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Abstract: Industrially manufactured titanium dioxide nanoparticles have been successfully radiolabelled with 48-V by irradiation with a cyclotron-generated proton beam. Centrifugation tests showed that the 48-V radiolabels were stably bound within the nanoparticle structure in an aqueous medium, while X-ray diffraction indicated that no major structural modifications to the nanoparticles resulted from the proton irradiation. In vitro tests of the uptake of cold and radiolabelled nanoparticles using the human cell line Calu-3 showed no significant difference in the uptake between both batches of nanoparticles. The uptake was quantified by Inductively Coupled Plasma Mass Spectrometry and high resolution gamma-ray spectrometry for cold and radiolabelled nanoparticles, respectively. These preliminary experiments indicate that alterations to the nanoparticles' properties introduced by proton bombardment can be controlled to a sufficient extent that their further use as radiotracers for biological investigations can be envisaged and elaborated.

Response to Reviewers: Since we received comments from so many referees we had to structure their comments and questions in a table which is annexed to a letter to the editor that is uploaded as pdf file separately as attachment to the manuscript with the file name "Abbas et al R1 - resubmission letter.pdf"



EUROPEAN COMMISSION  
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection  
Nanobiosciences Unit  
Uwe Holzwarth

Ispira, October 26<sup>th</sup> 2009

Editors

Journal of Nanoparticles Research

via on-line submission system

**Resubmission manuscript # NANO2128 now entitled “Radiolabelling of TiO<sub>2</sub> nanoparticles for radiotracer studies”**

Dear editors,

we were quite surprised to get so many referee reports concerning our manuscript, something we have never experienced before. It took some time to structure, answer and implement them in a revised version. We grouped the comments according to common arguments in a table (see below) trying to make our revision procedure more efficient and to provide the proper answers, and we tried to revise the manuscript taking into account as many of the comments as possible.

The radio-activation of nanoparticles (NPs) to enable radiotracer studies is quite a new application and highly interdisciplinary. To carry out studies in an entirely comprehensive and complete manner would require characterisation of the activated NPs using a wide variety of techniques. However, compliance with radioprotection legislation requires that the activated NPs have to be kept and investigated in a controlled area. This implies that we are very restricted in moving in and out the equipment for a state-of-the-art complete characterization of the radioactive NPs. In the manuscript we clearly pointed out this current weakness and some referees accepted the preliminary and suggestive nature of the results, and others criticized it.

Referee #9 explicitly accepted our approach to publish anyway, and to publish now, when writing “Although these results are somewhat preliminary in nature, it is important to make the community aware of this technology and its potential use ...” It is indeed our intention to motivate scientists working on possible environmental and/or health effects of NPs to check whether this approach might be useful for them and whether they have the possibility to collaborate with a nearby cyclotron laboratory. In order to facilitate such activities we also included the design of our target system which of course is less interesting for everybody who wants simply to use radioactive NPs. We would for sure have much less impact on nanoparticles research with a manuscript dedicated to radioisotope and accelerator scientists and published in one of the pertinent journals.

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Below you will find a table that contains all comments of the referees grouped according to the various aspects of criticism. Also the referee number is reported to facilitate traceability. Our answers to comments and criticisms are presented.

A lot of confusion has been created using two types of NPs which is an issue that repeatedly shows up in the table below. To make the explanation shorter: From a materials science point of view radiation damage and phase transitions are easier to see in material with a size between 20 and 50 nm, therefore we started the activation studies with P25 (21 nm). The biologists preferred smaller NPs for the uptake studies possibly below 10 nm, since they had experience with this size from earlier toxicity studies, and they ordered Alfa Aesar material accordingly. The material with a stated size of 5 nm turned out to have an average size between 15 and 20 nm when examined with XRD. Thus, the size is so similar that both materials behave identical under irradiation. Nevertheless, the lessons learnt from the P25 studies, i.e., that cooling may matter, have been fully implemented with the Alfa Aesar material using a smaller NP volume to be on the safe(r) side.

During the revision we had extensive discussions about the topic with Dr. Wolfgang Kreyling from the German Research Center for Environmental Health, from which we received valuable input and comments and who we added as an author. An increasing overlap with the European Commission's 7th Framework Programme, "NeuroNano" project (contract NMP4-SL-2008-214547) became obvious, for which the present work was a type of pilot and feasibility study that started well before the project has been approved. This is now reflected in the acknowledgement.

We would like to acknowledge the referees' efforts in improving the manuscript, and we hope that we could satisfy their essential requirements as far as possible within our current experimental possibilities and considering our motivation to publish such preliminary results.

Looking forward to your feedback I remain on behalf of all authors with

Best regards

A handwritten signature in black ink, reading "Uwe Holzwarth". The signature is written in a cursive, flowing style.

Uwe Holzwarth

<b>Compilation of referee comments according to problem fields</b>		
Referee comment	Ref. Num.	Authors comments and actions
<b>Appropriateness of the title</b>		
The manuscript was entitled "Radiolabelling of TiO <sub>2</sub> nanoparticles for use in nanotoxicology studies". But the authors just discussed the potential application of the radiolabelled TiO <sub>2</sub> NPs as radiotracers. I cannot find any experimental results or discussions related with nanotoxicology. So I suggest the authors to rewrite a more appropriate title.	10	Indeed in order to check to which extent the irradiation process would alter the interaction of cells with such NPs only a simple uptake experiment was performed in this investigation.  Taking this into account the title has been changed into " <b>Radiolabelling of TiO<sub>2</sub> nanoparticles for radiotracer studies</b> ".
<b>Questions concerning the effects of irradiation</b>		
P6, para1, line10: the authors indicated "The NP volume thickness used was 2mm for the P25 material, and 0.4mm for the Alfa Aesar material. The reduced thickness in the latter case allowed an increased activity concentration and better sample cooling to be achieved." Why not use the same design for the P25 materials? Is it unimportant for the P25 materials to increase the activity concentration and to cool the sample?	10	On one side heating has to be limited in order to avoid an aggregation of the nanoparticles, phase changes or even the sintering into large non-nano particles. The heating in a powder of material with poor heat conductivity can be controlled better if the volume is small and the distance to the heat sink is short. Hence for biological experiments we wanted to avoid heat effects on the NPs.  P25 was used for studies on radiation damage for reasons specified below. These experiments we performed with the thicker capsule to allow enough material for structural studies. The fact that little radiation damage was seen for P25 indicates the in the worst case of cooling there is little structural change due to radiation or thermal effects. The referee is correct that for subsequent tests (e.g. uptake) the P25 would have to be cooled better, but this material was not used for such studies.
I noticed that the same energy (25 MeV) of proton beam was used for the irradiation both P25 and Alfa Aesar, but these two types of TiO <sub>2</sub> -NPs are in the different thickness, in which P25 is definitely much thicker than Alfa Aesar. Then when there is a minor structural change for P25, a more obvious change should be gotten for Alfa Aesar under the same irradiation energy. I am wondering whether the structure of Alfa Aesar will be damaged after being radiolabelled, at least partially, and whether such damage will limit its application in biological system.	7	The concern is justified in any case, but the thinner the powder/nanoparticle layer is, the better is the cooling efficiency and the lower the risk of damage due to thermal effects. Hence the opposite is true, i.e., the irradiation conditions for the Alfa Aesar material lead to less thermal damage than those for the P25.  Regarding the actual NP dimensions an extensive and in depth study needs to be performed to check the radiation/thermal damage as a function of particle size. However this was not carried out in this preliminary study, the objective of which was only to highlight the possibilities of cyclotron radiolabelling of NPs.  The text of the paper in has been modified appropriately.

