Table 3. Effect of TiO_2 nanoparticles exposure on the skin sensitization.

		Fractional response*	Mean response	S.D.
24 h	Control	2/4	0.75	0.96
	DNCB	4/4	4.00	1.15
	Sudan I	4/4	2.00	0.82
	TiO_2 5%	3/4	0.75	0.50
	TiO_2 25%	1/4	0.25	0.50
48 h	Control	2/4	0.50	0.58
	DNCB	4/4	4.00	1.83
	Sudan I	4/4	1.75	1.50
	TiO_2 5%	4/4	1.75	0.96
	TiO_2 25%	2/4	0.50	0.58

*Fractional response (= positive/total)

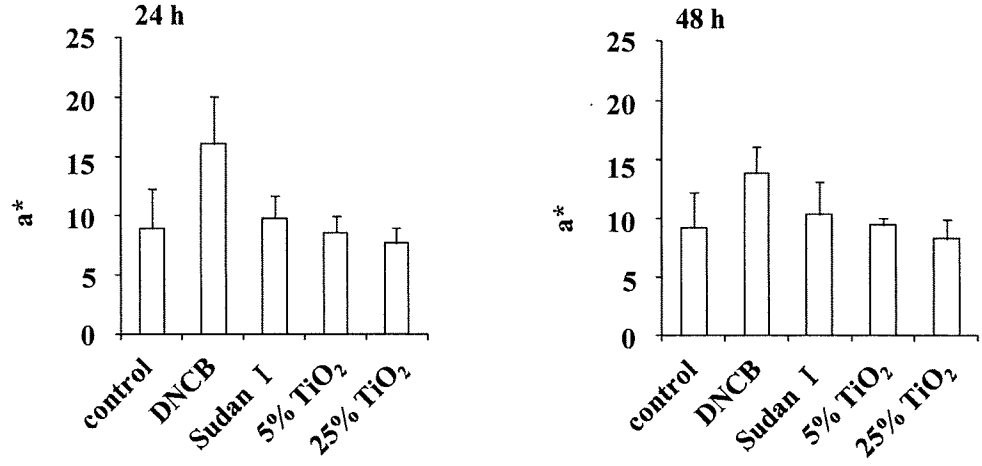

Fig. 6 Effect of TiO_2 nanoparticles exposure on the a* value.
Data are shown as mean ± S.D. (n = 4).

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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IV.　研究成果の刊行物・別刷

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Letter

Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin *in vitro*

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ABSTRACT — It is important for toxicological assessment of nanoparticles to determine the penetration of nanoparticle in skin qualitatively and quantitatively. Skin penetration of four different types of rutile titanium dioxide (TiO_2) (T-35, 35 nm, non-coating; TC-35, 35 nm, with almina/silica/silicon coating; T-disp, 10 x 100 nm, mixture of almina coated and silicon coated particles, dispersed in cyclopentasiloxan; T-250, 250 nm, non-coating) was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs to study the effect of dispersion and skin conditions. The TiO_2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose 2 μl/cm^2 for 24 hr, followed by cyanoacrylate stripping. The Ti concentration in skin was determined by ICP-MS. T-35 and T-250 easily aggregated in suspension with a mean diameter greater than 1 μm. TC-35 and T-disp showed good dispersion properties with a mean diameter in suspension of approximately 100 nm. No penetration was observed regardless of TiO_2 type in intact and stripped skin. The concentration of Ti in skin was significantly higher when TC-35 was applied on hair-removed skin. SEM-EDS observation showed that Ti penetrated into vacant hair follicles (greater than 1 mm below the skin surface), however, it did not penetrate into dermis or viable epidermis.

