols seems to be of great interest, especially for workers involved with nanomaterials. A novel method is proposed to detect the presence of nanoparticles in the nose. Different materials, including paper and polycarbonate, have been tested for their sampling efficiency towards ZnO nanoparticles. The detection of nanoparticles is performed by elemental analysis using Total Reflection X-Ray Fluorescence (TXRF). For an optimal sensitivity of the TXRF signal, samples have to be deposited on a smooth surface. Therefore, different materials have been compared as sample carrier. The results indicate that pure paper is the most efficient material for sampling nanoparticles (ZnO), while the use of polycarbonate, and even more flexible polyester, as sample carrier allows a better sensitivity for TXRF analysis. Therefore, the sampling device made of paper is digested under slightly acidic conditions in a microwave oven after collection, and the resulting solution is deposited on polyester for TXRF analysis. Internal calibration is performed thanks to an addition of Rb to the samples. The proposed method allows the chemical identification of nanoparticles deposited in the nose: a qualitative signature of a potential nanoaerosol inhalation.

1. Introduction
The market of engineered nanoparticles is expanding very fast worldwide, and should reach a thousand billion dollars in 2011-2012, according to the estimates of the National Science Foundation in the United Stated [1]. It has been estimated in 2009 that more than a thousand products containing nanomaterials were already internationally commercialized [2].

Along with the increasing use of nanomaterials, there is a growing concern for potential health impact following inhalation due to the known adverse health effects of atmospheric particles and more precisely of ultrafine particles [3-5]. Toxicological studies have increased over the past few years, but there is still a lack of clear answer on the potential toxicity of nanomaterials.

The precautionary principle can still be applied by lowering the exposure to zero, and currently, its application is recommended in France [6-7]. In this approach, exposure monitoring of individuals occupationally exposed to nanomaterials is of major importance. The assessment of external exposure thanks to air sampling is essential, but this type of measurement does not take into account the use of
individual protective devices, and individual parameters such as absorption, distribution and elimination. This is why, it is admitted that biomonitoring is the best approach to assess individual exposure to occupational and environmental toxicants [8]. Concerning nanoparticles, as shown by the congress on Nanomaterials and Worker Health held in Keystone (Colorado, USA) in July 2010, there is now a general agreement for the necessity to develop research studies on the establishment of biomonitoring [6, 9-10]. Pending the results of toxicological studies, a first step in this research area is the determination of biomarkers of exposure in order to identify the situations of exposure.

In general, biomonitoring is based on blood and urine analyses, but in the case of insoluble nanoparticles, toxicokinetics data would tend to prove that very few nanoparticles would be found in those matrices following inhalation [6, 11-14]. This is why other methods have to be explored.

In the first place, a test able to verify the non exposure to nanoaerosols seems to be of great interest, especially at work places where workers might be exposed to nanomaterials. Therefore, nasal sampling could be proposed for the evaluation of potential inhalation to nanoparticles.

The objective of the present work is therefore to develop a method that would allow the sampling and the analysis of nanoparticles deposited in the nose. Ideally, this method should be fast and convenient in order to give a quick indication on the existence, or not, of an inhalation situation.

Microscopic analyses allow nanoparticles identification according to their shape, size, aggregation state, and even their composition using EDX additional technique, but such analyses are time-consuming and hardly adequate for routine measurements. Chemical analyses, such as atomic adsorption spectrometry or inductively coupled plasma mass spectrometry, allow the identification of particle composition but they often require fastidious pretreatment protocols due to the necessity to mineralize particles before analysis. Interestingly, total reflection X-ray fluorescence (TXRF) allows elemental analysis of surfaces without fastidious pretreatment procedures. This method is already used for diverse biological applications as referred in the review of Szoboszlai et al. [15]. Therefore, TXRF was evaluated for its ability to analyze nanoparticles after nasal sampling, according to their chemical composition. In the present work, this evaluation was performed on ZnO nanoparticles.

