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XANES AND SR-XRF STUDY OF SKIN AS A BARRIER TO ULTRA-FINE NANOCRYSTALS OF TiO_2

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Abstract

Nanocrystalline TiO_2 is commonly used in cosmetic industry as a photoprotective agent. With recent advances in nanomaterial processing, the size of TiO_2 crystals decreased into the nanometre regime. There is no satisfactory evidence that crystals of such small size are harmless to the human population. An EU project NANODERM has been launched where several techniques have been applied to investigate the possiblity of particle penetration through the protective horny layer into vital skin regions. Skin biopsies of the animal and human skin have been collected after exposition to formulations containing TiO_2 nanocrystals. The Ti depth distributions were measured by electron and ion microscopy. The microscopy studies did not detect penetration into vital tissue of healthy skin what does not exclude a possibility that TiO_2 could penetrate pathological skin with lowered barrier efficiency.

Due to literature the physical effect of the UV irradiation of the TiO_2 nanoparticle is the shift from 4th to 3rd oxidation state of the Ti. Titanium at 3^{rd} oxidation state interact with environment producing free radicals and Reactive Oxygen Species. In order to quantify the oxidation state shift, XANES experiments were carried out with commercially available TiO_2 nanocrystals (6–100 nm size), both in anatase and rutile phase. The samples were irradiated with X-rays with, and without accompanying UV illumination at the NSLS X27A beam line. The corresponding XANES spectra were registered and the absorption edge was compared in UV–illuminated and not illuminated spectra. A shift of about 1 eV in the absorption edge position of the rutile sample exposed to UVA light (365 nm, 20 mW/cm^2) has been measured and attributed to the changed electron configuraion. However, the direction of the shift detected in measured samples was opposite to the expected.

Introduction

TiO_2 nanoparticles (down to 20 nm size and less), due to the excellent match of UV light energies and the band gap, are commonly used in sun screens as UV-filters. With recent advances in nanomaterial processing, the size of TiO_2 crystals decreased into the nanometre regime thus eliminating the undesired white appearance. Literature data show that TiO_2 nanoparticles are able to generate free radicals and Reactive Oxygen Species (ROS) under UV irradiation [Fujishima2000]. There is no satisfactory evidence that crystals of such small size are harmless to the human population. In other words, the question is if such nanoparticles could penetrate the skin, passing the protective stratum corneum layer and reaching the vital tissue [Tan1996]. Therefore, in the 5th Framework Program of the European Community, the NANODERM project has been launched with the goal of studying the "Quality of skin as a barrier to ultra-fine particles". In this project, several complementary techniques have been applied to investigate the possiblity of particle penetration through the protective horny layer (stratum corneum) into vital skin regions. In frames of the research consortium techniques like ion microscopy, high resolution transmission electron microscopy, and autoradiography were applied, accompanied with cellular response studies. From three crystallographic types in the TiO_2 group: rutile, anatase, and brookite, only two first ones have been chosen for the studies due to their technological importance.

If the nanocrystals do not penetrate the outermost horny layer, consisting of dead cells (without nuclei) tightly glued together, their presence is harmless for the organism. Penetration towards lower strata, especially to regions where cells divide, may lead to malignant processes due to the increase of free radicals concentration in the presence of UV light.

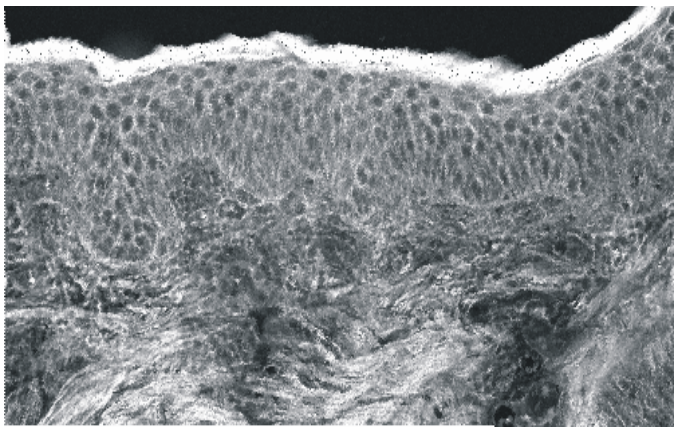


Figure 1. Cross section through the skin showing its subsequent strata

Distribution of TiO_2 nanocrystals in upper layers of skin.

