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Detection of titanium dioxide particles on frozen tissue sections using synchrotron radiation X-ray fluorescence analysis

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Received March 25, 2011, accepted May 2, 2011

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Pharmazie 65: 808–809 (2011)
doi: 10.1691/ph.2011.1041

Recent studies into the *in vivo* absorption and biological influence of particulate matter, especially nanomaterials (NMs), have raised worldwide concerns over their safety. However, it is often technically difficult to conduct these studies because NMs are too small to be observed by optical microscopy. Here, we attempted to establish a new method to visually detect NMs on tissue samples. Specifically, we have analyzed titanium dioxide particles with a diameter of 5 μm, which are widely used in cosmetics, using frozen tissue sections by synchrotron radiation X-ray fluorescence analysis.

With the recent development of the nanotechnology, nanomaterials (NMs) have been successfully employed in various industrial applications such as medicine, cosmetics, and food (Kaur et al. 2007; Cormode et al. 2010). NMs display a number of useful properties, such as high levels of electrical conductivity, tensile strength, electronic reactivity and tissue permeability, which are not evident in bulk materials (Rutherglen et al. 2009). However, recent studies suggest that NMs may pose a serious risk to human health, which differs from the toxicity of bulk materials (Nel et al. 2006; Shvedova et al. 2010). For assessing the safety of NMs, it is very important to identify their location within the body. In particular, NMs show unique dynamics that allow them to penetrate biological barriers (Nabeshi et al. 2011). Microscopic studies using transmission electron microscopic (TEM) analysis have been used by various researchers (Jin et al. 2008; Chen et al. 2010; Zhang et al. 2010). Many research groups are attempting to develop imaging techniques that will facilitate the visual detection of NMs in tissue samples. Such an approach is expected to greatly assist in the assessment of the biological effects of NMs.

Here, we describe the development of a method to detect titanium dioxide (TiO_2) using frozen sections by synchrotron radiation X-ray fluorescence (SR-XRF) analysis sourced from a synchrotron radiation facility (SPring-8 in Japan). Nanometer sized TiO_2 particles are widely employed in cosmetic products. Here, we used TiO_2 particles with a diameter of 5 μm as a model material for our investigations. Firstly, we observed TiO_2 particles on a frozen section of mouse lung using an optical microscope. We prepared the lung sample by injecting a large dose of TiO_2 into the tail vein of mice. As expected, the agglomerates of TiO_2 particles were observed as black areas (Fig. A). Subsequently, we conducted SR-XRF on the same section in order to verify that SR-XRF could detect titanium (Ti) at the corresponding location (see photomicrograph in Fig. B). These results indicate that SR-XRF is useful for detecting TiO_2 particles in frozen tissue sections.

SR-XRF-mediated detection of TiO_2 particles is a novel technique that has several advantages as described below. Firstly, our method is highly versatile given that SR-XRF can more readily detect elements as their atomic number increase. Thus, NMs such as nano-platinum, nano-silver and nano-zinc oxide will be easily detected by this method. Furthermore, our technique facilitates a direct correspondence between the localization of NMs in the tissue and pathological findings in the tissue samples. However, unlike TEM analysis, our method cannot observe the actual appearance of single NMs in the tissue sample. Moreover, unlike inductively coupled plasma mass spectrometry (ICP-MS), our method cannot quantify the amount of NMs in the tissue sample. It must be emphasized that this study examined the utility of SR-XRF for the detection of particulate matter on frozen tissue sections using only micro-TiO_2 particles. Therefore, further studies using nano-TiO_2 are required. Nonetheless our new method, in conjunction with established complementarily approaches such as TEM or ICP-MS, is expected to provide valuable information concerning the safety of NMs.

In conclusion, we present a new method for detecting and localizing nano-TiO_2 in frozen tissue sections. Although further investigations are needed, we believe our novel technique will complement conventional methodologies to help assess the biological effects of NMs.

Experimental

1. TiO_2 particles

Rutile TiO_2 with a diameter less than 5 μm was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TiO_2 particles were dissolved in solvents and subsequently sonicated for 10 min and vortexed for 1 min prior to use.

2. Animals

Female BALB/c mice were purchased from SLC, Inc (Shizuoka, Japan) and used at 7 weeks of age. All of the animal experimental procedures used in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals.