2. Materials and methods

2.1. Nanoparticles, chemicals and materials

Nanoparticles used in the study were ZnO nanoparticles in a commercial aqueous suspension from Evonik (Antwerp, Belgium). Scanning electron microscopy indicated that these nanoparticles are spherical with a diameter around 40 nm, and nanoparticles are essentially aggregated in water (data not shown).

Ultrapure water was used for solution preparation (MilliQ, Millipore). Suprapure nitric acid (65%) from VWR (Fontenay-sous-Bois, France) and H2O2 (≥ 30%) from Sigma-Aldrich (Lyon, France) were used for microwave digestion. Rb ICP standard (1000 ppm) from Merck (Martillac, France) was used as an internal standard. Polycarbonate membranes were from Ecomesure (Janvry, France). Rigid polyester was cut in laser transparency film from 3M (Cergy-Pontoise, France), and flexible polyester was from Avery (Ecullly, France). A new paper without impurities and a recycled paper used as sampling materials were from Fiorini (Ingre, France). They were pasted on wood sticks forming sampling devices in the shape of paper flags.

2.2. Determination of nanoparticle sampling efficiency

Ten µL of ZnO nanoparticles in water (1000 ppm) were deposited on glass slides and air-dried at room temperature. Glass slides were then rubbed 10 times with a polycarbonate membrane, a new paper flag, or a recycled paper flag, in triplicate for each material.

In a second experiment, 10 µL of ZnO nanoparticles in water at different concentrations (500, 1000, 2000, 4000, and 6000 ppm) were deposited on glass slides in duplicate, and air-dried at room temperature. Each series was then rubbed with either new paper flags or recycled paper flags.
Afterward, the polycarbonate membranes and the two types of paper were washed in 1 mL of HNO$_3$ 0.65% in a three dimensional agitator (Turbula) during 2 hours in order to dissolve ZnO nanoparticles. The washing solutions were then quantitatively analyzed by inductively coupled plasma mass spectrometry (ICP-MS) so as to determine the percentages of nanoparticles recovered by each material.

2.3. TXRF analysis
TXRF analyses were performed on a Rigaku NanoHunter bench top apparatus. Prior to TXRF analysis, the sampling devices were digested in Teflon vessel containing 1 mL of HNO$_3$ 1M + 1 mL of H$_2$O$_2$, in a microwave oven during 1.5 min. Afterward, 10 µL of the samples were deposited at the centre of the sample carrier and dried at 50°C on a hot plate. The materials used as sample carrier (paper, polycarbonate and polyester) were pasted on glass slides for rigidity. Zn and Rb analyses were performed on a Mo X-ray tube with an angle of the incident beam at 0.75° during 200 sec, and both elements were analyzed on their K$_\alpha$ lines.

2.4. Comparison of sample carriers for TXRF analysis
Prior to ZnO analysis all materials were tested for their absence of background fluorescence on Zn K$_\alpha$ line. Ten µL of nanoparticles of ZnO in water (1000 ppm) were deposited on different materials in triplicate, including polycarbonate membrane, pure and recycled paper, rigid and flexible polyester. All the samples were dried on a hotplate at 50°C. The intensities of the obtained TXRF responses were compared in order to determine which material shows the best sensitivity.

2.5. Calibration and limit of detection of the method
Calibration was performed using Rb as an internal standard. Ten µL of Rb (1000 ppm) were added to the sample prior to the microwave digestion step. A calibration curve was obtained by the TXRF analysis of solutions containing ZnO and Rb at different ratios in water.

The detection limit of the method (signal to noise = 3) was determined following the analysis of six blank paper flags. The limit of detection was calculated as three times the standard deviation of the six Zn/Rb blank values.

3. Results

3.1. Selection of the material for the nanoparticle sampling device
In order to choose the material for the sampling device, we compared the sampling efficiency of a polycarbonate membrane, known to be an adequate material as a substrate for TXRF analysis, and of two types of paper flags, a pure and a recycled one.