The skin studies have been carried out in the following way. Animal (pig) and human skin has been exposed for few hours to several pharmaceutical formulations containing TiO_2 nanocrystals (typically 20 nm in size) and, after subsequent washing, skin biopsies have been collected. Size of biopsy was 3 mm in diameter. To avoid artefacts, biopsies were freezed in Isopentane cooled in liquid Nitrogen. Next, they were cut into slices in a cryo-microtome. Section thickness was 20 μm to assure sufficient mechanical resistance and to facilitate skin morphology (strata identification). To avoid contamination of inner layers with material at skin surface, during cutting biopsy orientation was such that the cutting knife was moving from dermis to epidermis. Self-supported thin samples were mounted (glued by the edges) on conductive carbon tape. This assured no interference from substrate no sample charging during beam bombardment, and enabled STIM (Scanning Transmission Ion Microscopy) analysis. After freeze-drying, sections are stored in airtight boxes in a freezer or in a dessicator.

The depth distributions of Ti and other elements were extensively measured by electron and ion microscopy by several NANODERM participants [Menzel2004, Verissimo2007]. This latter technique proven its usefulness in many earlier, similar studies [Moretto1999] and was the main tool used in the whole NANODERM consortium (six European microprobes). Sample density was either controlled prior and after the PIXE experiment using STIM technique in the reduced beam conditions and/or RBS (Rutherford Backscattering) method simultaneously with PIXE. It has been shown that TiO_2 is not uniformly spread onto the horny layer but often clusters in traps like furrows. For healthy, undamaged skin there was no penetration through the horny layer and the presence of Ti was usually restricted to 3-5 corneocyte layers.

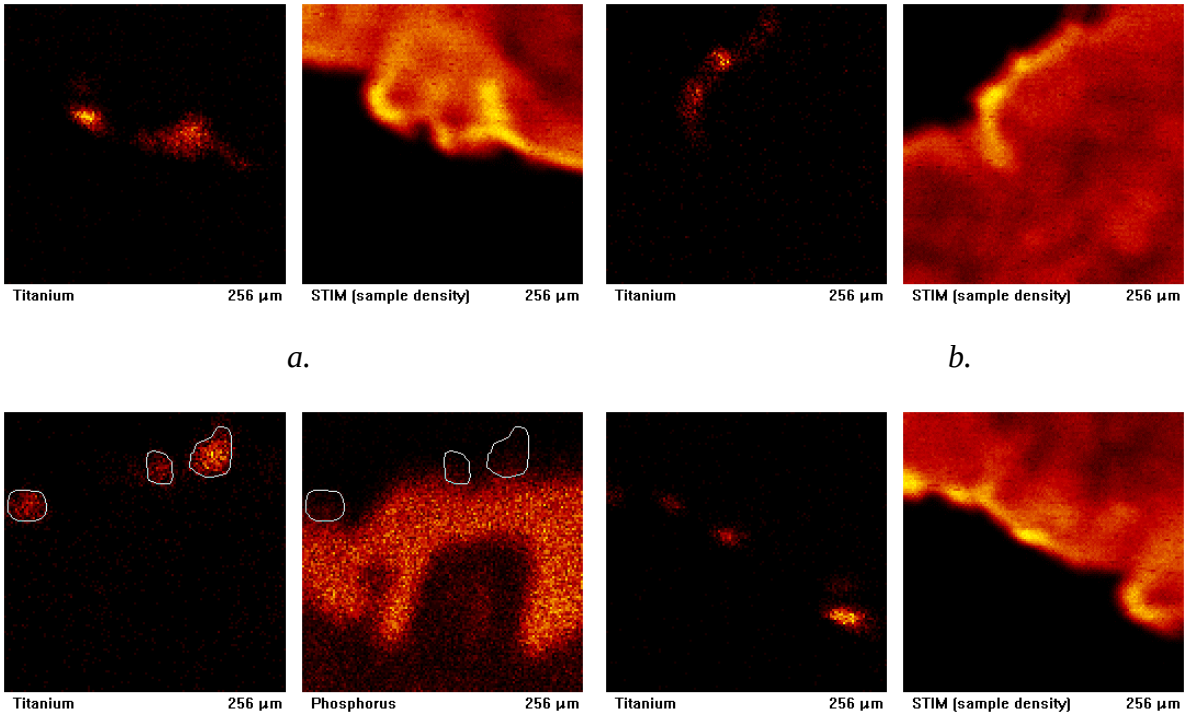


Figure 2 (a, b, and c): STIM and micro–PIXE maps of an upper layers of human skin exposed to the formulation containing TiO_2; (d) Distribution of Ti and P in the upper layers of skin. Ti signal is clearly located above the P–rich layer

Figure 2 presents images of human skin cross sections collected with the use of STIM and micro–PIXE (Proton Induced X-ray Emission) techniques. Skin was previously exposed to the formulation containing TiO_2. In the STIM figures bright parts of the image correspond to the high sample density regions. In micro–PIXE elemental maps Ti–rich areas are represented also by the bright color. The Ti signal is visible only in uppermost skin layer (stratum corneum, corresponding to the high density regions). The Figure 2(c) shows the comparison of distribution of Ti and P in the skin cross section. The location of Ti signal, distinctly above the P–rich region corresponding to strata below the horny layer, indicates no penetration into deeper skin layers.

