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Title	Behavior of in vitro, in vivo and internal motion of micro/nano particles of titanium, titanium oxides and others
Author(s)	Koyama, Chika; Akasaka, Tsukasa; Esaki, Mitsue et al.
Citation	Journal of the Ceramic Society of Japan, 116(1349), 1-5 https://doi.org/10.2109/jcersj2.116.1
Issue Date	2008
Doc URL	https://hdl.handle.net/2115/59708
Type	journal article
File Information	Yonezawa-JCSJ116_1.pdf



Behavior of in vitro, in vivo and internal motion of micro/nano particles of titanium, titanium oxides and others

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To clarify the effect of micro/nanosizing of materials onto biological organism, the particle size dependence of reaction of cells and tissue as investigated by both biochemical cell functional test and animal implantation test. Especially for nanoparticles the behavior of invasion and internal diffusion inside body was visualized using an XSAM (X-ray Scanning Analytical Microscope). The increase of specific surface area is usually counted as nanosizing effect which causes the enhancement of chemical reactivity and therefore toxicity of materials such as carcinogenicity found in 500 nm Ni particles for the long term implantation in the soft tissue of rat. Even biocompatible materials such as Ti and TiO₂ shows stimulus with the decrease of particle size. They cause phagocytosis to cells and inflammation to tissue when the size of particles is below 3 μ m. For the size below 50 nm, they may invade into the internal body through the respiratory or digestive system and diffuse inside body. After compulsory exposure test of 30 nm TiO₂ particles through the respiratory system, the Ti mapping by XSAM showed the internal diffusion inside the whole body. Nanoparticles injected from caudal vein diffused with time course to lung, liver and spleen. The uptake of 30 nm TiO₂ particles through the digestive system and diffusion into these organs was also confirmed. These phenomena observed in biocompatible or bioinert materials are the nonspecific, physical particle and shape effects which occur independent of materials. Nanoparticles might be the objects whose existence has not been assumed by the living body defense system.

Key-words : Nanoparticle, Phagocytosis, Inflammation, Internal diffusion, Biocompatibility, Nanotoxicology, Cytokine, Size effect

[Received October 15, 2007; Accepted November 18, 2007] ©2008 The Ceramic Society of Japan

1. Introduction

1.1 Development of nanotechnology

Nanotechnology has been intensively developed and applied in the fields of electronics, chemistry and others. For materials nanosizing brings in quantum effect for less than about 1.5 nm and the formation of activity points such as contained in some catalysts. However the most unambiguous and influential effect is the surface area effect. It is well-known that the specific surface area which is defined as surface area for unit volume is increased with the decrease of particle size and chemical reactivity is pronounced. Therefore high throughput is expected in the functions of material properties and performances of devices. In these developments it is usual that the merit side is emphasized and demerit is neglected.

1.2 Necessity to establish principle for biomedical application

However it is natural that demerit appears as well as merit since merit and demerit are dependent on whether they work as usefulness or not for the purpose of human beings, although both originate from the same mechanism. Especially for the biomedical application of nanotechnology it is necessary to elucidate the phenomena and establish the proper principle in advance to step out to human application. The reaction of biological organism to proteins and saccharides including virus, bacteria, enzyme, and pharmacological agents has been investigated in biology and medicine. For materials the reaction to the usual cases, that is, the macroscopic size is well investigated. But the reaction to the micro/nano sizing is not so clear.

1.3 Chemical reactivity enhancement effect

One of the most important factors to affect on the biocompatibility of materials in macroscopic size is ionic dissolution and this is also true for micro and nano size. This is closely related to the specific surface area and becomes apparent in most cases as stimulus or toxicity to nanosizing. Ni is known to cause allergy in macroscopic size. We found that 500 nm Ni particles cause the formation of tumors after one year when implanted in the soft tissue of rats. This is the typical example and nanosizing effect onto biological organism has been usually interpreted from this aspect.

1.4 Stimulus effect in non-soluble materials

On the other hand corrosion-resistant and biocompatible Ti causes inflammation in abraded fine particles^{1),2)} which are produced from artificial joint, and asbestos,³⁾ a kind of clay minerals, induces mesothelioma after a long-term, large quantity of exposure. These phenomena cannot be explained by the specific surface area effect and understood as the different effect from the material properties of either toxicity or biocompatibility, that is, physical size and shape effect. The abraded fine particles may diffuse inside the body through the cardiovascular system. There is also the possibility that the uptake of nanoparticles occurs through the respiratory and digestive systems.