<p>P6, para2, line1: the author described "As energy for the proton beam 25MeV has been used, since attenuation in the two aluminium windows and the front face water cooling circuit results in a beam energy of about 15MeV on the NPs." How to determine the beam energy on the nanoparticles? Is it measured by some equipment or just estimated?</p>	10	<p>It is difficult to measure, but it can be calculated with good accuracy using the stopping power of the protons in the attenuating materials and knowing the thicknesses of the Al windows and the water layer. There are convenient and well-accepted computer codes that can be downloaded for such calculations (see Ref. Ziegler et al.).</p>
<p>It would be helpful if the crystal phase(s) and their ratios were reported for the two TiO<sub>2</sub> particles types. If there are differences in crystallinity, please discuss if there would be expected differences in crystallite defects or stability of the radiolabel. It does not appear that the Aesar particles, which were used for the in vitro particle uptake studies, were tested for structural alterations following irradiation.</p>	9	<p>The XRD signal to noise ratio was not good enough for very accurate determination of phase ratios. Even the structural changes mentioned as a result of irradiation are not easily quantified, and this is simply a qualitative observation. In addition, heating would not be uniform throughout the NP powder, so differences in radiolabel stability due to structural changes would also not be uniform.</p> <p>Referee 9 is correct that the Alfa Aesar material was not checked for structural changes, but (as noted above) structural changes were much less likely given the improved capsule design and sample cooling. The result of a subsequent XRD scan on Aesar material is now noted in the text, and it can be noted that the XRD is not consistent with the stated particle size, thus this material (or at least that batch) is not a good candidate for study of structure modifications.</p>
<p>P12, para2, line7: the authors concluded that "There is no obvious major structural change to the TiO<sub>2</sub>-NPs, though minor differences in relative peak heights indicate that a small reduction of the anatase to rutile ratio could have occurred." The presence of the rutile peaks in the irradiated NPs XRD patterns indicates the formation of the new rutile phase, means anatase transforms to rutile. The authors should carefully state this result.</p>	10	<p>Following the suggestion of ref. 9 a section discussing radiolabel stability and crystallinity is now included in the text.</p> <p>Following ref. 10 the qualitative observation is now more carefully stated in the text.</p>
<p>How many <sup>48</sup>V radiolabels were conjugated with TiO<sub>2</sub>-NPs?</p>	13	<p>In the present case from an activity of 850 kBq in 12 mg NPs one can derive that only one out of 10 billions of Ti atoms have been transformed into <sup>48</sup>V. This means that only a tiny fraction of the NPs has a radiolabel. This is not an important consideration for statistical radiotracer studies unless too low an activity is achieved (though it would be very important for radiolabelled NPs for medical diagnosis or therapy).</p>
<p>Is it possible to control the binding efficiency of <sup>48</sup>V to TiO<sub>2</sub>-NPs by change of irradiation intensity?</p>	13	<p>"Binding efficiency" is not the issue here since implantation is the primary radiolabelling process. The radiation intensity will change the activation rate and also modify the sample temperature during irradiation. The stability of the radiolabel might be modified due to this, but this is a play off between thermal annealing and thermal damage. The text has been modified to note this point.</p>
<p><b>Questions concerning the use of 2 types of NPs</b></p>		

<p>P8, para3, line1: the authors mentioned "In order to determine if any structural changes to the NPs were induced by the proton irradiation, X-ray diffraction was performed on the irradiated P25 material both before and after irradiation." Why not measure the XRD of 5 nm nanoparticles to determine the possible structural changes? Are there any special reasons to select the P25 nanoparticles to measure the XRD? During the cellular uptake tests, the 5 nm nanoparticles were selected to perform the tests. Why did the authors design the experiments like this? Better to use the same sample to determine the effects of the proton irradiation on the sample structure and the cellular uptake tests.</p>	10	<p>The width of a XRD peak is given by the inverse of the crystal size, i.e. the smaller the particles the broader the peak. The radiation damage in terms of atomic defects in a NP induced by the irradiation contributes to a broadening of the XRD peak. Calculations indicate that the radiation damage to be expected is small and hence there is little effect to be expected on the XRD peaks. In order to recognize a small effect, a narrow peak is desirable, i.e., a larger NP size. The XRD peak of small particles is already large and any deviation due to damage would be even more difficult to recognize. Therefore P25 is more suitable than a 5nm material for this purpose.</p> <p>For this reason the first steps to investigate irradiation effects on the NPs, i.e., radiation damage (XRD peak width) and undesired temperature increase (formation of new XRD peaks due to phase changes) were started with P25. The biologists however preferred a smaller particle size for their uptake experiments and ordered a material with a nominal size of 5 nm.</p> <p>However, from the peak width in a XRD study on the Alfa Aesar material that has been purchased with a size of 5 nm, surprisingly a size of 15-20 nm was derived. Hence, it turned out to be a very similar material as the P25 with a mean particle size of 21 nm. A comment has been inserted in the text on this.</p>
<p>Why the authors used two types of nanoparticles? Why these nanoparticles were not submitted to the same experimental characterization? In our opinion, the results are a miscellaneous of both types of nanoparticles. The experimental for the preparation of irradiated TiO<sub>2</sub> nanoparticles as well as the in vitro characterization of nanoparticles is different. This is the major aspect to be revised in the manuscript.</p>	12	<p>We agree with the various referee comments that it is important to study the effects of radiation and temperature rise on all NPs that are used for subsequent radiotracing studies (XRD, DLS, Zeta potential, etc.), and have initiated such a study. However, given the inconsistency in primary particle size of the Alfa Aesar material, this was not considered an appropriate material to repeat the study done on P25. Appropriate comments have been inserted in the text.</p>
<p>P5, para2, line12: the authors mentioned "This sample was used for subsequent centrifugation tests and cellular uptake studies." Why only the irradiated 5 nm nanoparticles were selected to perform the centrifugation tests and cellular uptake studies? Is there any special consideration?</p>	10	
<p>It is unclear how leaching of the radiolabel would become apparent in vitro studies, as mentioned on pg. 15. If more activity appears in the cell-free fraction, one could not immediately conclude that this was leached from particles without doing another separation step. Perhaps this is what the authors were alluding to, but it would be helpful to include details of a plan to make this</p>	9	<p>Indeed, the passage was misleading and has been modified. Various additional separation steps would be required that are usually related to studies on the intracellular distribution of the NPs which is beyond the scope of the present uptake studies.</p>