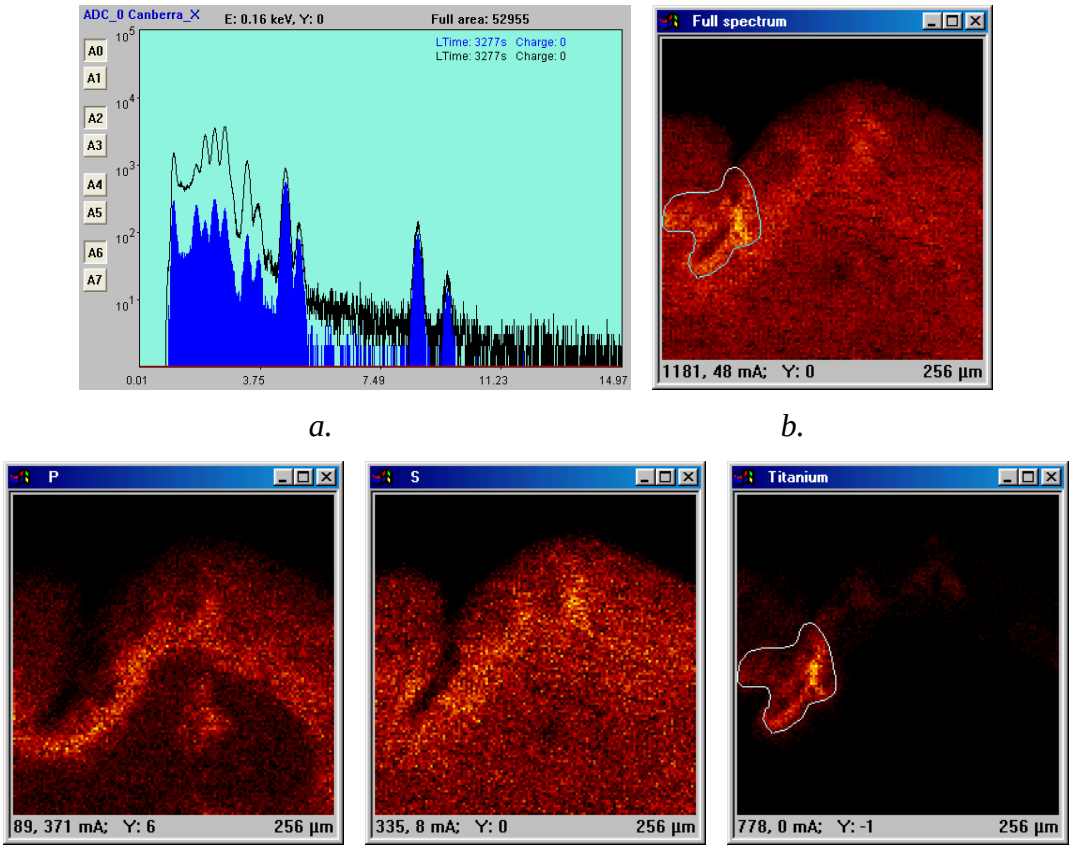


Figure 3 (a) The total X-ray spectrum with the Ti signal (blue) and the corresponding micro-PIXE map (b) of the pig skin cross section; Distribution of Phosphorus (c), Sulphur (d), and Titanium (e), measured with micro–PIXE.

As the direct penetration through the horny layer was not observed, the further possible pathway could proceed via hair follicles or sebaceous and sweat glands. The follicular pathway was again investigated by the complementary methods of ion microscopy (PIXE, RBS, STIM) and autoradiography [Lekki2007]. Ion microscopy images on sagittal cuts through follicles in skin occasionally show nanoparticles as deep as few hundreds of nm. No diffusion pathway was observed, strongly suggesting mechanical incorporation rather than diffusive transport. The following Figure 3 shows the accumulation of Ti in a hair follicle.