Key words: Nanoparticle, Skin penetration, Hair-removed skin, Stripped skin, Titanium oxide

INTRODUCTION

Titanium dioxide (TiO_2) has been used for cosmetics and is considered to be safe for topical use. Recently, TiO_2 nanoparticles (particle size < 100 nm) are used commonly in cosmetics because of their high transparency in visible wavelengths but high attenuation for UV wavelengths (Popov *et al.*, 2005). However, the safety of different conventional size particles is a concern. Safety concerns are based on not only its toxicity characteristics, but also on the potential increase in amount of absorption. In theoretical, materials with an appropriate octanol/water partition coefficient and low molecular weight (< *ca.* 500) penetrate skin through the stratum corneum (SC); therefore, penetration of inorganic particles into intact skin is not possible (Roberts *et al.*, 2002). Some studies indicate that TiO_2 and other inorganic particles, even on a nano-grade scale, do not penetrate skin (Schulz *et al.*, 2002; Pinheiro *et al.*, 2007; Nohynek *et al.*, 2008). However, some nanoparticles can penetrate viable skin (Ryman-Rasmussen *et al.*, 2006; Menzel *et al.*, 2004). Inorganic particles are often lyophobic in both water and oil, dispersed particles easily aggregate to form large (micro-grade) particles. A few studies investigating both dispersibility and skin penetration have been reported (Bennat and Müller-Goymann, 2000). The present study focused on skin penetration of TiO_2 *in vitro* with different dispersibility of TiO_2 and skin condition.

MATERIALS AND METHODS

Materials

All types of TiO_2 used in this study were rutile-type (Table 1). Cyclopentasiloxane (silicone, KF-995, Shin-Etsu Chemical, Co., Tokyo, Japan) was used as the dispersing medium. Purified water and nitric acid used in TiO_2 quantitative analysis were ultra-microanalysis grade from Wako Pure Chemicals Industries, Ltd. (Osaka,

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Table 1. Titanium dioxide used in this study

Abbreviation	Primary particle size*	Coating
T-35	35 nm	uncoated
TC-35	35 nm	alumina · silica · silicone
T-disp	10 nm x 100 nm	mixture of alumina coated and silicone coated
T-250	250 nm	uncoated

All TiO_2 are rutil-type.
* from catalogue of source company.

Japan). A stock solution of titanium containing 1,000 mg/l (Kanto Chemical Co., Inc., Tokyo, Japan) was used to produce standards for calibration curves for Ti analysis. All other chemicals were of reagent grade.

Preparation of suspensions

Drops of silicone were added to a weighed amount of TiO_2 powder in a tube, followed by kneading. Additional silicone was added to bring the concentration of TiO_2 samples to 10%, followed by sonication in a bath-type sonicator (US-3, Iuchi, Tokyo, Japan) for 30 min. The T-disp was diluted with silicone for a final TiO_2 concentration of 10%, followed by sonication.

Evaluation of TiO_2 suspensions

The particle size of TiO_2 in suspension was measured using a dynamic laser scattering apparatus (DLS-8000HL, Otsuka Electrons Co., Osaka, Japan) after a thousand-fold dilution with silicone.

Skin conditions after application of TiO_2 was observed using two methods. Two μl of suspension were applied to an area of skin of approximately 1 cm^2. After drying, the skin surface was observed by digital fine scope microscopy (VC-3000, Omron, Tokyo, Japan) with a magnification of 80. The epidermis of YMP skin prepared by a heat separating method (Kligman and Christophers, 1963) was mounted on a scanning electron microscopy (SEM) stage with adhesive tape. One μl of the suspension were spread over approximately 0.5 cm^2 and dried *in vacuo*. Then, the skin sample was coated with Pt/Pd and examined using SEM (JSM-5200LV, JEOL, 20 kV).

Skin penetration

Yucatan micropig (YMP) skin was used as the model because of its similarity with human skin (Lavker *et al.*, 1991; Fujii *et al.*, 1997). YMP skin (YMP skin set, Charles River Japan, Kanagawa, Japan) removed the subdermal tissue and fat was used as full-thickness skin (intact skin). The SC was removed from intact skin with adhesive tape (Scotch 313, 3M) (stripped skin). Hair was removed from intact skin using tweezers (hair-removed skin).

Two μl of suspension were applied to an area of skin of approximately 1 cm^2. Then the skin was placed on a modified Franz-type diffusion cell. After 24 hr, the receptor phase (pH 7.1 isotonic phosphate buffer solution) was collected, the skin was removed from the diffusion cell and cut off the rim for mounting the cell. Residues on the skin surface were removed by two cyanoacrylate (Aronalfa, Toagosei, Tokyo, Japan) stripping and Ti in the skin was determined. Application amount and period were in accordance with Standard SPF Test Method of Japan Cosmetic Industry Association (1999) and OECD (2004): Test Guideline 428 (skin absorption: *in vitro* method), respectively. For some samples, the epidermis and dermis were separated by heating after cyanoacrylate stripping. A similar procedure was used for obtaining SEM pictures with energy dispersed X-ray spectrometry (SEM-EDS).