distinction.		
<b>Questions concerning the biological procedures</b>		
During the cellular uptake tests, Calu-3 human cancer cells were selected as the in vitro model. Why use the human cancer cells rather than human normal cells to perform the tests?	10	<p>Primary cultures face major limitations such as the lack of availability of normal human airway tissue, the limited number of cells and a certain donor variation.</p> <p>We consider Calu-3 cells to be suitable for this study as they are readily available and differentiate into monolayer of polarized cells. Moreover, Calu-3 cells form tight junctions, produce mucous and express many of the characteristics of human native epithelium, despite being derived from a human bronchial adenocarcinoma.</p>
P5, para2, line13: the authors mentioned "It should be noted in both cases that cold and irradiated particles would be agglomerated to some extent." Is there any adverse effect of the agglomeration on the cellular uptake of the irradiated nanoparticles?	10	<p>It is clear that both the irradiated and non-irradiated particles are agglomerated. And agglomeration changes the effective size of the particles for uptake studies. As noted in the text, we did not perform DLS yet regarding this aspect. The simple uptake studies indicated a similar uptake for both samples, so the conclusion in this case was that there was no massive extra agglomeration associated with the irradiation. This is noted in the text.</p>
Page 5: "For the cellular uptake studies both cold and irradiated samples were dispersed in exactly the same way prior to the in vitro comparative tests." The conditions of such dispersion should be described.	12	<p>This was an error. The particles did not undergo a dispersion process. They were simply suspended in water when removing them from the capsule and the suspension was then buffered with PBS and aliquots were taken and diluted to the nominal concentrations by adding further PBS.</p>
Page 12: The authors say: The results show that the radiolabelling by proton irradiation of the TiO <sub>2</sub> -NPs did not significantly alter the uptake behaviour.". Which statistical analysis was employed? At which significance level?	12	<p>The experiments were repeated 3 times in 3 replicates for each concentration. The error margin is presented as the standard deviation.</p> <p>This criticism of referee #10 is justified. – Since the differences lay within the standard deviations they are not significant.</p> <p>In this respect Referee #7 is doing the same as we did when trying to exploit differences that lay within the error margins of the sets of experiments with cold and labeled NPs.</p>
P12, para3, line4: the authors stated "The slightly lower uptake observed in both cases for the radiolabelled material is within the experimental error." The data are presented in Table 1. But from the data shown in Table 1, it is difficult to conclude this statement. How many times the cellular uptake tests were repeated? From the biological points, firstly, the data of the two groups mentioned in this table should be obtained from the sextuplicate experiments at least. Secondly, the obtained data should be analyzed through statistically analysis to compare whether there is significant difference between the two groups.	10	<p>The text has been modified to specify more precisely what we did, and also to indicate that more statistically relevant results would be required to identify any real small differences in uptake that might be so far hidden within the error margins.</p>

So better to present the statistically analysis result to support the conclusion.		
My concern above is also from the results of Table 1. A lower uptake by Calu-3 cells was observed for TiO <sub>2</sub> -NPs after being radiolabelled. The authors think such alteration results from the experimental error, but I think it may be from the modification of the structure of TiO <sub>2</sub> -NPs. Please address whether such difference is statistical meaningful or within the experimental error.	7	
<b>Missing characterization methods</b>		
Better to confirm the particle sizes and the size distribution of the as received TiO <sub>2</sub> NPs through electron microscopy analysis	10	<p>Except for the size distribution already the XRD is a quite powerful method, and indeed indicated that the Alfa Aesar material did not match specifications. Moreover, the average obtained involves a much higher number of diffracting NPs than those that could be observed by TEM or SEM. The required high magnifications in microscopy are appreciably reducing the visual field and require taking and evaluating a significant number of micrographs.</p> <p>We consider XRD to be better for average primary particle size analysis (or DLS if the NPs are perfectly dispersed) and DLS to be appropriate for size analysis when the NPs are in aggregated form.</p>
There are many particle characteristics that could affect outcome following exposure - including agglomerate state, as mentioned by the authors, surface charge, and in vivo dissolution rates, to name a few. It will be important to thoroughly characterize the labeled particles and to assess outcomes other than cell uptake over a 24-hr time period in order to conclude that the radiolabeling process does not fundamentally change the ways in which nanoparticles interact with biomolecules, cells, and tissues.	9	<p>We agree with the comment of ref. 9. However, as noted in the text we do not have all the equipment available in the cyclotron controlled zone (in fact, for us the experiments give a justification to duplicate equipment in order to characterize radioactive NPs). If already a simple uptake experiment would have resulted in significantly different uptake data for cold and activated NPs questions were arising whether such an approach would have been feasible at all. The finding that under exactly the same conditions with except for the prior activation the uptake behaves the same justifies a further in depth investigation that has to comprise necessarily methods like DLS and zeta-potential measurements.</p> <p>In any case the point of ref 9 is addressed now in the text.</p>
As the authors pointed out, results from stability, DLS and zeta potentials would be very interesting in this paper	12	We agree with ref 12, but cannot comply with ref 13 without withdrawing the paper and repeating the entire series of experiments after a DLS and Zeta potential system has been procured!! We believe it is enough to note this and publish the results in this preliminary form.
The colloidal stability of labeled nanoplatfoms (TiO <sub>2</sub> -NP) should be examined. Size and surface charge data is essential for publication of	13	



this paper.		
When the cellular uptake experiment conduct, authors should be proceeded the cell viability test as variation of 48V-TiO <sub>2</sub> -NP and TiO <sub>2</sub> -NP, respectively	13	This suggestion goes probably in the same direction as that of referee #10 to change the title because we do not present any toxicology data. Indeed cell viability would be required to assess toxicity of the NPs. But this is beyond the scope of the present manuscript. – Based on many years of experience with radiotracer studies a difference due to the simple presence of a radiolabel can be excluded
Do the prepared nanoparticles exist at intracellular region?? TEM image or confocal microscope image data for NP treated cells need to obtain.	13	We do not consider this necessary for such comparative studies, the objective of which was to look at differences between irradiated and non-irradiated NPs.
Electron microscopic images for TiO <sub>2</sub> -NPs with/without 48V can be obtained to prove the absence of morphological change.	13	It is highly unlikely that SEM or TEM could reveal actual radiation damage effects in such studies. For larger effects due to thermal damage, XRD and DLS would be sufficient.
<b>Language Deficiencies</b>		
There are some sentences in the text with awkward structure. Examples of this are the first sentence of the second paragraph on pg. 6 and the second-to-last sentence of the top paragraph on pg. 14.	9	We have tried to simplify the structure of “awkward” sentences.
<b>Technical Deficiencies</b>		
The 'Introduction' section is quite perfect, however, the 'Experimental Methods' section is a little vague and expatiatory. Please describe the experiments in legible and straightforward way, avoid pleonastic description. In addition, my concern is the length of the 'Conclusion' section, it seems to be too long, please just give the primary conclusions, don't give the detailed information about the study. Some paragraphs could be moved the 'Results and Discussion'.	7	This criticism has been considered and the manuscript has been critically revised.
In my point of view, the paper is not organized according to the instructions for authors. Figures and tables are inserted in the text.	12	We used the LaTeX template provided by the publishing company which can be used to create a preview of a final print version. For the version available to the referees the “referee”-style has been used supplied by the publisher.
Figure 1 could be omitted	12	The figure has been modified to give more technical details and to enable possibly discussions elsewhere between interested users of NPs and accelerator people which could possibly provide them.
In Figure 2 and 3 it is very difficult (may be impossible) to distinguish the lines of spectrum a and	12	In Figure 2 the main message is that there is no transfer of contamination from the capsule material (Co, Zn) into the NPs and there is no need to distinguish the other peaks. In Figure 3