In the autoradiography experiment radiolabeling of TiO_2 crystals was performed by the 17 MeV proton irradiation, where radioactive ^{48}V isotope was produced in the reaction ^{48}Ti (p,n) ^{48}V at the IFJ cyclotrone. The activated TiO_2 was carefully mixed with hydrophobic basis gel and applied to large pieces of human skin samples (3 cm x 5 cm), delivered 2–3 hours after surgery of breast cancer. Samples were exposed to the radioactive formulation by gentle rubbing it to the outer part of the skin. After 2 hours of exposure the formulation was washed from the skin until no activity in the working solution was detectable. Next, several

biopsies were taken from the skin sample and fixed for 24 hours in formalin, then dried, dipped in paraffin and cut with a microtome perpendicular to the skin surface into 5 μm thick slices. Slices were stretched on a microscopy glass, covered with the nuclear emulsion and left in the dark for exposure for more than two weeks. After fixation and development the emulsion was investigated with an optical microscope.

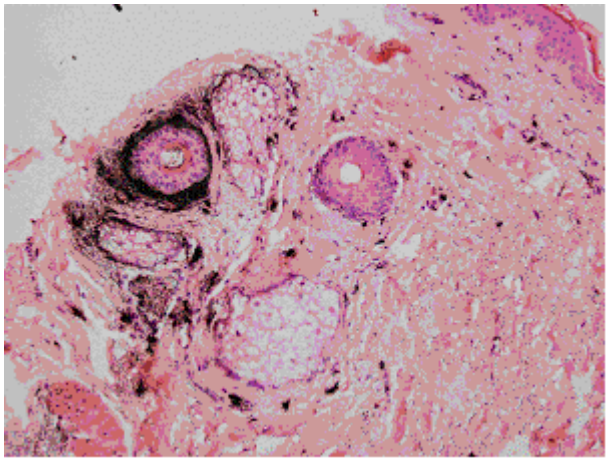


Figure 4. Autoradiography of the follicle area with underlying image of skin section, after staining. Sebaceous glands surrounding the hair are well outlined.

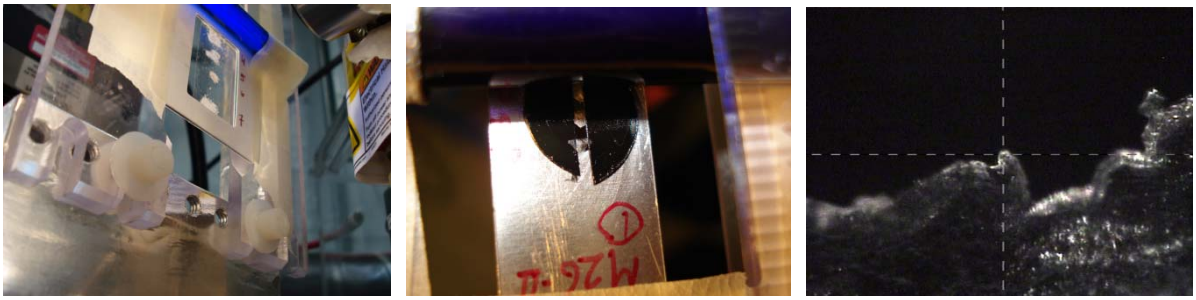
In Figure 4, the transversal cut through the skin containing a hair follicle is shown. TiO_2 (dark spots) is located around the hair in the follicle. Particles observed further away from the follicle in vital tissue were mechanically moved there because for a transversal cut contaminations are unavoidable.

XANES studies of the Titanium oxidation state

The physical effect of the UV irradiation of the TiO_2 nanoparticle as the first step involves the formation of an electron / hole pair in the bulk of the semiconductor nanoparticle. E.g., for the TiO_2 in the rutile phase, the band gap is about 3 eV what corresponds to a wavelength of about 400 nm. In normal conditions recombination of this complex is very fast (10^{-12} s), but in the presence of defects the resulting hole may become trapped and thus its lifetime becomes extended for several orders of magnitude. Such situation is particularly frequent for very small crystals, where the relation between surface and volume (and thus number of defects) becomes enormous. As a consequence, the modification of the electron structure produces the shift from 4^{th} to 3^{rd} oxidation state of the Ti. This process is then

followed by a sequence of events occurring in the particle's bulk or surface, thus leading to the formation of superoxide anion (O_2^{*-}) and hydroxyl (OH^*) radicals.

In order to quantify the oxidation state shift, XANES experiments were carried out with commercially available TiO_2 nanocrystals (6–100 nm size), both in anatase and rutile phase.



a. *b.* *c.*

Figure 5 (a) Irradiation holder with the miniature UV lamp (blue tube in the upper part of the image, lamp is on); (b) skin sample glued to carbon tape; (c) optical microscope image of the irradiated skin sample.