These strongly suggest the necessity to reveal the micro/nanosizing effect other than the specific surface area effect,⁴⁾ such as the biological reactivity of micro/nanoparticles and their internal dynamics.

1.5 Nanotoxicology and DDS originated from internal particle diffusion

Meanwhile Drug Delivery System (DDS) is one of the most typical biomedical applications of nanoparticles. The development of DDS is expected for the administration of anticancer agent and gene transfection. The behavior of nanoparticles in the internal body is necessary to investigate for the assessment of nanotoxicology and this is, in turn, essential to comprehend the diffusion path of DDS to reach the diseased target. Thus internal diffusion is significant from both demerit and merit aspects of nanotechnology.

1.6 Purpose

In the present study both biochemical cell functional test and animal implantation test were done to clarify the particle size dependence of reaction of cells and tissue, and micro/nanosizing effect with the primary attention focussed on non-soluble materials such as Ti and TiO₂.^{5,6)} In addition for nanoparticles, the behavior of invasion and internal diffusion inside body was visualized using XSAM (X-ray Scanning Analytical Microscope)^{7,8)} for the level of the whole body and organs.

2. Materials and methods

2.1 Specimens

99.9% pure Ti, and TiO₂ particles of the various size were principally used throughout. For in vitro and in vivo implantation tests Fe, Ni, TiO₂ and carbon nanotubes^{9,10)} were also used. The particles of nominal size from 500 nm to 150 μ m were used for Ti. Usually these contain the size distribution to the considerable amount. To reduce the size distribution as small as possible and equalize the experimental conditions among materials such as metallic Ti, Fe and Ni, the particles of 0.5, 3, 10 μ m were extracted by sedimentation method and those less than 300 nm were extracted by ultrafiltration from particle powders of nominal size.

2.2 Dissolution test of Ti particles

After Ti particles were immersed in HBSS (Hanks balanced salt solution) at 37°C for 1 month, the supernatant was filtered through a 0.45 μ m membrane to remove Ti particles and then elemental analysis was done by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using ICPS - 8100, Shimadzu, Tokyo.

2.3 Biochemical analyses of cellular reaction to materials

Human neutrophils, which play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies, were used as probe cell. Particles smaller (0.5, 3 μ m) and larger (10, 50, 150 μ m) than neutrophils were used to determine the relationship between cell and particle size with respect to cytotoxicity.

Cell survival rate and lactate dehydrogenase (LDH) values, superoxide anion (O₂⁻) production per 10⁶ neutrophils were measured. Cytokines of TNF- α and IL-1 β were measured using ELISA kits (Endogen, Inc. USA). Morphological change of neutrophils mixed with HBSS containing various particles was observed by optical microscopy (OM: Zeiss, Axioskop, Germany) and scanning electron microscopy (SEM: Hitachi S-4300, Tokyo).

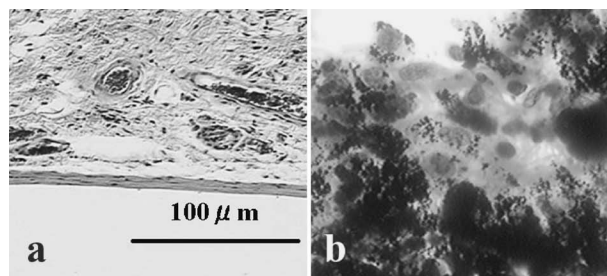


Fig. 1. Comparison of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μ m Ti particles (b) by histological observation.

2.4 Animal experiments

Particles were inserted in the subcutaneous connective tissue in the abdominal region of Wistar rats aged between 11 and 12 weeks (weight 350–380 g). Specimens were prepared through the usual process of fixation, embedding, sectioning, staining with hematoxylin-eosin, and then histopathologically observed.