Determination of Ti

Approximately 0.1 g of skin or 1 ml of receptor phase was transferred to a Teflon digestion vessel and 5 ml of nitric acid plus 1 ml of purified water was added to each vessel. The vessels were placed in a microwave oven (MARS5, CEM Co., Matthews, NC, USA). The microwave-assisted digestion consisted of increasing the pressure to 80 psi over 20 min and then maintaining that pressure for 20 min by applying 100% power at 1,600 W. For separated epidermis and dermis, approximately 1 cm^2 of skin (*ca.* 0.01 g of epidermis, 0.3 g of dermis) was used. After digestion, the resulting solution was fixed with 20 ml of purified water. The Ti concentration in the samples was measured by ICP-MS (7500, Agilent Technologies, Santa Clara, CA, USA). The amount of Ti was calculated using a standard curve of Ti created with the peak area at mass number 47.

Ti distribution in skin

The skin sample was fixed with Karnovsky solution, dehydrated with ethanol, and replaced with resin. Horizontal cuts were made in the skin from the surface to the dermis and observations were obtained every 50 μm using SEM-EDS (JSM-6700/JED2300, JEOL, Tokyo, Japan).

Statistical analysis

The amount of Ti in skin was determined using at least 3 samples and the data subjected to analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Particle size of TiO_2 in suspension and on the skin surface

Both oil in water (O/W) and water in oil (W/O) creams are used as sunscreen formulations with TiO_2. TiO_2 is usually formulated in the oil phase; the oil suspension using silicone, which is often used for the base of sunscreens because of volatile and repels water characteristics, was used for this study. Many types of TiO_2 particles exist, with differences in crystalline type, size, shape, and surface coating characteristics. Four types of rutile TiO_2 shown in Table 1 was used for this study. T-35 was used to represent typical nanoparticles, with a round shape and no surface coating. TC-35, which has lipophilic coating features, was chosen to produce a good dispersion in silicone. T-disp is a pre-formulated product consisting of TiO_2 dispersed in silicone. The T-250 was used for comparison because it does not form nanoparticles.

Even if the primary particle size is less than 100 nm, lyophobic colloidal dispersions are unstable and aggregation occurs easily. Particle size distributions of TiO_2 in silicone are shown in Fig. 1. Mean particle size of T-35 was 1,700 nm, which was larger than that of T-250, 1,200 nm. Although the possibility exists that large particles shielded small particles, few nanoparticles were found. In contrast, suspensions of TC-35 and T-disp contained nanoparticles with mean diameters of 80 and 130 nm, respectively. The TC-35 suspension contained large particles that were easy to precipitate.

After skin application of suspensions, silicone was spread and vaporized so that only TiO_2 particles remained on the skin. Fig. 2(a) shows microscopic pictures of the surface after application of each suspension to skin followed by drying. T-250 and T-35 suspension showed aggregated white powder. After application of the TC-35 suspension, the skin was covered with white film that was thicker in furrows. Silicone spread easily on the skin and tended to collect in furrows because of low viscosity and interface tension. No particles were observed but the skin was slightly white after application of T-disp suspension. The SEM pictures showed large agglomerated TiO_2 (about 5 μm) for T-35 and T-250, although their primary particle sizes were different. The TC-35 also aggregated into particles of approximately 1 μm, although many small particles stuck to the skin. The T-disp formed uniformly agglomerated particles that differed from other preparations, TiO_2 particles appeared to be covered with dispersing agent (Figs. 2(b), (c)).

Skin penetration of TiO_2

Cosmetics and sunscreens are usually used only on intact skin. However, skin can be injured slightly by objects or through physical force. Thus, skin penetration of TiO_2 was investigated *in vitro* with intact skin and with stripped skin as a model of injured skin. Previous reports have indicated that hair follicles are important in the skin penetration of nanoparticles (Lekki *et al.*, 2007; Zvyagin *et al.*, 2008). Therefore, hair-removed skin was used to represent skin damaged by hair-removal treatments often done for the pursuit of beauty.