In case of the pure rutile/anatase samples a droplet of water with the suspended TiO_2 powder was deposited on a mylar. After evaporation of water, sample was mounted in a holder with the miniature UV–A (365 nm) lamp attached at the distance corresponding to the illumination power density of about 20 mW/cm^2 (Fig. 5a). UVA radiation (320–400 nm), constituing more than 90% of the ultraviolet radiation reaching the earth's surface, has been found to penetrate human skin and be responsible for severe damage. The value of 20 mW/cm^2 is few times higher than the average power density on the Earth surface and was chosen to increase the effect and thus facilitate the measurement. For skin samples the mounting used in ion microscopy studies was used (Fig. 5b, c).

Next, samples were irradiated with X-rays with, and without accompanying UV illumination at the NSLS X27A beam line. The corresponding XANES spectra were registered and the absorption edge was compared in UV–illuminated and not illuminated spectra.

SR–XRF experimental set-up and method

Due to advantageous such as high brilliance (high intense photon) and tunable (energy variability) synchrotron radiation (SR), synchrotrons become an ideal X-ray sources to investigate trace elements in biological samples. Synchrotron Radiation Induced X-Ray

Emission (SRIXE) and X-ray Absorption Near-Edge Structure (XANES) spectroscopy with a SR microbeam are two complementary techniques: SRIXE determines existence and distribution of trace elements even in a single cell while XANES spectroscopy provides information about electronic structure and the binding configuration of the probing element [Jones1992, Bianconi1988].

In this study the measurements by SRIXE and XANES spectroscopy were carried out on the X27A beamline at the National Synchrotron Light Source (NSLS) in Brookhaven National Laboratory, Upton, New York, USA. This beamline is equipped with the microprobe setup, described by Ablett [Ablett2006], dedicated for geological and biomedical applications. Fig. 6. presents the photograph of the experimental set-up. Using the highly brilliant 2.8 GeV synchrotron source with its white and linearly polarized radiation for fluorescence excitation it is routinely possible to detect the elements with atomic numbers between 13 and 92 with minimum detectable limits c_{MDLi} of down to 0.1 pg/µg [Falkenberg2001].

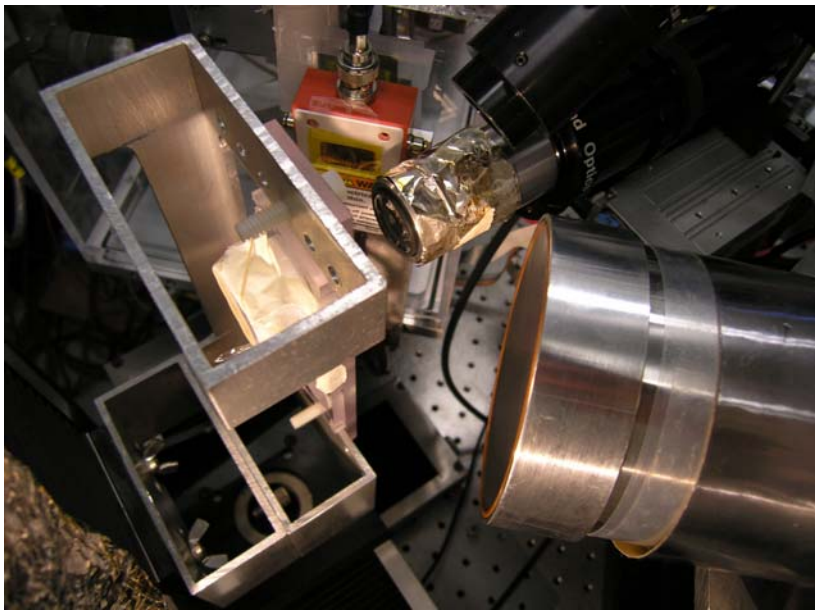


Figure 6. The photograph of the experimental set-up at X27A beam line at the NSLS

The microbeam arrangement at the Z27A beamline was set to provide highly intense beam on the sample with the size of 10 µm x 20 µm. During our measurements 2-dimensional (2D) scans were made on selected areas on prepared tissues samples in order to obtain distributions of titanium and to select the areas of its high concentration. In addition to 2D map a typical single X-ray fluorescence spectrum with the monochromatic synchrotron radiation beam of 10 keV was taken from each spot of pre-selected area.

