The use of PIXE for engineered nanomaterials quantification in complex matrices

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Abstract. Engineered nanomaterials (ENMs) quantification in complex media is an area under development, much demanded by stakeholders due to the introduction of a myriad of consumer products containing ENMs. In this work, Particle-Induced X-ray Emission (PIXE) will be shown as capable of quantifying ENMs in complex media for both in vivo and in vitro assessments using minimal conditioning. ENM quantifications (SiC, TiC, SiO2) were performed on complex media (rat feces, rat lungs and cell culture), with applied ENM concentrations corresponding to ranges of interest for in vivo or in vitro assessments. The case studies presented in this work show the capability and versatility of PIXE measurements for biopersistence, biodistribution and dose assessment studies.

1. Introduction
With the advent of engineered nanomaterials (ENMs) discovery and their fundamental properties different to those of bulk materials, there has been a rush in the industry to produce and use these new materials in different products, touching wide and diverse areas such as semiconductors [1], medicine [2], cosmetics [3], and food [4]. Correspondingly, stakeholders have raised health concerns due to the unprecedented progress uncoupled with a proper understanding of the possible associated risks. Reports point out that ENMs may be more toxic than their larger counterparts [5]. An important area is ENM quantification, not only in ideal conditions (i.e. ENMs dispersed in ultrapure water) but rather in complex media, should it be for biopersistence, biodistribution or dose assessment purposes.

The current recommendations about the definition of a nanomaterial [6] considers them to be anything with a dimension lower than 100 nm and with a greater than 50% ratio in number in a product. A current challenge in the nanosafety field is the quantification of ENMs, especially when reaching small sizes such as 20 nm or less [7]. In this regard, ion beam techniques, particularly Particle-Induced X-ray Emission (PIXE), can overcome this challenge given that an ion beam interacts on an atomic scale basis. PIXE requires a minimal sample conditioning and yields a detection limit down to a few ppm [8-10]. In addition, special set-ups allow to probe the local concentrations on the...
micron range, and therefore build spatial elemental composition maps with micrometre lateral resolution [9, 11]. Non-vacuum PIXE is also possible, making this technique suitable to analyse the contents of NPs in liquids [12, 13].

The aim of this work is to present case studies of ENM quantification by PIXE in complex media: rat feces and lungs exposed to ENMs, and ENMs dispersed in cell media and ultrapure water. The presented case studies are part of larger studies focusing on the nanotoxicology or fate of ENMs.

2. Materials and methods

2.1. Engineered Nanomaterials
SiC and TiC ENMs were purchased from Ionic Liquids Technologies GmbH & Co. KG, Germany [14, 15] and used as received. Mesoporous SiO$_2$ ENMs synthesized via sol-gel were supplied by Nanologica$^\text{TM}$ through the NanoValid project (FP7 2007-2013).

2.2. Sample preparation

2.2.1. Rat feces. Female Sprague–Dawley rats (Charles River, France) were orally administered with 50 mg/kg of SiC or TiC ENMs in an acute assessment. The administration was done following the OECD (Organization for Economic Cooperation and Development) 420 guideline for acute toxicity study of chemicals [16] using tap water as delivery media. Feces were collected 24 hours later. The procedure of feces preparation for PIXE analysis has been described previously [10]. Briefly: feces were dried in a 60 °C oven for 24 hours; chromium nitride (Cr$_2$N) powder (Goodfellow, 99% purity, 45 µm particle size) was added as 7-10 % of the feces weight. Both feces and Cr$_2$N powder were ball milled to produce a homogeneous powder mixture. This mixture was hard pressed into a 2 cm diameter, 1-2 mm thick pellet. Chromium was added as an internal standard; it is not present in the biological matter and does not interfere in the PIXE measurements.

2.2.2. Rat lungs. Female Sprague–Dawley rats (Charles River, France) were exposed to a nanoaerosol of SiC ENMs in an acute assessment during 6 hours and sacrificed 6 hours later. The exposure was done according to the OECD 403 guideline for chemicals [17]. The lungs were prepared into pellets following the previously described procedure [10]. Briefly: lungs were dried in a 37°C oven for 24 hours, then they were froze dried [18] and chromium nitride (Cr$_2$N) powder (Goodfellow, 99% purity, 45 µm particle size) was added as 7-10 % of the feces weight. Both lungs and Cr$_2$N powder were ball milled to produce a homogeneous powder mixture. This mixture was hard pressed into a 2 cm diameter, 1-2 mm thick pellet. Chromium was added as an internal standard; it is not present in the biological matter and does not interfere in the PIXE measurements.