After 24-hr application, the skin surface was stripped with cyanoacrylate to remove surface TiO_2. A tape stripping technique is often used to remove residual materials on skin; however, materials in furrows or on hair follicles cannot be removed by this technique (Pflücker *et al.*, 1999). In this study, the amount of Ti varied greatly when

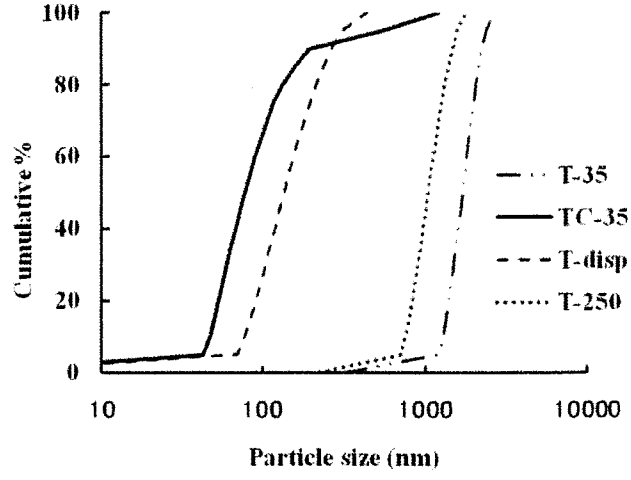

Fig. 1. Particle size of TiO_2 dispersed in silicone. Particle size was measured by dynamic laser scattering using a thousand-fold dilution of 10% TiO_2 silicone suspension.