spectrum b. The type of TiO <sub>2</sub> nanoparticles should be informed in the legend		the message is that the curves in terms of counts, i.e. activity, fall apart by a factor of 100 or more to support graphically the statement that only about 1% of V-48 is lost by leaching. We believe that for this purpose the Figures are adequate. The figure captions have been modified to indicate this.
In Figures 2 and 3, it is difficult to distinguish the individual spectra and to see if and where they overlap (in Fig. 2). Because of the size of the figures, it would be helpful to use different colors or place the spectra side by side.	9	Concerning the size of the Figures, they are present in the size which corresponds nearly exactly the final print version and not as enlarged copies for submission since we used the LaTeX style files supplied by the publisher.
Description of the materials should be inserted in the beginning of the "Experimental Methods"	12	The relevant phrases have been relocated accordingly.

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## Radiolabelling of TiO<sub>2</sub> nanoparticles for radiotracer studies

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**Abstract** Industrially manufactured titanium dioxide nanoparticles have been successfully radiolabelled with  $^{48}\text{V}$  by irradiation with a cyclotron-generated proton beam. Centrifugation tests showed that the  $^{48}\text{V}$  radiolabels were stably bound within the nanoparticle structure in an aqueous medium, while X-ray diffraction indicated that no major structural modifications to the nanoparticles resulted from the proton irradiation. *In vitro* tests of the uptake of cold and radiolabelled nanoparticles using the human cell line Calu-3 showed no significant difference in the uptake between both batches of nanoparticles. The uptake was quantified by Inductively Coupled Plasma Mass Spectrometry and high resolution  $\gamma$ -ray spectrometry for cold and radiolabelled nanoparticles, respectively. These preliminary results indicate that alterations to the nanoparticles' properties introduced by proton bombardment can be controlled to a sufficient extent that their further use as radiotracers for biological investigations can be envisaged and elaborated.

**Keywords** Nanoparticles · radiolabelling · titanium dioxide · *in vitro* · cell uptake

## 1 Introduction

Nanotechnology and nanoparticles hold enormous potential in many areas, from environmental remediation, to energy efficiency, novel consumer products and more efficient treatment of disease. However, many of the same physico-chemical properties that give nanomaterials such promising possibilities, also open the possibility that they could have adverse effects on human health and the environment (Balbus et al. 2007). For example the ability of certain nanoparticles (NPs) to penetrate through cell membranes and into the cell nucleus (Pantarotto et al. 2004), to bind to and potentially damage DNA (Zhao et al. 2005) makes them a promising vehicle to deliver therapeutic agents

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into specific cells and body compartments (Kam et al. 2005, Florence and Hussain 2001). On the other hand, the possibility that industrially fabricated NPs, which are released into the environment from consumer products or waste, and that unintentionally penetrate into and accumulate in the body, and/or pass through biological barriers or cell membranes could turn the benefit of this new technology into a major risk (Balbus et al. 2007, Reijnders 2009). Since the exploitation of nanoparticulate materials for commercial applications is rapidly increasing (e.g. OECD 2008, Roco 2008, Aitken et al. 2006, Anselmann 2001), as is the concentration of NPs in the environment (Handy et al. 2008a, Wiesner et al. 2006, Owen and Depledge 2005, Kreyling et al. 2004), a sound risk assessment of this new technology is overdue (Keller 2007, Maynard et al. 2006). Indeed, governments, industries and research organizations are beginning to address how the benefits of nanotechnologies can be realized while minimizing their potential risks (Maynard et al. 2006, OECD 2006). However this attempt is hampered by scientific knowledge gaps. The lack of toxicological data and the absence of adequate testing methodologies has triggered much research world-wide on *in vitro* and *in vivo* testing methods in order to assess possible health effects of NPs to humans and has created a new scientific term: nanotoxicology (Oberdörster et al. 2005).

The mechanistic basis of exposure and effect are poorly understood in many cases (Handy et al. 2008b) and experimental methods to investigate absorption, distribution, metabolism and excretion of NPs are incomplete or insufficient. Recent investigations of the toxicity of nanomaterials in cell cultures and animals have shown that size, surface area, surface chemistry, solubility and shape may all affect the toxicological potential of NPs (Poland et al. 2008, Maynard et al. 2006, Oberdörster et al. 2005). In addition, it has been observed that NPs can translocate from the tissue where they have been absorbed to other target tissues adding further complexity to the assessment of their

potential toxicity (Semmler-Behnke et al. 2008, Oberdörster et al. 2005). In this context the development of fast and reliable *in vitro* and *in vivo* test methods is essential, and for this purpose the use of radiolabelled NPs as tracers can be very advantageous. Radiolabelling of NPs for biological and toxicological experiments is however a challenge because modifications of their physical, chemical, and surface properties have to be avoided in order to exclude unwanted effects on their bio-distribution and cell uptake characteristics. Moreover for certain studies regarding the safety of industrially fabricated NPs the radiolabelled particles should not be synthesized in laboratory experiments from radioactive precursors but should be used as they emerge from the industrial production process. Under such circumstances the only way of radiolabelling, apart from chelation of a suitable radiotracer which might modify the NP behaviour or indeed present stability problems, is via neutron or ion bombardment of the NPs in reactors or accelerators. Both methods involve subjecting the NPs to significant radiation fields and high rates of energy deposition.

The present work deals with radiolabelling of NPs by irradiation with a proton beam produced by a cyclotron and the comparison of the uptake of radiolabelled and cold (as received) NPs *in vitro* using a human cell line. Industrially fabricated TiO<sub>2</sub>-NPs have been examined due to their favourable physical properties and because protocols for *in vitro* testing using radioactive and cold NPs have been developed earlier to assess their skin toxicity, since they are used as an important component of sun creams (Di Giacchino et al. 2007, Di Giampaolo et al. 2004). It can therefore be expected that the *in vitro* cell uptake obtained with cold and proton irradiated TiO<sub>2</sub>-NPs can be compared with high accuracy. Moreover, recent studies on the cytotoxicity of TiO<sub>2</sub>-NPs in fish (Handy et al. 2008b, Vevers and Ija 2008) raise questions about their ecotoxicological risk, and the large quantities involved in industrial production processes suggest

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that a further assessment of some aspects of airborne exposure in occupational health is necessary (Liao et al. 2009, Garabant et al. 1987).

