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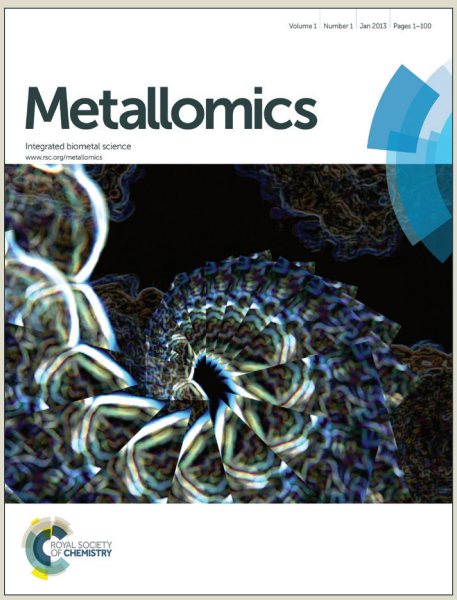

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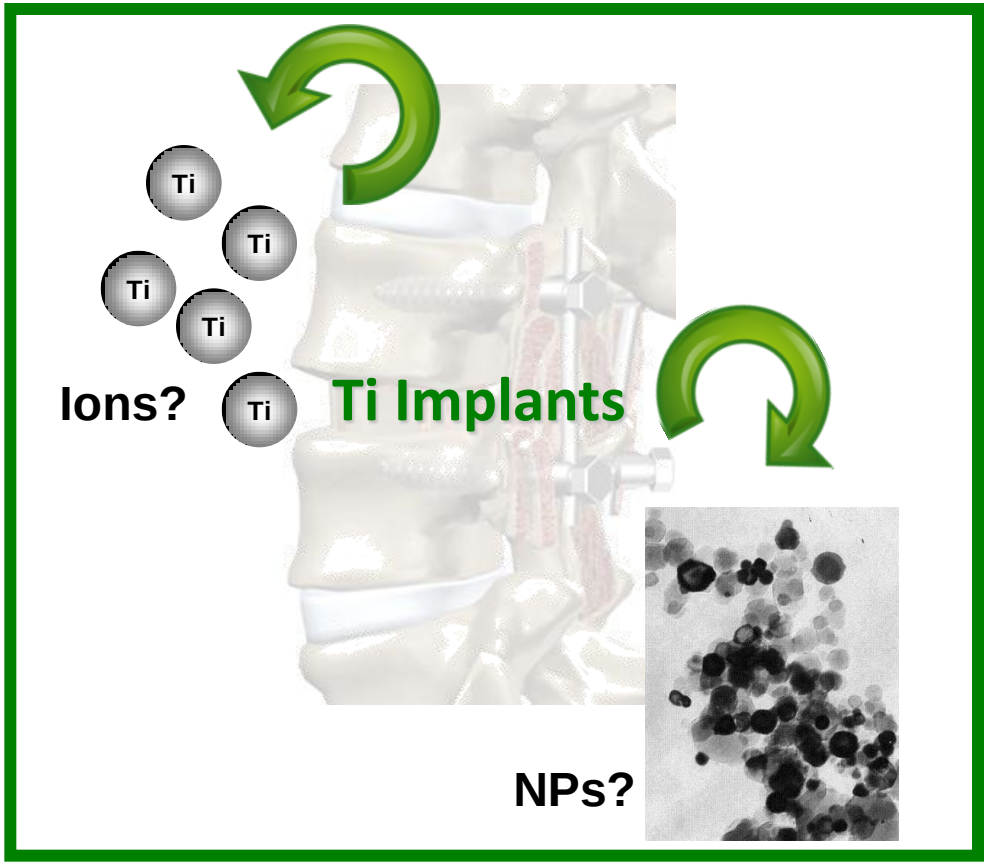

EVALUATION OF THE BIOLOGICAL EFFECT OF Ti GENERATED DEBRIS FROM METAL IMPLANTS: IONS AND NANOPARTICLES.