2.2.3. Cell media and ultrapure water. SiO$_2$ ENMs, at a 20 µg/mL concentration, were dispersed in minimum essential medium (MEM, Gibco), containing 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (BioWhittaker). These ENMs were also dispersed in ultrapure water at the same concentration. A drying method was used to prepare the samples and has been described elsewhere [19]. Briefly: ten 20 µL droplets were placed in a sample holder and each droplet was left to dry during one hour in a 60 °C oven before placing the next droplet.

2.3. Particle-Induced X-ray Emission (PIXE)
PIXE measurements were performed with the University of Namur ALTAÏS accelerator. PIXE is an ion beam technique, its physical principles can be found extensively explained elsewhere [9]. Briefly: ion-matter interactions occur on an atomic scale basis, meaning that, ion beam techniques like PIXE are not limited by particle size in an ENM quantification context. At the same time, ion beam techniques cannot separate particles sizes, but can be coupled with other size distribution techniques.
like Centrifuge Liquid Sedimentation (CLS) to provide precise quantification and size distribution in toxicological assessments [10].

Ion-matter interactions can produce several phenomena, amongst which characteristic X-ray emissions for each element present, due to the disturbance in its electronic shell. In this disturbance, an electron emitted from a shell in the atom is then replaced by an electron from an upper shell. During this transition, the difference in energy between the shells is emitted as an X-ray. The X-ray yield of a specific Z element is described by the following equation [9]:

\[
Y(Z) = QHe^\varepsilon_i t_Z C_Z Y_t(Z)
\]  

(1)

where Q is the accumulated charge from the ion beam, H is the solid angle, \(\varepsilon_i\) is the detector intrinsic efficiency, \(t_Z\) the transmission through any absorber, \(C_Z\) is the concentration of element Z, and \(Y_t(Z)\) is the theoretical X-ray yield production of element Z. The GUPIXWIN software was used to analyze the PIXE spectra [20]. The measurements calibration was validated using two standards from the International Atomic Energy Agency [21, 22] and a standard from the Institute of Reference Materials and Measurements [23] following an H-value adjustment methodology [24].

PIXE, as an ENM quantification tool, offers several advantages: concurrent multi-element acquisition in a single measurement, minimal sample preparation when compared to other techniques, parts per million (ppm) levels sensitivity, fast measurements (a few minutes per measurement). Also, when compared to similar techniques like energy dispersive X-ray (EDX) spectroscopy, PIXE has a higher sensitivity due to lower background noise [10]. In addition, a high volume sampling for solid samples is achieved by the use of a rotating stage providing a total scan area of 140.5 mm\(^2\) [10].

In order to have fully quantitative PIXE measurements, a precise knowledge of the major elements present in any sample is needed. This means the knowledge of the carbon (C), oxygen (O), and nitrogen (N) contents for biological samples. This matrix of elements acts as an attenuator for X-ray emission. In our setup, all PIXE measurements are recorded simultaneously using Rutherford Backscattering Spectrometry (RBS), a technique that relies on backscattered ion beam energy loss due to its interaction with the samples. RBS can precisely quantify light elements like C, O, and N, whose values are then used as the main matrix for PIXE analysis. In the case of feces and organs, the amount of Cr\(_2\)N is added as part of the matrix and represents 7-10 % of the biological matter weight. This kind of methodology is known as ‘Total Ion Beam Analysis’ [25].

The geometry setup for the reported measurement was the following: with respect to the beam direction, the samples are tilted at 45°, a Canberra LEGe (Low Energy Germanium) detector is located at 90° for PIXE measurements, and a Canberra PIPS detector is positioned at 145° for RBS measurements. The current PIXE detector setup is suitable for X-ray measurement on elements of Z\(\geq13\) (sodium and heavier elements). The PIXE detector is calibrated with a \(^{57}\text{Co}\) source and the RBS detector is calibrated with a SnO\(_2\) thin film on glass. The used ion beam was composed of protons, although it is possible to work with alpha particles [9]. Two sets of parameters were used: Protons of 2 MeV and an aluminum collimator for the LEGe detector of 3 mm in aperture and 1 cm thick, or protons of 2.5 MeV and an aluminum collimator for the LEGe detector of 1 mm in aperture and 0.2 mm thick.

3. Results and discussion

3.1. Oral administration of SiC or TiC to rats

Within the framework of possible effects to SiC or TiC ENMs exposure, the oral exposure route was carried on. One of the purposes was to assess the ENMs biodistribution in feces and the possibility of crossing the intestinal barrier [10]. Figure 1 shows the PIXE spectra of feces from control rats and rats administered with 50 mg/kg of either SiC or TiC ENMs. Table 1 summarizes the ENM quantification in both cases and their expulsion rate on day 1. Figure 1 highlights the ability of detecting the Si-K\(\alpha\) or Ti-K X-rays coming from the SiC or TiC ENMs respectively. In a single measurement, all the elements present in the feces were measured, a feature which allows matrix changes evaluation due to
the presence of ENMs. PIXE showed that feces composition is altered due to orally administered ENM [10].