The *in vitro* model chosen for this study was Calu-3, a human cell line derived from a bronchial adenocarcinoma (Forbess 2000). Calu-3 is known as a good cell model of the airway epithelium, as cells are able to form a polarized confluent monolayer with tight junctions and the production of mucous under air-interfaced culture conditions (Grainger et al. 2006, Steiner et al. 2005, Wan et al. 2000, Berger et al. 1999). The presence of the main characteristics of the airway epithelium in Calu-3 suggests the relevance of this cell line as *in vitro* model for studying the effect of micro- and nano-sized particles on the lung.

## 2 Experimental Methods

### 2.1 Preparation of irradiated TiO<sub>2</sub> nanoparticles

Titanium dioxide (TiO<sub>2</sub>) in nanoparticulate form is manufactured worldwide in large quantities for use in a wide range of applications, from paint to sunscreen, to food coloring. Two types of TiO<sub>2</sub>-NPs have been proton irradiated. The first batch was P25 (Degussa) with a stated average primary particle size of 21 nm. This batch was used for initial determinations of activation yield, and a small amount was also used for XRD (X-ray diffraction) comparison of as-received and proton irradiated material to examine if the irradiation caused gross structural changes to the sample. Whereas phase changes can be detected by XRD due to additional diffraction peaks appearing in the XRD spectra, radiation damage will manifest in a slight broadening of the XRD peaks. Additionally the full height half width of the XRD peaks depends inversely on crystal or grain size, i.e. in the present case on the NP size. In order to be able to recognize

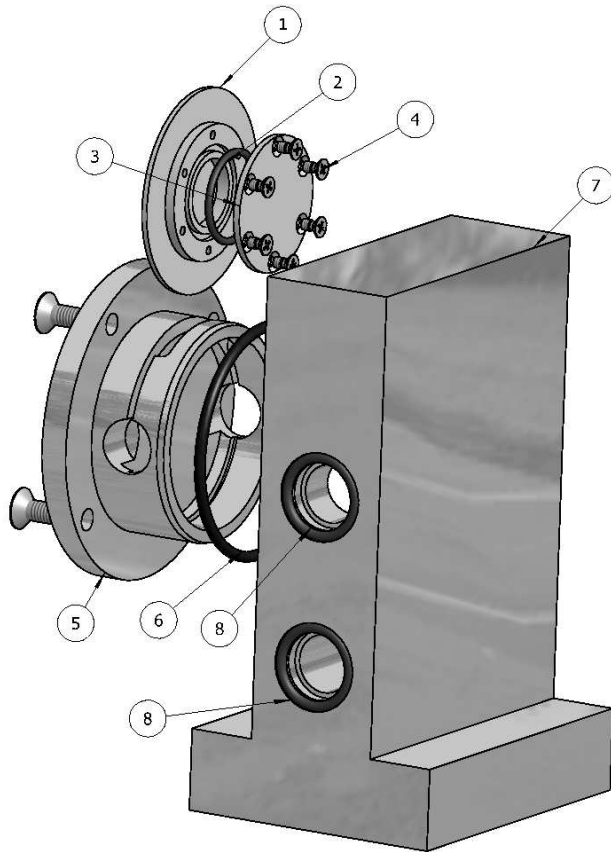
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radiation damage and to discern it from the effect of small particle size, narrow XRD peaks and thus larger NPs are preferred to examine this problem. More interesting for the envisaged radiotracer applications are however particles smaller than 20 nm. Therefore, a second type TiO<sub>2</sub>-powder of 99.9% purity purchased from Alfa Aesar (Johnson Matthey) with a supposed size of 5 nm was activated. An XRD scan on this material indicated however an average crystallite size somewhat larger, between 15 nm and 20 nm. This means that the particle size used to address the alteration of the NP properties and that foreseen for the cell uptake experiments was so similar that no additional assessment of radiation effects on the Alfa Aesar NPs was required.

The irradiations were performed with the Scanditronix MC 40 cyclotron of the Joint Research Centre (Ispra, Italy), which is able to accelerate positive ions such as protons, deuterons, alphas and <sup>3</sup>He<sup>2+</sup> to variable energies. The radioisotope <sup>48</sup>V with a half-life  $T_{1/2} = 15,97$  d can efficiently be produced by the nuclear reaction <sup>48</sup>Ti(p,n)<sup>48</sup>V and its half-life is suitable for biological tracer experiments. In order to preserve the properties of the NPs during the proton bombardment it is essential to limit the heat load and radiation damage by a reasonable limitation of the particle beam intensity and by assuring efficient cooling. The samples were irradiated in aluminium capsules as shown in Fig.1 with an inner diameter of 10 mm. The capsules were inserted in a holder that allowed direct water cooling from both the rear and the front side. Consequently the most critical cooling problem was the heat transport inside the NP volume to the confining aluminium surfaces.

The cyclotron was set to a proton energy of 25 MeV. Due to the attenuation of the proton beam in the two aluminium windows and the front face water cooling, the available beam energy for the activation of the NPs was reduced to about 15 MeV. This energy still covers the range of the maximum reaction cross section of the <sup>48</sup>Ti(p,n)<sup>48</sup>V





**Fig. 1** The powder capsule (1) is completely filled with  $\text{TiO}_2$ -NPs and sealed with an O-ring (2) when fixing the cover (3) carefully with its screws (4). The capsule holder (5) keeps the capsule in its irradiation position and assures all-around water cooling. The target body (7) is sealed by an O-ring (6) when the capsule holder is fixed in its proper position. The upper O-ring sealed water connection (8) directs the incoming cooling water on the powder capsule; the water flows back through the lower connection (8). All parts are fabricated from pure aluminium that is itself getting less activated by ion beams than Al alloys. The thickness of the components (1) and (5) is reduced to 0.3 mm each within a radius of 5 mm around the beam axis, referred to as *windows*, in order to keep the attenuation of the proton beam in energy and intensity at reasonable values and to allow for an efficient activation of the  $\text{TiO}_2$ -NPs without compromising mechanical stability.

reaction (IAEA 2008, EXFOR database). The beam attenuation inside the TiO<sub>2</sub>-NP volume leads to a temperature increase of the NPs, especially since the density of the NP powder and the thermal conductivity of TiO<sub>2</sub> are low. This implies that the NPs in the center of the volume that have the largest distance from the water cooled surfaces suffer the most. In order to reduce this risk and in view of the small quantities of NPs that are required for biological radiotracer experiments, the volume of the capsule was reduced since shorter distances (higher temperature gradients) facilitate heat transfer, and a more efficient cooling is achieved. Leaving the inner diameter of the capsule constant, its useful thickness was reduced from initially 2 mm to 0.4 mm corresponding to a reduction of the activated TiO<sub>2</sub>-NP mass from 75 mg to about 12-15 mg. Additionally the required activity could be concentrated in a smaller NP volume since protons with attenuated energy and hence reduced reaction cross section deposit their energy now in the well cooled metallic material of the capsule cover (see (3) in Fig.1) after having passed the NP volume, instead of depositing their energy in the NPs while having low activation probability. Therefore, this modification increased both the efficiency of the NP activation and of their cooling.