2.5 Visualization of internal distribution of nanoparticles

The compulsory exposure test to the respiratory system was performed to rats using 30 nm TiO₂ particles. The uptake of nanoparticles through the digestive system was also tested for mice by mixing agar gelatin containing 30 nm TiO₂ particles to their foods. To inspect internal diffusion more simply, the experiments were done for mice by injecting nanoparticles directly to the cardiovascular system from caudal vein. The observation of internal distribution of nanoparticles was conducted for the whole body and each organ by elemental mapping in air using X-ray Scanning Analytical Microscope (XSAM: Horiba XGT-2000V, Tokyo) without the pretreatments of fixation, dehydration and staining after sectioning. The distribution inside the organ was inspected by elemental mapping using energy dispersive X-ray spectroscopy (EDS) installed to SEM. The experiments of internal diffusion were also done for the particles Ti, Fe, Ni, Pt, TiC, Fe₂O₃.

3. Results

3.1 Comparison of tissue reaction to macroscopic and nanosize materials

Figure 1 shows the histological observation of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μ m Ti particles (b) after 8 weeks, comparatively. For the macroscopic size, Ti implant was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk Ti. For 3 μ m Ti numerous inflammatory cells appeared. The macrophages and adjacent collagen show degenerative changes in morphology. Ti particles, observed as small black dots, were phagocytized into the cytoplasm by a macrophage.

3.2 Particle size dependence of cell reaction

Figure 2 shows the SEM images of human neutrophils in HBSS (Hanks balanced salt solution) (a) and exposed to 500 nm Ti particles (b). Figure 2b showed the neutrophil extending its pseudopod to phagocytize Ti particles for the size below 3 μ m. For the particles larger than 10 μ m, phagocytosis was not observed.

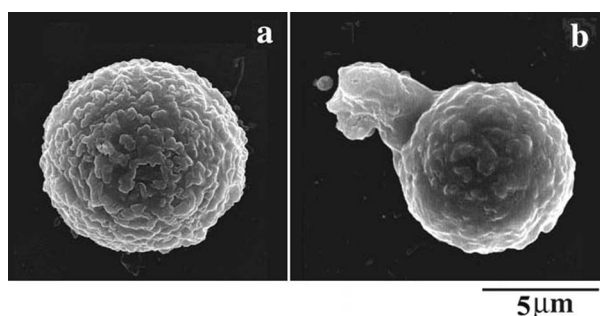


Fig. 2. SEM images of human neutrophils. a: control, b: exposed to particles of 500 nm Ti.

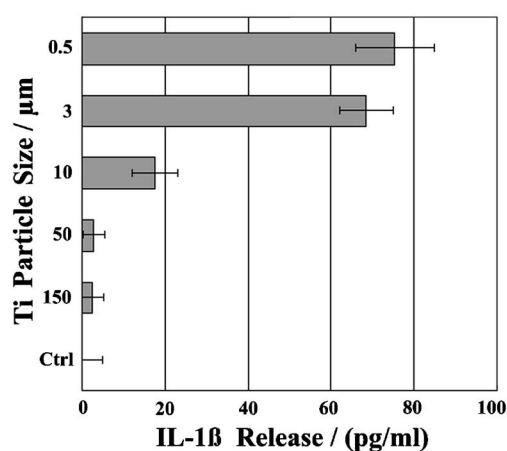


Fig. 3. Dependence of IL-1 β release from neutrophils on Ti particle size.

Figure 3 shows the amount of IL-1 β released from neutrophils in HBSS containing Ti particles. IL-1 β is one of the most representative cytokines of inflammation. IL-1 β showed the increase against the decrease of particle size. The increase was pronounced for 0.5 and 3 μm . The release of LDH, superoxide and cytokine TNF- α showed the similar behavior as IL-1 β , while cell survival rate showed the inverse decreasing tendency. ICP elemental analysis showed that the dissolution from Ti particles was negligible below detection limit. The pronounced phenomena of biochemical cell reactivity observed for the particle size below 3 μm in Fig. 3 are closely related to the phagocytosis shown in Fig. 2.

3.3 Particle size dependence of tissue reaction

The histological image of tissue reaction of rat to the different size of Ti particles for the long term implantation test showed the similar size dependence to those in vitro shown in Figs. 2 and 3. **Figure 4** is the tissue reaction to 10 μm (a) and 150 μm Ti (b) particles after 30 week implantation. For 150 μm Ti, each particle was surrounded by fibrous connective tissue layer, which is similar to the case of macroscopic Ti implant shown in Fig. 1a. Tissue reaction to 10 μm Ti was inflammatory where there was inflammatory cell infiltration as well as fibrous connective tissue formation.