![Figure 1](image.png)

**Figure 1.** PIXE rat feces spectra from oral administration studies: (a) control, (b) SiC administered at 50 mg/kg, (c) TiC administered at 50 mg/kg. Chromium (Cr) is due to the standard added to the feces. It is clear to the eye that the increase on Si-Kα and Ti-K X-rays yield for the feces where rats were instilled with SiC or TiC respectively.

**Table 1.** Administered ENMs, quantity found in feces and expulsed amount. The administered quantity is based on rat weight average (n=3). The amount found in feces subtracted the background Si or Ti signal found in control feces. The expulsed amount is the average ENM ratio found in feces with respect to the average administered quantity.

| ENM administered | Quantity administered (ppm) | Amount found in feces 1 day later (ppm) | Expulsed amount on day 1 (%) |
|------------------|-----------------------------|--------------------------------------|-----------------------------|
| SiC              | 13000 ± 209                 | 9652 ± 604                           | 74.25                       |
| TiC              | 10250 ± 700                 | 5932 ± 35                            | 57.87                       |

### 3.2. SiC nanoaerosol exposure to rats

The respiratory pathway is one of the most important routes of exposure to ENMs [26]. A whole body exposure system for rodents has been developed and validated at the University of Namur, using SiC ENMs as the produced nanoaerosol [27]. Lungs from control and exposed rats are shown in Figure 2. SiC ENMs presence in the exposed lungs was clearly seen as a raise in the Si-Kα X-ray yield, showing a load of 1413 ± 477 ppm (n=3) of SiC ENMs in the lung 6h after exposure. The elemental lung composition was not affected by the presence of SiC ENMs.
Figure 2. PIXE spectra of rat lungs from inhalation studies: (a) control, (b) exposed to SiC nanoaerosol. Chromium (Cr) is due to the standard added to the lungs. The presence of Si-Kα X-rays is seen only for the exposed lungs.

3.3. ENM dispersion into cell media and ultrapure water

ENM quantification in cell media is important as a mean to understand cell-nanoparticle dynamics, especially with incubation times where the quantity of ENMs present in the media can vary with time [28]. Firstly, the drying method route was preferred to a droplet measurement, given that the detection limits can be greatly improved. For example, taking the case of SiO$_2$ ENMs dispersed in water, the limit of detection (LOD) was estimated in 500 µg/mL using a droplet measurement methodology [13]. In a recent set of studies 20 µg/mL of SiO$_2$ were dispersed in ultrapure water and was measured by PIXE using the drying method (see Figure 3). The estimated LOD was 435 ng/mL, i.e. ~3 orders of magnitude lower than using the droplet methodology. This dramatic improvement in the LOD is due to 2 factors: the lack of other elements generating a high background noise like argon in air, and the fact that the matrix is evaporated using the drying method and thus leaves only ENMs for measurement. Given the ion beam penetration depth, a proton beam of 2 MeV penetrates 76.92 ± 2.98 µm of water [29], the drying method is preferred when going to low concentrations. The droplet measurement methodology still remains relevant when droplets cannot be dried (i.e. oils) or when the drying method may aerosolize the ENMs (i.e. a very volatile matrix). While the matrix effect is not important when analyzing ENMs on droplets of different matrices [13], it is an important issue in the drying method for complex matrices which partially evaporate. For example, this is the case for cell media where proteins remain when dried. In a recent study of the dynamics of ENMs in cell media, it was found that 20 µg/mL dispersed in MEM present a LOD of 16.2 µg/mL [19]. While this result was clearly not achievable using the droplet method, it is still two orders of magnitude higher from the same concentration measurement in a fully evaporable matrix. Table 2 summarizes the SiO$_2$ ENM value measured in water using both methods and in using the drying method in MEM.
Figure 3. PIXE spectrum of SiO$_2$ ENMs measured using the drying method. Dispersion was done in H$_2$O at a 20 µg/mL concentration.

Table 2. Summary of SiO$_2$ ENM detection using the droplet or drying method dispersed on H$_2$O or MEM. The detection limit (LOD) of each measurement is included.

| Preparation method | Matrix | Measured concentration (µg/mL) | LOD (µg/mL) |
|--------------------|--------|-------------------------------|-------------|
| Droplet            | H$_2$O | 750                           | 500         |
| Drying             | H$_2$O | 20                            | 0.435       |
| Drying             | Mem    | 20                            | 16.2        |

In conclusion, case studies of ENM quantification in complex media by PIXE were presented, where minimal conditioning for either solid or liquid sample was required. These examples show the capability and versatility of PIXE measurements for biopersistence, biodistribution and dose assessment studies.

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