3. Preparation of tissue samples

For the detection of TiO_2 in lung, BALB/c mice were treated with 2 mg/mouse TiO_2 in PBS *via* intravenous injection into the tail. Several minutes after injection of TiO_2 the lung sample was collected. Lung samples were frozen in O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan) by liquid nitrogen and cut into 20 μm thick sections using a cryomicrotome (Leica Microsystems Japan, Tokyo, Japan) and laid on a Kapton® film.

4. SR-XRF

Tissue sections were observed by optical microscopy. Images were captured in order to determine the area under investigation. Subsequently, SR-XRF was performed using precisely the same area of tissue section. Experiments were carried out at BL37XU of SPring-8, Hyogo, Japan (Terada et al. 2004). The sections were subjected to two-dimensional SR-XRF. The incident beam was monochromatized with a Si (111) double-crystal monochromator to an energy of 10 keV. The monochromatized beam was focused by total-reflection mirrors with Kirkpatrick–Baez configuration into a 0.9 (horizontal) × 1.1 (vertical)-μm^2 microbeam at the sample position. The samples were set on the XY stage and irradiated with this beam. The fluorescence X-rays of the Ti-Kα lines generated from the samples were

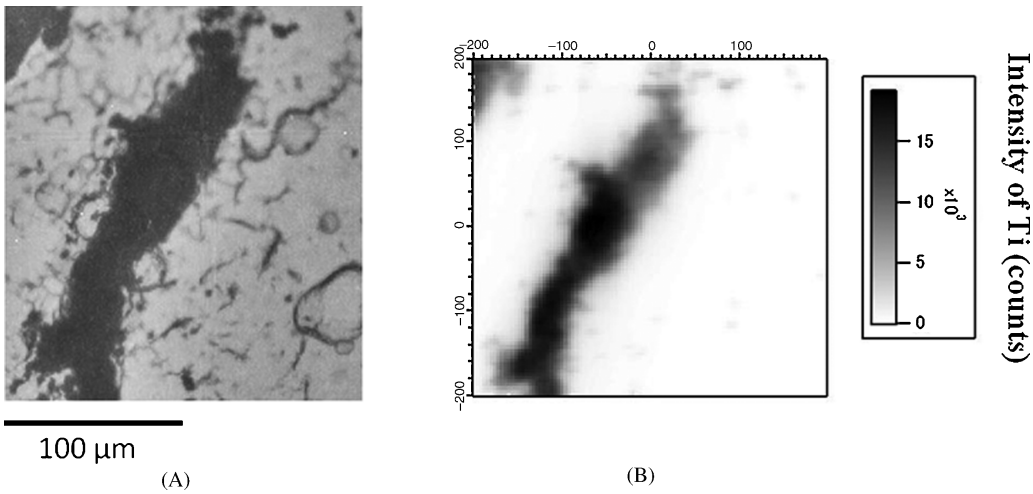

Fig.: Optical microscope image and the 2D-image of Ti distributions in the frozen section of lung. BALB/c mice were treated with 2 mg/mouse TiO_2 in PBS *via* intravenous injection. After sacrificing the mice, lung samples were collected and observed by optical microscopy (A) and SR-XRF (B). The imaging area was 40000 μm^2. Black areas observed in the microscopic image correspond to Ti.

measured by a silicon drift detector positioned perpendicular to the incident X-ray beam. The 2D-imagings of Ti distributions in the samples were obtained by scanning the sample stage along the x-y axes during fluorescent X-ray detections. The measurement time was 0.1 s/step. The scanning range was tuned to 200 μm^2 with 2 μm steps. The images were stored as digital data. These data were restored to images by IGOR Pro (WaveMetrics, OR).

Acknowledgements: This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Japan Society for the Promotion of Science (JSPS). This study was also supported in part by Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan; by Health Sciences Research Grants for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation; by a Global Environment Research Fund from Minister of the Environment; and by a the Knowledge Cluster Initiative; and by Food Safety Commission; and by The Nagai Foundation Tokyo; and by The Cosmetology Research Foundation; and by The Smoking Research Foundation; and by The Takeda Science Foundation. The synchrotron radiation experiments were performed under the approval of SPring-8 Proposal Review Committee (Proposal No. 2009B1036).

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