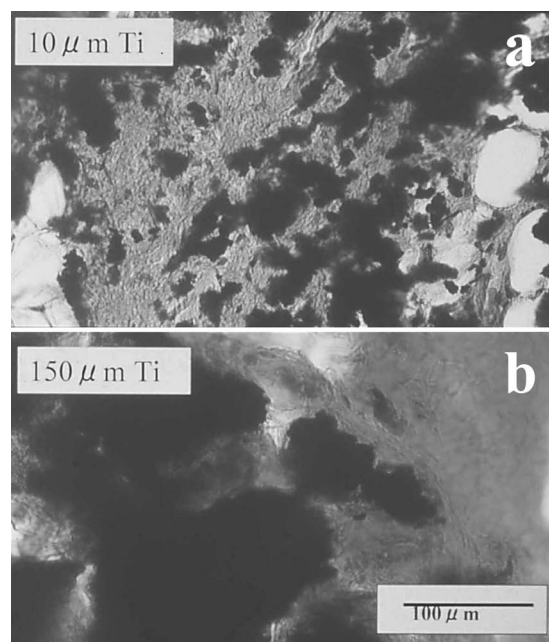


Fig. 4. Histopathological image of tissue reaction to 10 μm (a) and 150 μm (b) Ti particles after 30 week implantation.

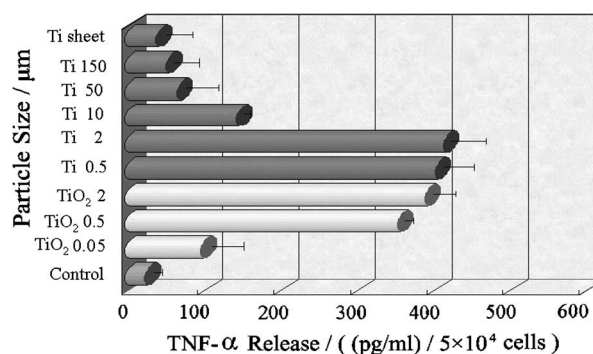


Fig. 5. Dependence of TNF- α release from neutrophils on particle size down to nm size.

3.4 Stimulus in nm size

Figure 5 shows the dependence of TNF- α release from neutrophils on particle size down to nm size. Stimulus, represented as amount of TNF- α release, which is pronounced below 3 μm , exhibited the maximum from around μm down to 500 nm, similar to the case of IL-1 β shown in Fig. 3, and then for further smaller size decreased below 200 nm. This means that the biophylactic system does not work well any more against the invasion of nanoparticles into the inside of body.

3.5 Internal diffusion of nanoparticles

Figure 6 is the Ti mapping of the internal whole body of rats by XSAM after compulsory exposure test to respiratory system, and reveals the distribution of 30 nm TiO₂ particles. The condensation occurred from the respiratory system to urinary bladder by diffusion in the body through the cardiovascular system after the direct uptake into blood

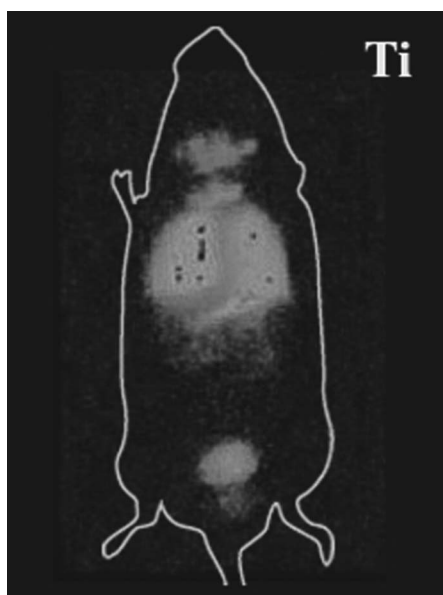


Fig. 6. XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test to respiratory system.