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ABSTRACT

Metallic implants placed in human's exhibit wear and corrosion that yield in the liberation of metal-containing by-products. In the case of titanium (Ti) containing implants, the metal containing debris may exist in a number of states, including metallic particles produced by mechanical wear and the products of metal corrosion in biological environments, such as the joints and surrounding fluids and tissues. In addition, these constituents may dissolve in both intracellular and extracellular solutions generating Ti ions. Both species, ions and nanoparticles show different cellular toxicity. In this work we have evaluated the possible evolution of TiO_2 nanoparticles (NPs) to soluble Ti metal ions by contact with biological fluids. For this aim, an *in vitro* study to address quantitative Ti solubilisation from TiO_2 nanoparticles (with a diameter of 21 nm) after incubation with human serum at different concentrations has been conducted. Total Ti determination revealed low solubilisation rates ranging from 0.53 to 0.82 % after just one week of incubation in the serum. The incubated serum was then subjected to speciation analysis by anion exchange liquid chromatography using inductively coupled plasma mass spectrometry (ICP-MS) as elemental detector for Ti monitoring. The obtained results revealed a significant increase in the Ti signal associated to the fraction of the protein transferrin and preferentially to one of the metal binding sites of the protein, the N-lobe. Thus, the effect of Ti at the cellular level has been evaluated by considering that it can be present either as ions or as nanoparticles using two different cells lines: human enterocytes HT29 and murine osteoblasts MC3T3. Significant toxicity was found at the highest concentration assayed (50 $\mu g\ mL^{-1}$) for both Ti species (ions and NPs) and slightly higher for the ionic species at lower concentrations (1 and 10 $\mu g\ mL^{-1}$).

Key-words: titanium, TiO_2 nanoparticles, Ti(IV) ions, toxicity, ICP-MS, HPLC.

INTRODUCTION

Immediately after implantation in the human body all implants start to degrade through two mechanisms: corrosion and wear. Wear is a mechanical/physical degradation of materials (producing particles) whereas corrosion is a chemical (or electrochemical) form of degradation (mainly producing solubilized metal ions).[1] Thus, the use of titanium alloys for biomedical applications has introduced the possibility of generating microscopic Ti particles together with Ti metal ions that are present in the tissues of the surrounding implants (peri-implant environment).[2,3] The particles are commonly of nanometer size (nanoparticles, NPs) and activate macrophages that increase bone absorption and, eventually, inflammatory reactions leading to the implant failure.[4] In addition, there is growing evidence that metal debris can directly affect cells of the osteoblast linage.[5, 6] As previously stated, all metal implants *in vivo* undergo corrosion which results also in the additional production of metal ions that reach, eventually, the circulatory system. The behavior of metal ion released into biofluids can be diverse, showing combination with biomolecules (e.g. proteins) or with a water molecule or an anion to form an oxide, hydroxide, or an inorganic salt.[7] Metal liberation from metallic implants has been reported in the body fluids of patients carrying metal on metal total hip replacements,[8] intramedullary nails[9] and, more recently, in patients undergoing instrumented spinal arthrodesis.[10] Locally, these metal ions might also enter the cells and alter intracellular processes.[11 12]

As previously described, the release of metal ions and nanoparticles to the peri-implant environment should not be considered as independent phenomena since they might occur simultaneously. In fact, metal ions detected in the biological fluids of patients carrying metallic implants can be additionally coming from the partial dissolution of released NPs.[13] Although few documents exist on this topic, the formation of metal ions

from dissolution of NPs has been documented by several authors, for instance, having Ag or NiO-NPs.[14, 15] In these cases, the rate of dissolution depends not only on the type of nanoparticle but also on the particle size, concentration and surface functionalization. In addition, the temperature and the nature of the immersion medium (e.g., the presence of salts or binding biomolecules) will be major factors affecting the dissolution of NPs. In the case of Ti alloy implants, the production of nanoparticles (<50 nm) has been observed in the synovial fluids and peri-prosthetic tissues of patients.[16] The Ti ions dissolved from the oxide nanoparticles (e.g. TiO_2) originated from wear as well as the Ti ions directly produced by corrosion of the implant will influence biological characteristics of the cells in contact with the peri-implant environment; thus, it is necessary to measure the ion concentration in the nanoparticle dispersion. Particularly the determination of released metal ions from nanoparticles dispersion is very important for the evaluation of the cytotoxicity of nanoparticles.[17]