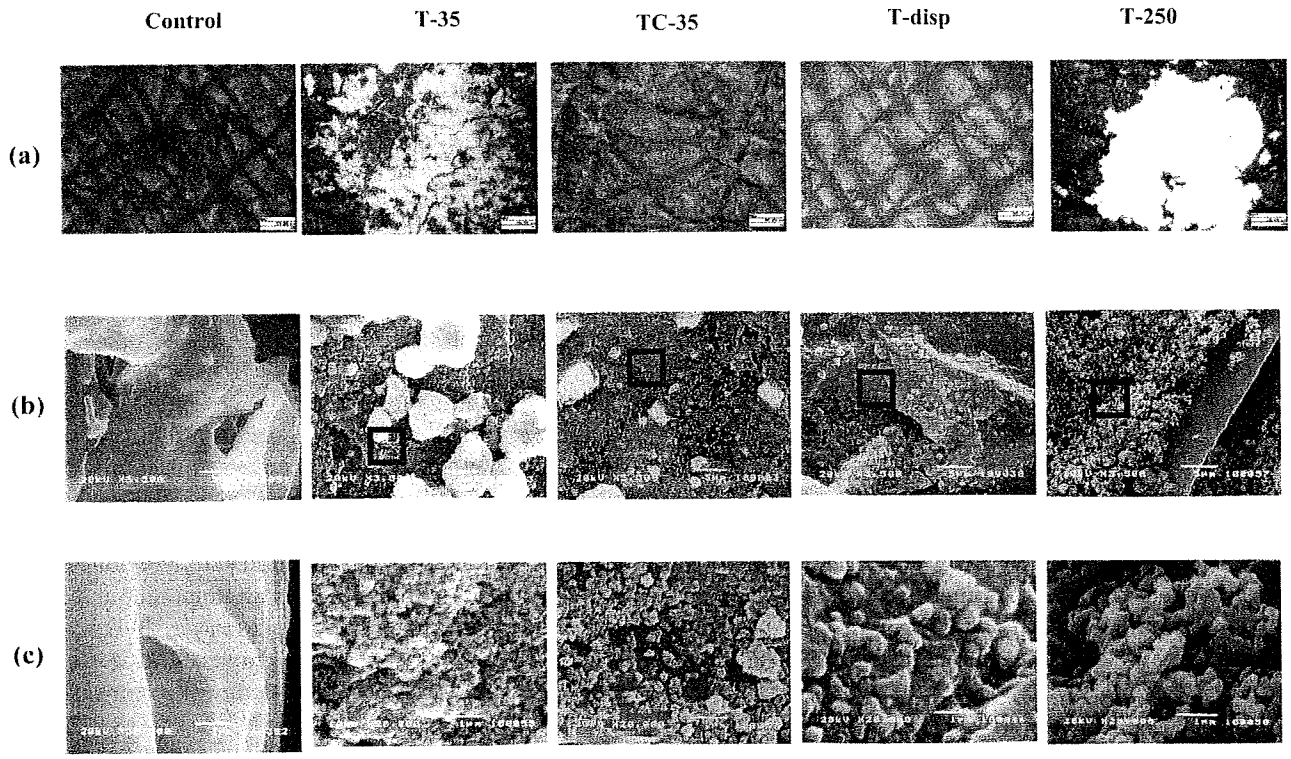

Fig. 2. The conditions of TiO_2 after skin application of a 10% silicon suspension of TiO_2 at a dose of 2 μl/cm². The skin surface was observed using a digital fine scope at a magnification of (a) 80. The surface of the epidermis was observed by SEM at a magnification of (b) 3,500 and (c) 20,000.

Ti remained in furrows (data not shown), so cyanoacrylate stripping was used. In this procedure, the surface SC layer of intact skin and hair-removed skin, and some hairs of intact skin and stripped skin were also removed.

Silicone is used to control formulation properties. Ti concentration in the receptor phase was similar in all skin conditions and formulations applied (Fig. 3(a)). Fig. 3(b) shows Ti concentration values in skin. For intact and stripped skin, no significant difference in Ti concentration was found between the control and suspension applications, which indicates TiO_2 did not penetrated into the skin regardless of particle size and even when the SC, which is the skin's primary barrier, was removed. For hair-removed skin, Ti concentration in skin after application of TC-35 suspension was significantly higher than that of the control, and after application of T-disp suspension, tended to be high. The Ti concentration in the dermis was no different than that of the control. Ti concentration in the epidermis after application of TiO_2 nanoparticles tended to be greater than that of the control, but the difference was not significant (Fig. 4). The epidermis consists of SC, viable epidermis and hair follicles. The horizontal sections from hair-removed skin after application of TC-35 suspension were observed using SEM-EDS. One of two SEM-EDS images showed the presence of Ti in the empty hair follicle after removal of the hair shaft 1 mm from the surface (Fig. 5(a)). Ti was detected in the hair follicle pocket, but not in the surrounding viable skin (Fig. 5(b)). We also found the similar distribution of 20 nm FITC-polystyrene (data not shown). The radius of a hair follicle is 0.05-0.2 mm (Otberg *et al.*, 2004), which would allow solvent to enter if the hair shaft and sebum did not fill the follicle space. When fluid enters a small space by capillary action, small particles of Ti in fluid may be able to enter the follicle. Large particles cannot be moved by such a small force, but TC-35 well dispersed in solvent might enter a follicle more easily than other types of TiO_2. For T-disp, the dispersing agent had some effect, resulting in particles left on the skin after drying of the suspension.

In conclusion, TiO_2 does not penetrate into viable skin, even if the particle size is less than 100 nm and the SC is damaged. However, immediately after hair removal, some TiO_2 particles penetrated relatively deeply into the