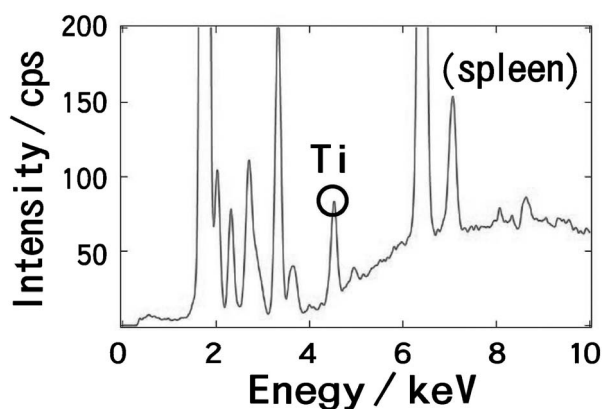


Fig. 7. Elemental analysis of spleen of mouse by XSAM after 10 days of oral administration of 30 nm TiO_2 particles.

vessels from lung cells.

Figure 7 is the XSAM elemental analysis from spleen for the case after 10 d of oral administration of 30 nm TiO_2 particles. Although peak height is small in this case, $\text{Ti-K}\alpha$ peak undoubtedly exists other than $\text{Fe-K}\alpha$ peaks around 6.5 keV and peaks of incident X-ray from Rh target below 4 keV. This confirms the phenomenon that nanoparticles were taken into the internal body through digestion system.

Figure 8 shows the X-ray transmission image and the corresponding Ti elemental mapping by XSAM for 5 min and 3 hr after injection of 30 nm TiO_2 particles to caudal vein. TiO_2 nanoparticles diffused to lung just after injected from caudal vein, then liver and spleen with time course.

Figure 9 shows the change of existence ratio of TiO_2 particles in each organ with time. Particles reach lung shortly after injection, then the content in lung decreases and the content in liver and spleen increases with time.

To observe the more detailed distribution of nanoparti-

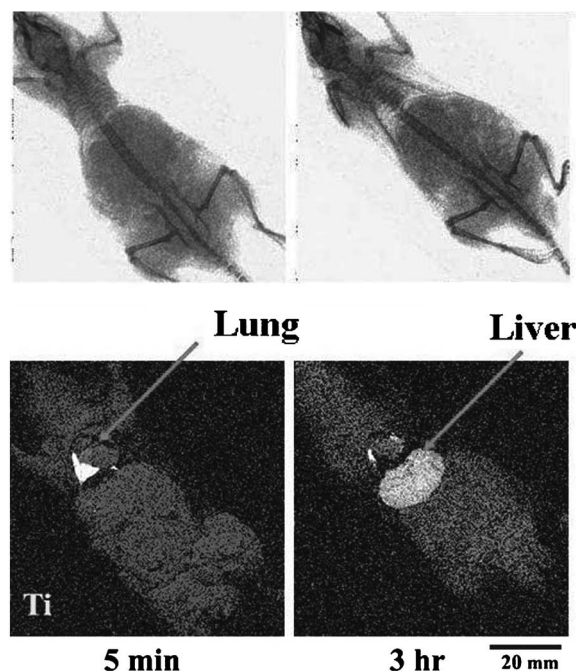


Fig. 8. Time course of internal diffusion of 30 nm TiO_2 particles after injection to caudal vein.

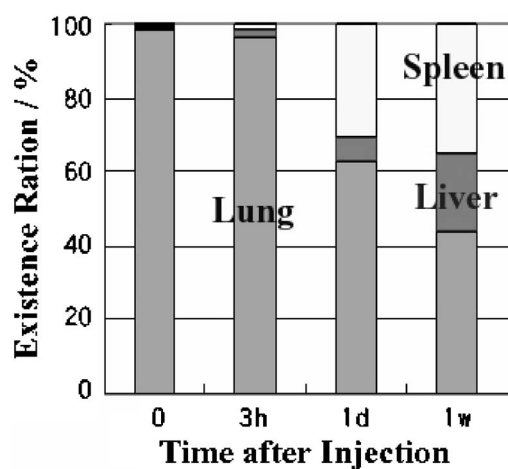


Fig. 9. Change of existence ratio of TiO_2 particles in each organ with time.

cles in each organ, EDS elemental mapping was applied. **Figure 10** is the SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein. The distribution is not uniform and in dotted manner.