Thus, the present work tries to evaluate, comparatively, the toxicological effect of Ti ions and Ti nanoparticles, which might be released from alloy implants in cell cultures of murine osteoblasts (bone cells). However, in order to evaluate if both situations can be treated independently, it is also necessary to address the putative presence of Ti ions released from titanium dioxide nanoparticles (TiO_2) once in contact with biological fluids (e.g. human serum). For this purpose, TiO_2 NPs (21 nm) were dispersed in human serum and after some days of incubation with mechanical stirring, the metal ions released were evaluated by total Ti determination using ICP-DF-MS (inductively coupled plasma-double focusing-mass spectrometry). Further studies to elucidate if some of the released Ti is associated to the main serum proteins metal speciation were conducted by anion exchange chromatography coupled on-line to ICP-DF-MS. In order to assess differential cell toxicity, proliferation and viability tests were used to evaluate the effect of TiO_2 NPs

and Ti (IV) ions in murine MC3T3 osteoblasts (bone cells that be directly exposed to the ions or particles produced during the implant surface degradation) and in HT29 enterocytes (intestinal cells and main route of absorption of most metals in the organisms) that could be in contact with the NPs due to their dissemination in the organism. The study was completed by confirming the incorporation of the TiO_2 NPs in the cells by endocytosis using transmission electron microscopy (TEM).

EXPERIMENTAL

Instrumentation.

A double focusing-sector field ICP-MS (ICP-DF-MS) instrument, ELEMENT II (Thermo Fischer Scientific, Bremen, Germany), was used for the total Ti determination using medium resolving power ($m/\Delta m=3000$). The instrument was equipped with a Meinhard concentric glass nebuliser and with nickel sampler and skimmer cones. Titanium monitoring was conducted at m/z 47 and m/z 49 using standard plasma operating conditions described somewhere else.[9] The transmission electron microscope (TEM) used was a Carl Zeiss (Oberkochen, Germany) (model Libra 120 Plus).

HPLC separations were carried out using a dual piston liquid chromatographic pump (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) equipped with a sample injection valve Rheodyne, Model 7125 (Cotati, CA, USA), fitted with a 100 µL injection loop, and an anion-exchange column, Mono-Q HR 5/5 (50 x 5 mm id, Pharmacia, Amersham Bioscience, Spain). The chromatographic conditions and mobile phases are similar to those previously described.[9] In summary, the separation involves a gradient of ammonium acetate (linear from 0 to 50%B in 15 min) at 1 mL/min. The exact composition of the mobile phases is described below. Before injection, serum was diluted

(1+1) in mobile phase A. This gradient permits the separation of the most abundant serum containing proteins (IgG, transferrin and albumin) with adequate resolution.

Chemicals

TiO_2-NPs (21 nm) were purchased from Sigma Aldrich (St. Louis, MO, USA). A stock suspension of TiO_2-NPs of 1 mg/mL was prepared in deionized Milli-Q water (Millipore Bedford, MA, USA) and sonicated for 15 min in an ultrasonic bath. Further dilutions were prepared for incubation with serum samples or addition to the cell cultures.

Mobile phases for HPLC containing: A) 50 mM tris-aminomethane (Merck, Darmstadt, Germany)/acetic acid (Merck), pH 7.4 and B) A + 1.5 M ammonium acetate (Merck) were prepared by dilution of the solid salts with the 18 MΩcm distilled de-ionized water.

Ti (IV) solubilisation in a compatible form for cellular studies was accomplished by formation of a non commercial Ti-citrate complex with a 1:3 stoichiometry, ($[Ti(cit)_3]^{8-}$) that was formed by using of Ti(IV) tetrachloride and citric acid monohydrate as described somewhere else.[18] The two solutions were mixed and the final pH was adjusted to 2.0 with the slow addition of dilute ammonia. Once the Ti (IV) complex was formed, the addition of the growing media (at physiological pH 7.4) did not originate any Ti visible precipitation.