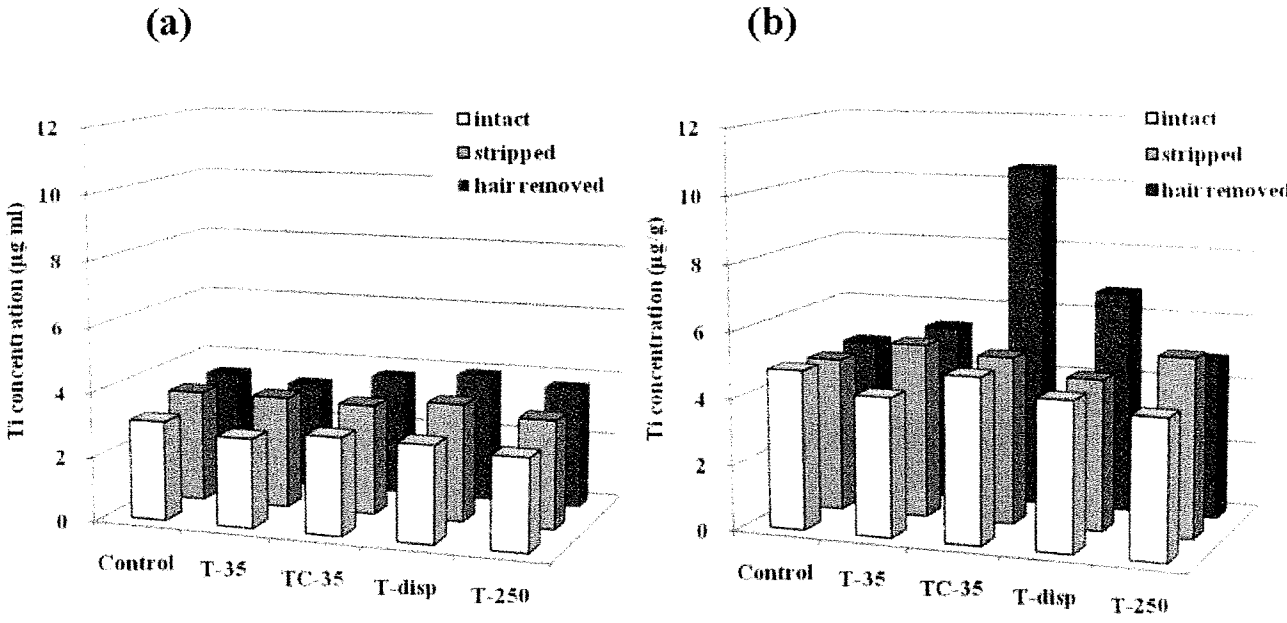

Fig. 3. Ti concentration in receptor phase (a) and in skin (b) after 24 hr application of a 10% silicone suspension of TiO_2 at a dose of 2 μl/cm² on intact skin, stripped skin, and hair-removed skin.
Silicon applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping.

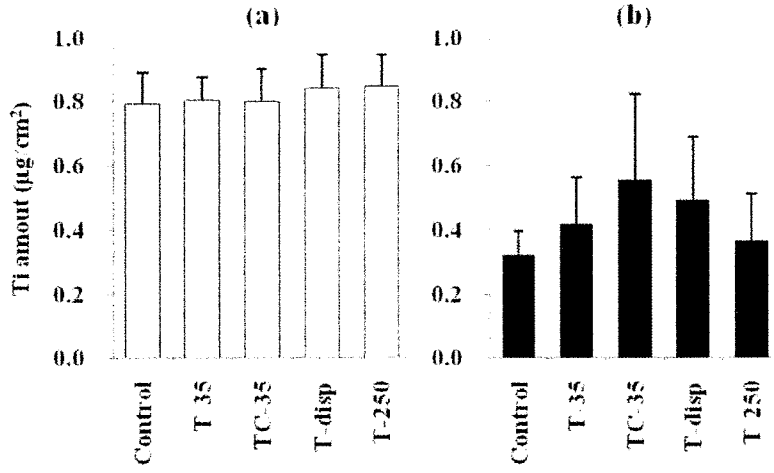

Fig. 4. Amount of Ti in (a) dermis and (b) epidermis after 24 hr application of a 10% silicone suspension TiO_2 at a dose of 2 μl/cm² to hair-removed skin.
Silicone was applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping, followed by separation of the dermis and epidermis by heat.

skin, possibly by entering the empty hair follicle. This was an *in vitro* study, so no inflammation was induced by the hair removal procedure. Inflammation could affect these results, therefore, further *in vivo* studies on viable skin with the hair removed are necessary.