In a series of test irradiations for fixed time, the proton beam current was escalated and the NP powder was checked for visible alterations afterwards. These tests were stopped at a beam current of 10  $\mu$ A without encountering any visible changes to the powder or any different behaviour when washing the NP powder out of the capsule and preparing an aqueous TiO<sub>2</sub>-NP suspension. In order to reduce radiation exposure during handling of the activated capsule, the whole holder was left for one day of radioactive decay of the mainly short-lived radioisotopes that are co-produced in the aluminium capsule, before the capsule was removed from the beam line.

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For subsequent centrifugation tests and cellular uptake studies 12 mg of the Alfa Aesar material material were irradiated. It should be noted that cold and irradiated particles would be agglomerated to some extent. This would make no difference to the XRD studies, but a large increase in agglomeration would be likely to show up in the cellular uptake studies. For the cellular uptake studies both cold and irradiated samples were suspended in exactly the same way prior to the *in vitro* comparative tests in water and mixed with PBS solution.

## 2.2 Post irradiation tests

The nanoparticle activation level was analyzed by  $\gamma$ -ray spectrometry using high purity germanium detectors calibrated in energy and efficiency by using certified radioactive standard sources. The calibration was performed in the same geometries used for the measurements of the irradiated TiO<sub>2</sub>-NP samples. The  $\gamma$ -ray spectra were analyzed using the Genie 2000 software package (CANBERRA, USA).

The irradiated capsules were handled in a glove box when opened, and activated TiO<sub>2</sub>-powder was washed out of the aluminium capsule and suspended in distilled water. For the NP sample (Alfa Aesar) destined for use in uptake experiments, the obtained suspension of 12 mg of irradiated TiO<sub>2</sub>-NPs in 2 ml of water was transferred into a quick seal vial (Beckman, Italy) and subjected to ultracentrifugation at 41000 rpm at a temperature of 4°C for one hour to check for any leaching or ionic release of the <sup>48</sup>V radiolabel into the aqueous phase.

In order to determine if any structural changes to the NPs were induced by the proton irradiation, X-ray diffraction was performed on the irradiated P25 material both before and after irradiation. The measurements were made on a dedicated glancing

angle diffractometer by mixing a small amount of material with poly(methyl methacrylate), short PMMA, and smearing this onto the surface of a silicon wafer over an area of about  $0.5\text{ cm}^2$ . Because of the high surface sensitivity of the glancing angle XRD technique, the amount of material analyzed could be maintained low enough so that no special precautions regarding sample radioactivity had to be taken during the XRD measurements. This limitation had however the disadvantage that the XRD signal-to-noise ratio was not optimal.

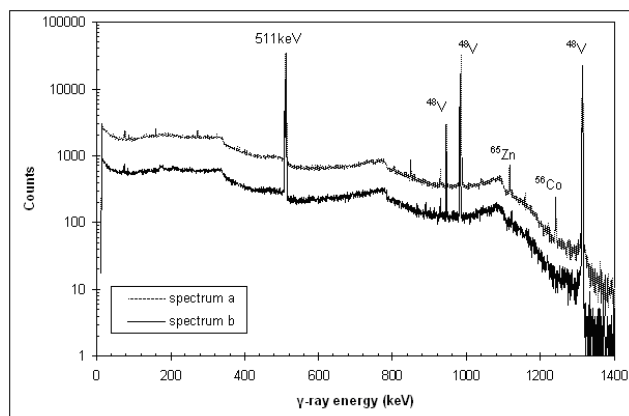
For the present uptake tests Calu-3 cells were chosen because they are readily available, in contrast to primary human cell lines of airway tissue, and differentiate into a monolayer of polarized cells. These cells form tight junctions, produce mucous and express many of the characteristics of human native epithelium despite being derived from a human adenocarcinoma. Calu-3 human bronchial epithelial cell line was purchased from American Type Culture Collection (ATCC, USA). Cells were maintained in Minimum Essential Medium Eagle (Sigma Aldrich), supplemented with 10% heat inactivated fetal bovine serum (Lonza, Italy), 0.1 mM non-essential amino acids (Sigma Aldrich), 1 mM sodium pyrovate (Sigma Aldrich), and 100 I.U./100  $\mu\text{g}/\text{mL}$  Pen/ Strep (Sigma Aldrich). The cells were grown at  $37^\circ\text{C}$  in an atmosphere with 5%  $\text{CO}_2$  in a humidified incubator. For the experiments the cells were seeded on Transwell cell culture supports (Sigma Aldrich) at a density of  $10^5$  cells/ $\text{cm}^2$  in 0.25 mL medium and with 1 mL medium added to the basolateral compartment. Cells were grown in air-interfaced culture for 14 days before the uptake experiments were performed. Cold  $\text{TiO}_2$ -NP or proton irradiated  $^{48}\text{V}$ - $\text{TiO}_2$ -NP suspensions were freshly prepared in phosphate buffered saline (PBS, Sigma Aldrich) and were diluted to appropriate concentrations (200 and 500  $\mu\text{M}$ ) with PBS. The radioactivity concentration expressed in counts per ng of NPs was used to determine the nominal concentrations by radioactive counting.

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At the day of exposure, the freshly prepared NP suspensions were applied apically to the cells and fresh medium was added to the basolateral compartment. After 24 hours of exposure, the cells were trypsinised and centrifuged and the obtained pellet was rinsed twice with PBS.

Percoll gradient (obtained by ultracentrifugation) was used to remove particles that were extracellular and not bound to the cell membrane. Finally, viable cells were counted using a hemacytometer, then the  $\text{TiO}_2$  and  $[^{48}\text{V}]\text{-TiO}_2$ , i.e. the NP uptake, was determined by ICPMS (Inductively Coupled Plasma Mass Spectrometry) and high resolution  $\gamma$ -ray spectrometry, respectively. The ICPMS instrument used was an ICPMS SCIEX ELAN DRC II (Perkin Elmer) equipped with a Dynamic Reaction Cell (DRC). 99.99% pure argon (Air Liquide) and anhydrous  $\text{NH}_3$  of 99.99% purity (Sigma Aldrich) were used for the ICPMS and the DRC, respectively. The analysis was done in 2-3%  $\text{HNO}_3$ . Before the instrumental analysis, the samples were mineralized in a microwave furnace (CEM-MSD 2000). In order to correct for matrix effects due to the different sources of samples and calibration standards 5 ppb of Re was added as internal standard to all biological samples, blank specimens and calibration standards.