Samples

For this study, serum samples from patients from the Hospital Universitario Central de Asturias (HUCA) have been provided. Samples were collected in accordance with protocols approved by the relevant institutional review boards (HUCA) and with the Declaration of Helsinki. They were taken in five millilitres vacutainer tubes (Greiner bioone, Madrid, Spain) for trace elements. The precautions taken to avoid metal contamination during blood extraction have been described somewhere else[8] and those

procedures were followed here. Samples were pooled to obtain a large sample volume and divided into different aliquots of 5 mL (for incubation with different TiO_2 NPs). For incubation with different TiO_2 concentrations (to achieve a final Ti concentration around 10, 30 and 50 μg/mL), the serum samples were maintained at room temperature (21 ± 2 ºC) and continuously shaking for 7 days. Then, the nanoparticles were separated by centrifugation of the nanoparticle suspensions for 30 min at 12,000 g and then filtrated through 0.45 μm. The supernatant containing the ions released from nanoparticles was analysed by ICP-MS using a previously optimized methodology based on isotope dilution analysis.9

Cell cultures

MC3T3 murine osteoblasts purchased in Sigma Aldrich were cultured in Alpha-Minimum Essential Medium (α-MEM) (Sigma), supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (PS) in a humidified incubator (37ºC and 5% CO_2) and media was replaced every other day. Cells were seeded in 96 well plates and attached overnight prior exposure to TiO_2 NPs or Ti(IV) solutions. Cells not being incubated with Ti species served as the control group throughout each assay.

Human colorectal adenocarcinoma cell line HT29, originally isolated from a colon cancer in a 72-year-old Caucasian male, was provided by the cell bank of the University of Granada (Spain). It was cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum (FCS), 10,000 units/mL penicillin and 10 mg/mL streptomycin. The cell line was maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were passaged in a solution containing 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma). Similarly to the MC3T3 cell line, the HT29 cells were treated independently with TiO_2 NPs or Ti(IV) solutions as described below for toxicity studies.

Cell viability and cytotoxicity assays

Two different assays have been used to address cellular viability in the different cell lines: the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction and trypan blue ((3Z,3'Z)-3,3'-[(3,3'-dimethylbiphenyl-4,4'-diyl)di(1Z)hydrazin-2-yl-1-ylidene] bis (5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid) assays. The MTT assay is based on the conversion of tetrazolium salt by mitochondrial dehydrogenases and is normally used to address cell viability. This method was complemented with the Trypan blue method also used to determine cell viability. In this latter method, the blue colorant crosses cell membranes only if cell membranes are compromised while intact cell membranes are not bypassed. That is, viable cells are not stained. For these assays a total of 9 x 10^4 cells/mL were seeded in 96-well plates. Cells were grown until confluence. Then, different concentrations of sterile solutions of TiO_2 NPs or Ti(IV) ions containing 0.01, 0.1, 1, 10 and 50 μg/mL were added to each well. Cells were incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2. Solutions containing NPs TiO_2 were placed in an ultrasonic bath for half hour before adding them to the cell cultures in order to minimize aggregation effects. After treatment with the NPs, the medium was changed, and the cells were incubated with 0.5 mg/mL MTT (Sigma-Aldrich) or trypan blue (Sigma-Aldrich) under normal culture conditions for 4 h. Subsequently, the medium was removed and 200 μL DMSO was added to each well. The plates were shaken for 10 min and the absorbance of each solution was measured at 490 nm via a spectrophotometer (BioTek, Elx 800, Winooski, VT, USA). Absorbance values were also corrected with a NP blank.