4. Discussion

4.1 Particle size dependence of reaction of cells and tissue

Comparison of the reaction of tissue to the macroscopic Ti and 3 μm Ti particles in Fig. 1 showed clearly the micro/nanosizing effect on biological organism. Both biochemical cell functional test and animal implantation test showed the toxicity due to fine particles and its size dependence. Both

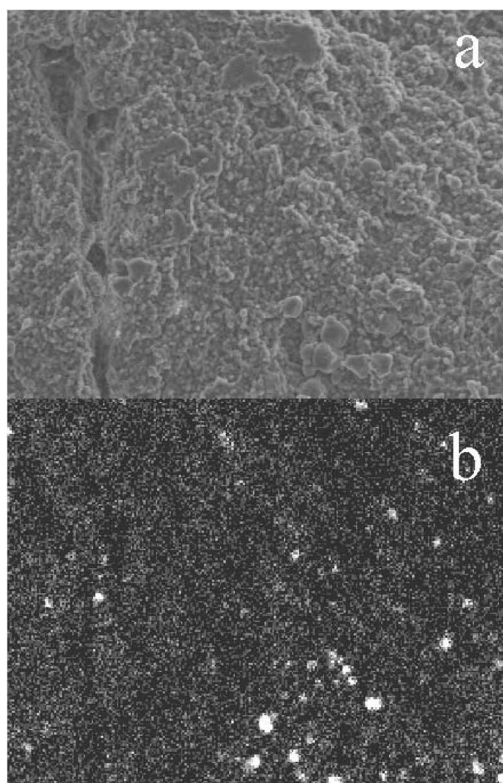


Fig. 10. SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein.

results of in vitro and in vivo are in accordance each other in their size dependence. Ti particles larger than approximately $100\text{ }\mu\text{m}$ was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk size of Ti implant.^{(11),(12)} As the particle size was smaller, stimulus was induced due to the physical size effect in the range less than about $100\text{ }\mu\text{m}$ as shown in Fig. 4. The inflammation was especially pronounced when the particle size was typically below $3\text{ }\mu\text{m}$ which is smaller than $10\text{ }\mu\text{m}$, about the cell size, where phagocytosis was induced.

These phenomena occur commonly in any bioactive and bioinert materials other than Ti, such as Fe and TiO_2 where particles induce nonspecifically phagocytosis to cells and inflammation to tissue for the size below $3\text{ }\mu\text{m}$. It is different from the usually observed toxicity due to the ionic dissolution effect in the macroscopic size.⁽⁴⁾

4.2 Stimulus in nm size

Nanosizing effect is usually interpreted by the increase of specific surface area, which pronounces chemical reactivity with the decrease of particle size. Effects related to the ionic dissolution correspond to this category, such as the acceleration of toxicity observed in Ni where tumor was generated in the long-term implantation for 500 nm particles, compared with necrosis occurred in short term for macroscopic size.

Specific surface area effect is based solely on the material properties, and indifferent from biological body, while physical particle size effect has the origin in the relative size relationship between particles and cell/tissue. Stimulus arises

by biological process which induces the occurrence of functionality of body defense system.

4.3 Internal diffusion of nanoparticles

Figure 5 shows that particles become less stimulative when the particle size becomes in the level of 50 nm or less and the recognition by body defense system becomes lower. The invasion of nanoparticles into the body occurs for this range of particle size. The present results showed both cases of uptake of nanoparticles through the respiratory (Fig. 6) or digestive system (Fig. 7). Figures 8 and 9 show that nanoparticles diffuse with time course to lung, liver and spleen after injection from caudal vein. SEM image and EDS elemental mapping in Fig. 10 show the distribution of TiO_2 nanoparticles inside the organ of spleen.

5. Conclusions

Particles cause nonspecifically phagocytosis to cells and inflammation to tissue for the size below $3\text{ }\mu\text{m}$. For the size below 50 nm particles may invade directly into the internal body through the respiratory or digestive system and diffuse inside body. Nanoparticles might be the objects whose existence has not been assumed by living body defense system. Thus the visualization of the internal dynamics of nanoparticles is essential for the proper treatments based on risk assessment and biomedical applications such as DDS. The present study could successfully visualize the internal diffusion of nanoparticles inside the whole body using XSAM.

Acknowledgements Present research was supported by Health and Labour Sciences Research Grants in Research on Chemical Substance Assessment of Ministry of Health, Labour and Welfare of Japan (H18-Chem-General-006).

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