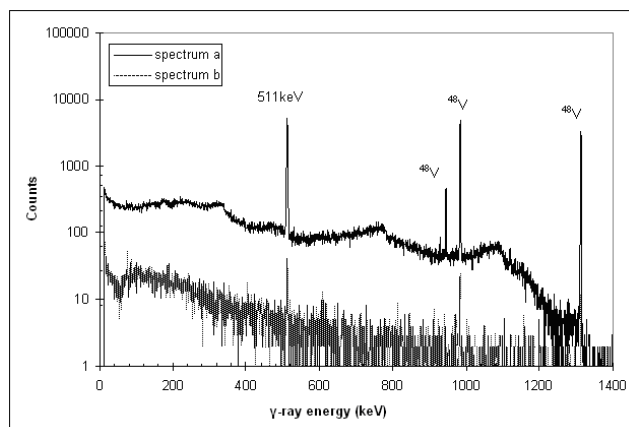
The uptake of  $[^{48}\text{V}]\text{-TiO}_2$  was determined by high resolution  $\gamma$ -ray spectrometry by quantifying the radioactivity of  $^{48}\text{V}$  emitted from the radiolabelled  $[^{48}\text{V}]\text{-TiO}_2$ -NPs using a high purity germanium detector. The acquisition and analysis of the  $\gamma$ -ray spectra was carried out by specific software (Nuclear Elements Digital Analysis, NEDA, Ascom, Milano). The measurements of the radiation emitted from the  $[^{48}\text{V}]\text{-label}$  are expressed in counts per minute (cpm) and converted into mass of  $[^{48}\text{V}]\text{-TiO}_2\text{-NPs}$ . The calibration factor for this conversion was determined from a comparison with standard suspensions of well known concentration of  $[^{48}\text{V}]\text{-TiO}_2\text{-NPs}$  and activity of  $^{48}\text{V}$ .



**Fig. 2**  $\gamma$ -ray spectra (spectra a and b) of the proton irradiated  $\text{TiO}_2$ -powder. Spectrum a) refers to the aluminium capsule containing the NPs, spectrum b) refers to the  $\text{TiO}_2$ -NPs removed from the capsule. The  $\gamma$ -ray peaks of  $^{48}\text{V}$  are well resolved in both spectra. The  $\gamma$ -ray peaks of  $^{56}\text{Co}$  and  $^{65}\text{Zn}$  visible in (a) are due to the impurities in the aluminium the capsule is made of. The recovered NPs (spectrum b) are not contaminated with such impurities.

### 3 Results and Discussion

Fig. 2 shows the  $\gamma$ -ray spectra of the proton irradiated aluminium capsule containing the Alfa Aesar  $\text{TiO}_2$ -NPs and the spectrum of these  $\text{TiO}_2$ -NPs after they were removed from the capsule, respectively. The  $\gamma$ -ray peaks of radioisotopes such as  $^{65}\text{Zn}$  and  $^{56}\text{Co}$  result from the activation of impurities in the aluminium material of the capsule. They are not present in the spectrum of the [ $^{48}\text{V}$ ]-labelled  $\text{TiO}_2$ -NPs extracted from the capsule. Under the present irradiation conditions with a beam intensity of  $10\ \mu\text{A}$  about 850 kBq of  $^{48}\text{V}$  were obtained within 45 minutes of proton bombardment of 12 mg of  $\text{TiO}_2$ -NPs. This activity concentration is more than enough for the *in vitro* uptake studies reported here. However, higher activities - up to 1 MBq/mg or more may be required for certain studies. Fig. 3 presents two  $\gamma$ -ray spectra of the  $\text{TiO}_2$ -NP pellet and the aqueous phase after ultra-centrifugation, respectively.



**Fig. 3**  $\gamma$ -ray spectra (spectra a and b) obtained after ultracentrifugation of the colloidal  $\text{TiO}_2$ -NP suspension. Spectrum a) refers to the  $\text{TiO}_2$ -pellet while the spectrum b) refers to the supernatant which shows only a minor fraction of the  $^{48}\text{V}$  activity.

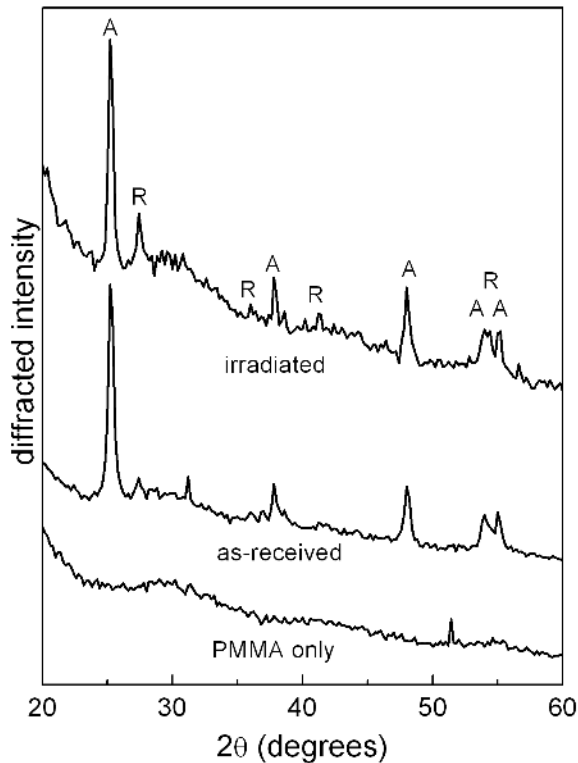
The quantitative evaluation of the  $\gamma$ -ray spectra of the pellet and the aqueous phase after suspending the NPs in water and subsequent ultracentrifugation shows that only a tiny fraction of less than 1% of the  $^{48}\text{V}$  activity was not retained in the NPs. The issue of radiolabel stability as a function of crystalline structure, primary particle size, and possible irradiation damage is a complex one however, and is best addressed on a case-by-case basis by experimental determination of radiolabel loss in appropriate environments. The simple test performed here indicated that in an aqueous environment only a little of the  $^{48}\text{V}$  was detached from the NPs. However, for follow-up studies in other environments, more suitable leaching tests would be appropriate.

Fig. 4 shows the XRD patterns obtained from the irradiated and non-irradiated P25 NPs, together with the background pattern due to the PMMA used to fix the NPs onto the Si surface. As expected the P25 material is composed mainly of anatase with a minor fraction of rutile (roughly 10% in this sample). There are no obvious major structural changes to the P25  $\text{TiO}_2$ -NPs caused by the irradiation, though minor dif-

ferences in relative peak heights indicate that a small increase in the fraction of rutile (to roughly 20%) could have occurred. Due to the small amount of material analyzed and the resulting suboptimal signal-to-noise ratio of the XRD scans, this observation needs to be further investigated. Computer simulations of radiation displacement damage (see below) indicate that this is unlikely to account for any structural change. But at high temperatures anatase converts to rutile, so the observed changes indicate some local heating of the NPs during irradiation. The thicker capsules initially used for the P25 irradiations with a large amount of material in the activated volume were in fact not optimal regarding cooling, so localized heating could have occurred in the center of the capsule volume, accounting for some anatase to rutile transformation. The cooling of the Alfa Aesar NPs that were activated in the thinner capsules, with much smaller sample volume and a much shorter beam path through the NPs, was much more efficient. Currently, even more efficient geometries for sample cooling are being developed, and further investigations of structural changes to NPs of different primary particle sizes, induced by ion beam irradiation over a range of different irradiation parameters, are warranted, given the sensitivity of NPs to increased temperatures. This holds in particular if higher activity concentrations are required for subsequent experiments, as would be the case for some *in vivo* biokinetics studies.