Hoechst assay was used to determine TiO_2 NP cytotoxicity because trials with more than one assay are recommended to determine cell viability and cytotoxicity. For the Hoechst assay, cells were seeded in a 96-well plate at a density of 25,000 cells/mL of

complete media. They were allowed to attach overnight and then incubated with the solutions of TiO_2 NPs or Ti(IV) ions containing 0, 0.01, 0.1, 1, 10 and 50 μg/mL of metal for the times indicated. After 48 hours, media was removed and cells were frozen at -80°C. After thawing, 100 μL of Milli-Q water was added to each well and cells were incubated 1 hour at RT. After that, cells were frozen once more to ensure cell lysis. Finally, cells were incubated with 20 μg/mL of Hoescht 33258 in TNE buffer (10 mM Tris pH 7.4, 2 M NaCl) for 1 hour at 37°C. Fluorescence was measured in an automatic microplate reader (μQuant, Biotek, Winooski, VT, USA) at 350nm excitation/ 460nm emission filters. Results are the media of six samples.

For cell cytotoxicity experiments, the data were expressed as the mean ± the standard deviation (SD). Data comparisons were performed using a standard analysis of variance (ANOVA). A value of $P<0.001$ was considered significant for the experiments conducted with the MTT, trypan blue and Hoechst assays. Every experiment was repeated at least five to seven times.

TEM studies

For the TEM studies, HT29 enterocytes were cultured at 37ºC in 96-well plates as described above. After 24 h, the cells were treated with TiO_2 NPs (10 and 100 μg/mL, moderately cytotoxic doses) for another 24 h and then fixed with glutaraldehyde and formaldehyde at 4ºC for 1 h. All this experiments followed the sample preparation protocol described by Megías et al.[19]

RESULTS AND DISCUSSION

Ti ion release from TiO_2 NPs

As described in the introduction, metal ions release from nanoparticles can be a cause of concern when evaluating their cytotoxicity since this can be ascribed to two different

sources: the physico-chemical nanoparticles-derived effect due to their high mass specific surface (related to the NPs size) and the effect of dissolved metal ions solubilised from the NPs. In order to evaluate the dissolution power (in terms of ion release) and complexation capabilities of human serum in contact with NPs, TiO_2 NPs (21 nm) were dispersed initially in water and then mixed with human serum at three different final concentrations of 10, 30 and 50 μg/mL. These concentrations were selected by taking into account the literature with respect to the concentration of particles in the peri-implant environment of patients carrying metallic implants.[20] The TEM picture of the mixture (containing 50 μg/mL TiO_2) is shown in Figure 1. It can be seen that even under these conditions the TiO_2 NPs agglomerate in small clusters, as previously described by other authors,[21] where the approximate 21 nm diameter of each particle can be observed. As compared with particles solubilisation in water (data not shown), human serum proved to be superior as dispersing media. The existing literature in this regard is in agreement with this finding and ascribes this fact to the presence of high abundant serum proteins (e.g. albumin) that may all compete for the limited space on nanoparticles surface and so contribute to a better disaggregation of the nanoparticles in solution.[21, 22]

To evaluate metal ion release, triplicates of the dispersed TiO_2 NPs (10, 30 and 50 μg/mL) in serum were analyzed and provided solubilised Ti concentrations that can be seen in Table 1. In this case, the blank of the serum before addition of the TiO_2 NPs contained 1.5 ± 0.3 ng/mL (n=3) of Ti. After incubation, this concentration increased but obtaining released percentages below 1% (in respect to the incubated Ti concentration as TiO_2 NPs) in all cases after 7 days. These results are consistent with the fact that Ti implants (normally covered by a passivation layer to form the oxide, e.g. TiO_2) are relatively inert in the body when compared with other like CrCoMo alloys and therefore only a very small fraction of Ti is released into the biofluids.