Results and discussion

After the selection of area with high Ti concentration the beam energy was set to 5 keV and the K-edge XANES measurements were performed in fluorescence mode by measuring the X-ray fluorescence line of 4,5 keV that corresponds to Ti-K_α line. The white synchrotron radiation beam was monochromatized and the monochromatic beam was scanned in the photon energy range from 4900 eV to 5500 eV with 0.2 eV or 1 eV steps. Selected compounds such as TiO, Ti_2O_3, TiO_2, $SrTiO_3$ were chosen as standards for an appropriate oxidation state of titanium (Ti(II), Ti(III) and two Ti(IV) respectively). Titanium XANES spectra were collected with 13-element liquid-nitrogen cooled high-purity germanium detector. The energy of incident beam was scanned with a step of 1.0 eV in the range (4900 eV – 4960 eV), with 0.2 eV in the range (4960 eV – 5000 eV) and again 1.0 eV in the range (5000 eV – 5500 eV). The acquisition time for tissue samples was 5 sec. per energy step, while for standards and titanium compounds was 1 sec. per step.

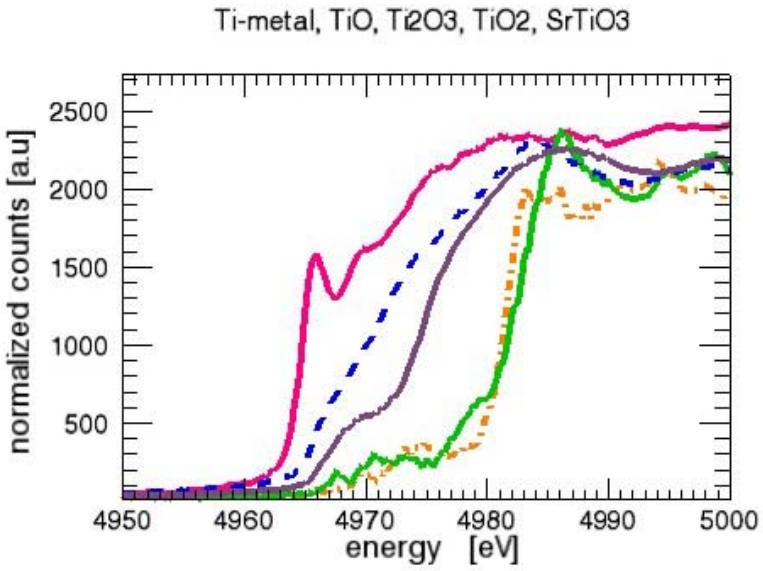


Figure 7. Shift of the energy absorption edge in Ti compounds characterized by the increasing oxidation state.

As can be seen from Figure 7, the total shift in the absorption edge position reaches the value of almost 20 eV for curves representing extreme different oxidation state of Titanium. The following Figure 8 presents this data as a linear fit.

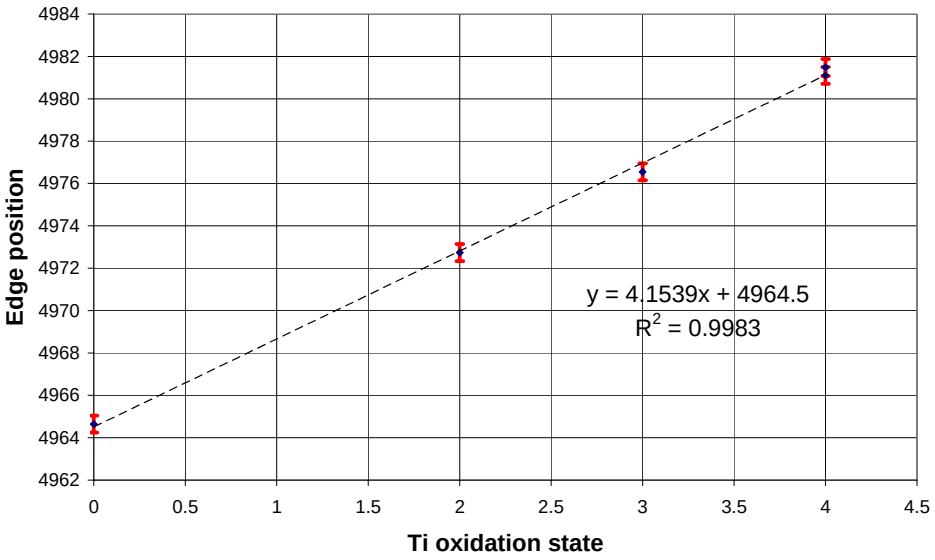


Figure 8. Calibration curve derived from data from Figure 7

However, it must be taken into account that the above result is obtained for the extreme conditions when the given oxidation state represents all Ti atoms in a sample. In real experiment, even during intensive UV irradiation, only a fraction of Ti atoms changes its oxidation state and the expected shift is much smaller.

Indeed, the subsequent measurements of human and skin samples treated with TiO_2–containing ointment did not show any difference in the position of the absorption edge. Obviously, the effect of change of oxidation state was too weak to be visible in the registered spectra, especially if taking into account relatively little amount of the TiO_2 nanocrystals still present on the skin.