The polycarbonate membrane allowed a recovery of only 5 ± 2% of 10 µg of ZnO deposited on glass slides while the pure paper and the recycled paper allowed recoveries of 46 ± 4% and 55 ± 6%, respectively, for the same quantity of ZnO. The polycarbonate membrane, as a sampling material, was therefore eliminated for the rest of the study.

The pure paper and the recycled one were further studied for their sampling efficiency using increasing quantities of ZnO deposited on glass slides. The results shown in Figure 1 indicate that the sampling recovery was almost constant, around 50%, whatever the quantity of ZnO, for both paper flags, the recycled paper being a little more efficient than the pure one.
3.2. Selection of a sample carrier for best TXRF sensitivity
Different materials were compared as sample carrier for TXRF analysis of ZnO nanoparticles. The results of the comparison are shown in Figure 2 and indicate that the flexible polyester showed the most intense signal for ZnO detection with a two orders of magnitude higher signal than the signals obtained with the two types of paper. Therefore, the flexible polyester was chosen as the sample carrier for TXRF analysis in the final method.

3.3. Proposition of an entire procedure
The entire protocol consisted in collecting nanoparticles with paper flags, digesting the paper flags and depositing the resulting liquid on flexible polyester for TXRF analysis. For that purpose, the digestibility of the two types of paper was tested (data not shown) and it appeared that the pure paper was more easily digested than the recycled one in acidic conditions described in part 2.3. Therefore,
the pure paper flag was selected as the sampling device. Following paper digestion, the resulting solution was deposited on flexible polyester for TXRF analysis.

3.4. Calibration of TXRF measurement
The calibration of ZnO TXRF measurements was obtained using internal calibration with Rb. In Figure 3, a calibration curve of ZnO in water is shown, in addition to a calibration curve obtained following the analysis of paper flags spiked with 4 to 80 µg of ZnO. It appears that the two calibration curves are perfectly superimposed, the slope of the two curves being very similar. This means that no matrix effect due to the digestion step of the paper flags is observed, and therefore, the calibration in water can be used for the analysis of paper flags.

![Figure 3: TXRF analysis of ZnO nanoparticles in water and of paper flags spiked with ZnO nanoparticles and treated according to the entire protocol, using Rb as an internal standard.](image)

3.5. Sensitivity of the method
The limit of detection of the method was calculated to be a little less than 1 µg of ZnO on paper flags. The TXRF spectrum obtained for 2 µg of ZnO is shown in Figure 4 in comparison with a blank spectrum.
4. Discussion
The objective of the present work was to develop a method including the sampling and the analysis of nanoparticles potentially deposited in the nose after inhalation. In order to develop a fast and convenient method, the analytical technique of TXRF was chosen. This technique is an elemental surface analysis that requires no pre-treatment of the samples. The main requirement of the technique is the use of a very smooth and flat sample carrier to allow a sensitive detection.

The first step of the study was to identify an adequate material for nanoparticles collection. The choice was guided by the fact that paper flags are already used and found to be efficient for radioactive particle collection [16]. Polycarbonate membranes were also tested because of their suitability for TXRF analysis as sample substrate. It was shown that the paper flags were more efficient for nanoparticles collection than the polycarbonate membranes. Moreover, pieces of paper were easily pasted on little wood sticks to form sampling devices, whereas polycarbonate membranes were very fragile and would easily tear.

The second step was to identify the best sample carrier for TXRF analysis. Indeed, the developed method had to be adequate for the analysis of traces, and therefore, had to be as sensitive as possible. It was shown that flexible polyester pasted on a glass slide allowed the best sensitivity of detection for ZnO nanoparticles. Glass slides themselves were also tested (data not shown) but the detection was less sensitive than with flexible polyester, probably due to the fact that glass surface is less hydrophobic than polyester.