There might be various nanoparticls to determine skin penetration, thus *in vitro* method is important for screening. If the materials have possibility to use on hair

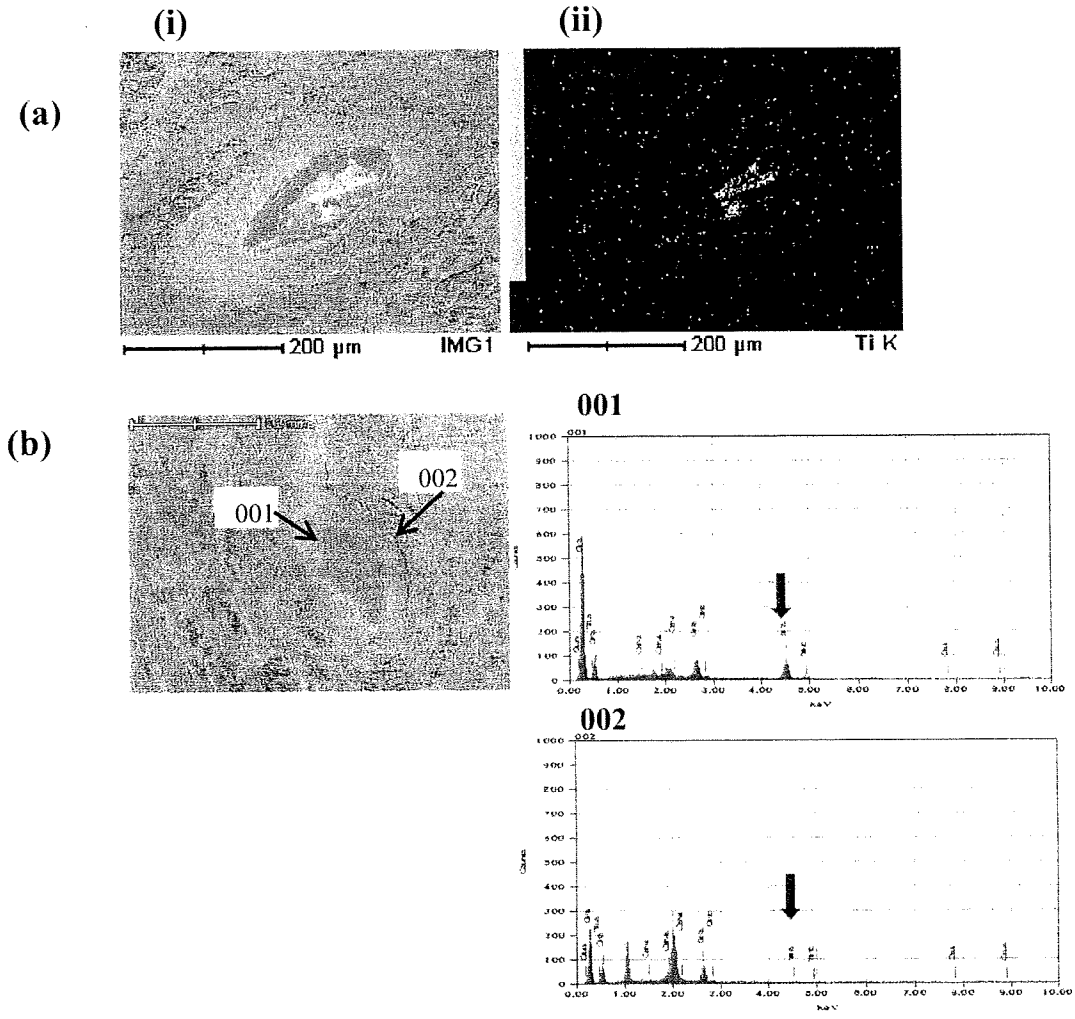

Fig. 5. SEM-EDS images (a) and elemental analysis (b) of a horizontal section of skin after 24 hr application of a 10% silicone suspension of TC-35 at a dose of 2 μl/cm² to hair-removed skin.
(a) at a depth of 1,050 μm from the skin surface. (i) SEM images, (ii) Ti distribution; (b) at a depth of 1,250 μm from the skin surface, (001) the hair follicle, (002) the dermis in contact with the hair follicle.

removed skin, hair removed skin should be taken into safety assessment of nanoparticles as well as stripped skin as a model of damaged skin. Also, this results indicate that the split skin (thickness 200~400 μm) which OECD Test guideline 428 recommended for skin absorption study *in vitro* have possibility to overestimate the skin permeation of nanoparticles because hair follicle is cut and nanoparticle in hair follicle is into receptor phase.

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