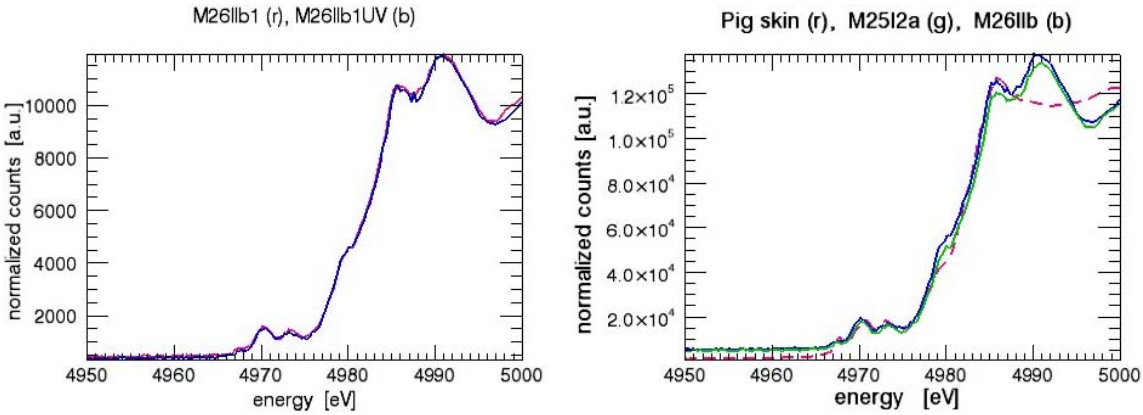


Figure 9. (a) Human skin with (blue) and without (red) UV light; (b) comparison of the pig (red) and human (blue, green) skin samples; UV light OFF

Similar measurements has been carried out for the pure anatase powder samples (TAYCA, product symbols JA-1 and AMT-100). The example spectra taken with (blue) and without (red) UV irradiation are shown in Figures 9-12. Again, the difference in the position of the absorption was negligible.

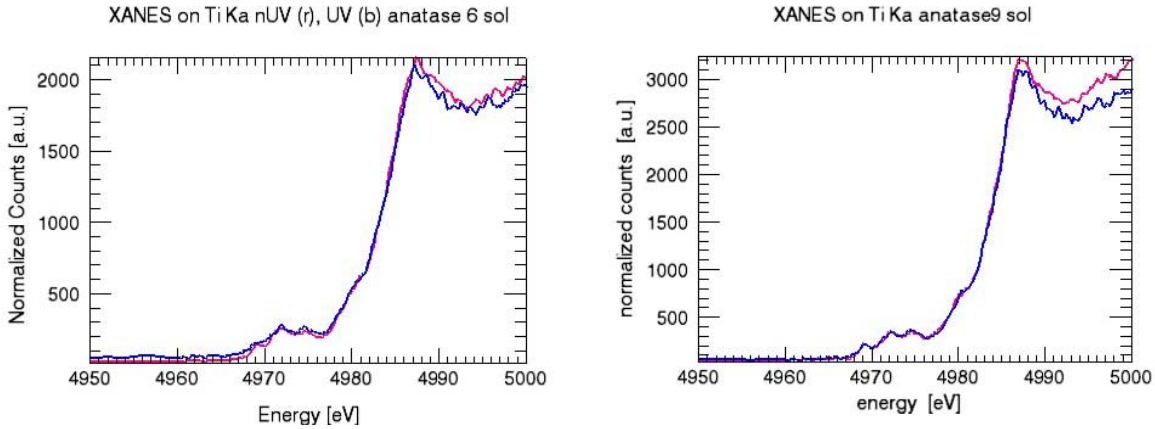


Figure 10. Anatase samples measured with (blue) and without (red) UV light for different nanocrystal sizes and sample preparation (a) anatase 6 nm; (b) anatase 9 nm.