Ideally, the easiest protocol would have been to directly analyze the sampling device after collection. However, we have shown that the sampling devices made of paper were efficient for nanoparticles sampling but they induced a poor sensitivity of TXRF signal when used as sample carriers. On the opposite, the polycarbonate membranes had a very poor sampling efficiency whereas they were better sample substrates than paper for TXRF analysis. This is directly due to the fact that sampling requires a fibrous material whereas TXRF analysis requires a very smooth material. As a consequence, the proposed protocol combines the use of paper as a sampling device and the use of flexible polyester as sample carrier for TXRF analysis. Polyester was shown to be even more interesting for TXRF sensitivity than polycarbonate.
As a consequence, the sampling device was digested in order to release the nanoparticles in liquid after collection. Then, the liquid was deposited on a flexible polyester piece pasted on a glass slide to make a rigid sample carrier for TXRF. Interestingly, it was not an obligation to dissolve nanoparticles since it was shown that TXRF response is the same whether an element is dissolved in solution or constitutive of a particle, provided that the size of the particle is very small, and that nanoparticle concentration is not too high.

Another important requirement of TXRF is the necessity to use an internal calibration for quantification purpose. Indeed, without internal calibration, no linear calibration curve could be obtained, even when homogeneous conditions of sample deposition were used prior to TXRF analysis. This is why internal calibration was performed using Rb. The choice of Rb resulted from the necessity to use a non-toxic element which is not ubiquitous in the environment, not used as nanoparticles, and with fluorescence lines distinct from those of Zn. With internal calibration, a linear calibration curve was obtained and it was confirmed that this calibration was similar whether Zn was analysed as ZnO in water or dissolved in acidic conditions (see Figure 3). The only restraint for quantification was the necessity to use low concentration samples because the linearity is deteriorated when using concentrated samples. This is in line with general recommendation for TXRF which is a technique that was first developed for ultra-trace analyses in the field of micro-electronics [17].

Concerning the sensitivity of the method (1 µg of ZnO collected on the paper flag), a rapid calculation was done to assess the magnitude of this value. Of course, the result has to be considered with precaution due to all the approximations it includes. First, we considered the occupational exposure limit of 0.1 mg/m³ proposed by NIOSH for TiO₂ on a working week of 40 hours (8 hours per day). Then, we applied this value for ZnO, and we considered the respiratory flow rate of an adult (11 L/min) on an 8 hour post shift. According to the CIPR model, the extra-thoracic fraction of nanoparticles deposited after inhalation is between 5 and 80%, depending on the size of the nanoparticles, which finally leads to 26 to 422 µg of ZnO deposited in the extra-thoracic part of the respiratory apparatus. We could therefore assume that at least 2 µg would be deposited in the nostrils (two times the detection limit, the sampling efficiency being around 50%). Of course, this assumption will have to be verified in the future with adequate studies.

As a conclusion, the developed method has the main advantage to be suitable for several kinds of nanoparticles, even those which are difficult to dissolve, like TiO₂ for instance. Nanoparticles are specifically detected according to their chemical composition. One resulting drawback can be that no information on the morphological characteristics of the nanoparticles is obtained, but in the case of positive detection with TXRF analysis, the second flag obtained in the second nostril could be used for scanning electron microscopy. Otherwise, the second flag could be used to perform a more sensitive TXRF analysis.

Also, it has to be kept in mind that this type of sampling can not be used for quantitative dose assessment. The sampling efficiency determined on glass slides should be different in the nose, and the sampling by itself is non-quantitative. However, the purpose of the method is to propose a qualitative indicator of potential inhalation which is a first step in the identification of exposure scenarios.

One limitation of the TXRF method is that some elements can not be analyzed by TXRF such as carbon, and some elements present a poor sensitivity, including alumina and silica. Other methodologies should be developed in the future for these types of nanoparticles. Improvements of the current method could also be envisioned such as the lowering of the limit of detection, or the improvement of the sampling device in terms of sampling efficiency.

The next step will consist in applying the method to other engineered nanoparticles, and then, in the validation of the method on non-exposed volunteers in order to determine the background levels in the population at large. This method provides a qualitative indicator of inhalation that could be useful in the case of accidental scenarios to verify the absence, or not, of any exposure.
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