Table 1 summarizes the average uptake of cold and radiolabelled NPs in Calu-3 cells under the conditions reported in Sect. 2.2. The data obtained at each concentration are the average of three independent uptake experiments with three replicas for each concentration, with the standard deviation being presented as the measurement error. The results show that the radiolabelling by proton irradiation of the TiO<sub>2</sub>-NPs did not significantly alter the uptake behaviour in Calu-3 cells for both concentrations used. Within the experimental error, the results on the non-irradiated and irradiated





**Fig. 4** X-ray diffraction patterns of as-received and proton irradiated P25 TiO<sub>2</sub>-NPs. Also shown is the background due to the PMMA used to fix the TiO<sub>2</sub>-NPs onto a Si surface. "A" and "R" indicate the positions of major anatase and rutile peaks, respectively. The patterns have been normalized to the same intensity value of the main anatase peak at 25.3° for easy comparison.

materials are the same. We conclude that the irradiation did not massively affect the state of agglomeration of the NP powder. This should become visible in a significant difference between the uptake of cold and radioactive NPs otherwise treated in exactly the same way. However, it cannot be excluded that small differences caused by the irradiation are still hidden within the given error margins. Nevertheless, our results serve as a first demonstration that proton irradiation is a viable technique for nanoparticle

**Table 1** Comparison of the uptake of cold TiO<sub>2</sub>- and radiolabelled [<sup>48</sup>V]-TiO<sub>2</sub>-NPs in Calu-3 cells in pg of TiO<sub>2</sub> per cell after exposure to NP suspensions of different concentrations. The error margin is given by the standard deviation of the average uptake determined in three independent experiments and three replicas for each concentration, respectively.

concentration	Uptake	
	pg TiO <sub>2</sub> / cell	
	TiO <sub>2</sub>	[ <sup>48</sup> V]-TiO <sub>2</sub>
200 μM	1.15 ± 0.12	0.96 ± 0.11
500 μM	2.25 ± 0.33	1.98 ± 0.2

radiolabelling, and that under the conditions used, no significant differences in uptake were observed. Further studies on a variety of different NP types are under way using a range of characterization methods in order to carefully study to what level the NPs may be activated before significant changes to the NP structure or *in vitro* behaviour are observed.

With regard to theoretical considerations, calculations of radiation damage based on a full cascade calculation, using the SRIM (Version 2008.03 by Ziegler et al. 2008), indicate that the direct displacement damage expected due to ion collision should be of the order of  $6 \cdot 10^{-4}$  dpa (displacements per atom) for an irradiation of 45 minutes at 10 μA at a proton energy of 15 MeV (the incident beam energy is reduced from 25 MeV to 15 MeV by the aluminium windows and water cooling), in a target with 400 μm thickness and an assumed density of 0.5 g/cm<sup>3</sup>. This reflects therefore the approximate amount of damage calculated for the activity level used for the uptake experiments reported here, and would not be expected to induce any major structural change. However, if much higher [<sup>48</sup>V]-TiO<sub>2</sub> activity levels are required, then damage

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levels might occur where several % of the NP atoms are displaced from their lattice positions. Likewise, if other NP types with lower activation cross sections, or irradiation with other particles such as deuterons or alphas is necessary, than it is clearly important to calculate and perhaps experimentally determine any possible structural damage to the NPs before using them for subsequent experiments. If thick targets are used where the ion beam is fully stopped in the nanoparticulate material, then the direct radiation damage near the end of the ion range may be several times higher than at the start of the trajectory. Additionally, the obtainable activity concentration of the irradiated material will be much lower because a considerable fraction of the NPs will be irradiated far from the energy of maximum reaction cross section. Furthermore, significant heating can be expected to occur within the sample. Such a situation is clearly undesirable.

Another important consideration relates to the kinetics involved in the activation reaction. Conservation of momentum means that it is most probable that any radiolabels produced during the irradiation will not in fact remain on the lattice site occupied by the target atom involved. While this is not necessarily true for all reactions, it can be expected that in the majority of cases the radiolabels are either displaced within the target nanoparticle or are in fact ejected from their source nanoparticle and implanted into a nearby one. Stability of the radiolabel within the nanoparticle therefore is an important issue, and it is important to determine if any leaching of activity occurs in the environment associated with any subsequent experiments. In this study, we limited ourselves to simple centrifugation studies in water to determine if the  $^{48}\text{V}$ -radiolabels were well bound to the  $\text{TiO}_2$ -NPs. In experiments going beyond the present simple uptake studies, e.g. for much longer exposure times or intracellular distribution or even more in *in vivo* studies, significant leaching would become a major concern and

it is highly important to establish the stability of the radiolabelled NPs in appropriate media on longer time scales.

We activated un-coated non-functionalised dry TiO<sub>2</sub>-NP powder, which is expected to be rather resistant to radiation or thermal damage. Direct ion-beam activation of liquid suspensions or of NP samples with organic coatings or functional layers will pose additional problems related to a lower incidence of "re-implantation" of radioactive recoil nuclei in adjacent NPs, lower achievable activity concentrations, radiation damage to the organic component which is much more radiation sensitive than the non-organic core, and possible interaction of the NPs with the liquid medium under irradiation.

#### **4 Conclusions**

In the present study we have demonstrated that industrially fabricated TiO<sub>2</sub>-NPs can successfully be radiolabelled with <sup>48</sup>V, to useful activity levels for radiotracing purposes, by proton irradiation. The *in vitro* uptake study in Calu-3 cells shows that the NP properties that determine the cell uptake can essentially be preserved at the activity level used. For higher activity concentrations, which may easily be achieved by longer irradiations and/or higher beam currents it will be important to carefully control any possible thermal heating of the samples under irradiation, as well as investigating whether direct structural radiation damage occurs. The characterization methods we applied here were limited to those available in the cyclotron controlled area. It should be noted that several particle characteristics other than crystalline structure might affect *in vitro* or *in vivo* studies with radiolabelled NPs, including state of agglomeration, surface charge and dissolution rates. The present work demonstrates the feasibility of radiolabelling NPs by proton bombardment. Future work will address a systematic

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study of the activation of different NP types, additionally employing other characterization methods such as DLS and zeta-potential determination, as well as radiotracer stability studies under different conditions.

In summary, our results demonstrate that dry TiO<sub>2</sub> NPs can be radiolabelled by direct proton irradiation in a suitable fashion for *in vitro* cellular uptake studies and other applications that require limited activity concentrations. For other NP types, activity concentrations, and applications, it will be important to examine on a case by case basis if the radiolabelled material is effectively equivalent to the non-activated material for the required experiments. In addition, stability of the radiolabels within the NPs should be determined by appropriate tests for each application.

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