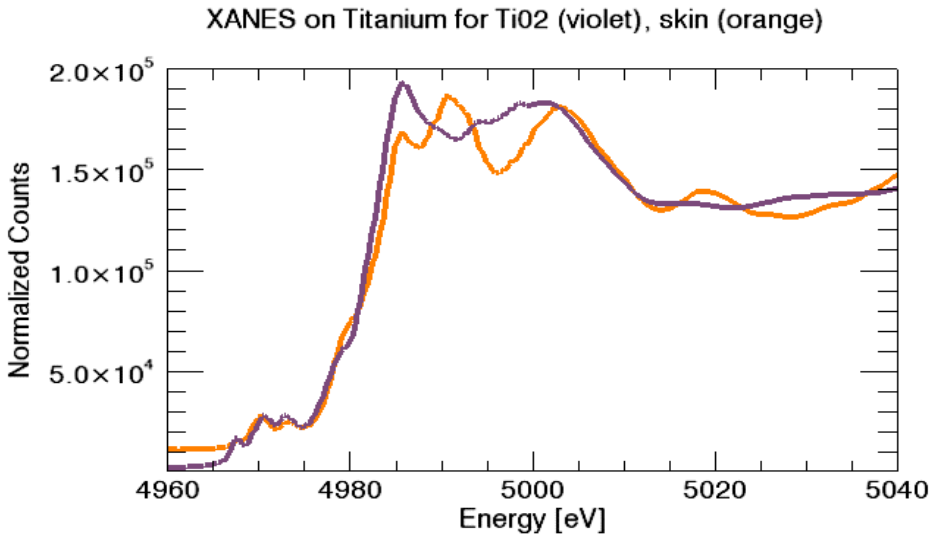


Figure 11. Comparison of the XANES spectra taken for pure TiO_2 deposited on mylar foil and the TiO_2 treated skin sample.

As shown above to the left edge of Ti XANES spectrum taken on skin sample corresponds to the edge of Ti XANES spectrum taken on Ti nano-particles in TiO_2 compound. But there is a significant difference in "white" peak structure what can be explained by different DOS (Density of State) structure. . Since the incident synchrotron radiation beam size was 10 μm x 20 μm, the measured X-rays provide the averaged

information about the irradiated area and that is the reason one cannot observe definite oxidation state change as in the case of standards shown in Fig 7 However, for rutile samples (Huntsman, product name R-HD2) there was a shift in the position of the absorption edge could be observed, what is shown in the subsequent Figure 12.

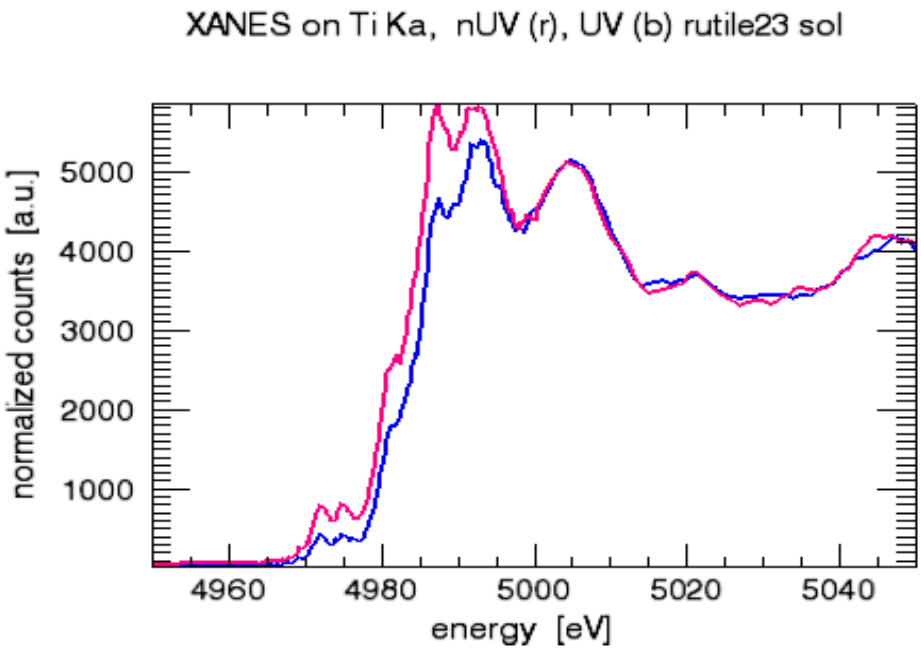


Figure 12. Rutile (23 nm) sample measured with (blue) and without (red) UV light.

As shown in Figure 12, there is a small shift between the absorption edges measured of titanium in rutile sample. As shown in Fig.11 the Ti edge position measured on the sample is very similar to the one measured for TiO_2. But the structure of the edge is more complex and seems to be composed of two fractions. In the rutile sample there are also two pre-edge peaks visible what could prove two component structure of the edge. The determined energy shift between Ti edge in the sample irradiated by UV (blue spectrum) and rutile not irradiated with UV (red) is about 1.2 eV ± 0.2 eV towards higher energy. Such a shift may not indicate changes in oxidation state of the measured sample but could be connected with the change of Fermi level in changed local structure of energetic states induced with UV irradiation. Also, there is the evidence for the local DOS change since the intensity of the white peak changes while the spectra above Kossel region are very like similar. Therefore this experiment proves the necessity of further study which could solve the problem of DOS calculation.

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