One question arising from the previous results regards to the nature of the solubilised Ti after incubation since this could be still due to the presence of dispersed TiO_2 NPs of smaller diameter than the initial ones (e.g. covered by the protein corona) to increase solubilisation. To further address the nature of the observed solubilised Ti, speciation studies using anion exchange chromatography and ICP-MS detection were performed. Thus, the serum samples before and after incubation with the TiO_2 NPs were diluted (1+1) in the mobile phase and injected into the chromatographic system as described elsewhere.[9] Figure 2 shows the chromatographic profile of the serum sample with the different monitored isotopes after incubation (^{34}S, ^{57}Fe and ^{49}Ti using resolving power $m/\Delta m=4000$). This chromatography permits the separation of the most abundant serum containing proteins with adequate resolution (IgG at 2 min, transferrin different isoforms between 18 and 22 min and albumin at 27 min). Here is possible to observe a single Ti peak (dark blue trace) corresponding the retention time of the protein transferrin (Tf) (22 min) in which Fe (green trace) and sulphur (pink trace) can also be observed. As reported in our previous publication,[23] Ti is found here preferentially associated to a single lobe of Tf (N-lobe) in contrast to Fe that shows an equal distribution between lobes (at 20.5 and 22 min respectively).

According to previous post-column isotope dilution experiments,[23] the column recovery for Ti-Tf associations can be considered quantitative. In this case, the Ti detected in the Tf fraction should correspond to a concentration of approximately 78 ng/mL (considering the total Ti concentration determined before and the fact that serum is diluted 1+1 before chromatographic injection). However, the observed Ti signal turned out to be a concentration below 5 ng/mL of Ti (too low even considering the low abundance of the monitored Ti isotope, 5.41%, and the medium resolution necessary to resolve the polyatomic interferences in the ICP-MS). Thus, it appears that the total Ti determined in

solution previously (158 ng/mL) could correspond to both Ti species: metal ions that get complexed with serum components (Tf in particular) and smaller nanoparticles that remained in solution stabilized by the proteins present in that media (they would not elute in the chromatographic system). However, this is the first time, to our knowledge, that the presence of metal ions associated to proteins can be seen in serum after incubation with TiO_2 NPs although at low solubilisation rate. Therefore, the slow solubilisation of TiO_2 NPs in the peri-implant environment could be the cause of the increased Ti concentrations in the blood of patients with Ti implants, which remain relatively constant with time in most of the patients.[9]

Cell viability and cytotoxicity studies

As we have observed, the release of Ti ions can occur either directly from the implant surface or after previous production of nanometre size particles that show a high surface area for electrochemical dissolution. Therefore, the presence of both species (ions and particles) is expected at the bone-implant interface and thus, their toxicity has to be evaluated in cell cultures, particularly, in MC3T3 murine osteoblasts and HT29 enterocytes. For this purpose, the previously used TiO_2 NPs were, in this case, dispersed in the growing media (at different concentrations as described in the experimental section) and the cell viability and proliferation studies were conducted. As previously reported, NPs aggregations were observed also in the cell culture media as can be seen in the corresponding TEM pictures observed for HT29 enterocytes of Figure 3. Figure 3A shows the cells and the cell growing media while Figure 3B and 3C show two magnifications of two specific areas. Figure 3B shows the particles partially agglomerated in the cell medium as well as the interface between the cellular membrane and the growing media where a small vesicle of NPs crossing the cell membrane can be observed (marked with a

red circle). On the other hand, Figure 3C shows clearly the presence of these particles close to the Golgi apparatus containing TiO_2 agglomerates. These TEM observations indicate cellular vesicle formation after treatment with TiO_2 NP as almost all metal oxide NPs can be endocytosed by cells.[24] Endocytosis occurs through different mechanisms, including phagocytosis, pinocytosis and receptor-mediated endocytosis. The formation of a coated vesicle, clearly visible by TEM, indicated that the uptake of TiO_2 NPs occurred most likely via receptor-mediated endocytosis.[6, 25]

In order to asses the level of NPs incorporation into such cells, a mass balance was established for the HT29 cell cultures exposed to the TiO_2 NPs (0.1, 1 and 10 μg/mL). Then, those cells were digested (in HNO_3 + H_2O_2 mixture) and analysed for total Ti by ICP-MS. Incorporation values calculated based on theoretical Ti concentration spiked to the cell media ranged from 2.3 to 3.8% for the three concentrations assayed and considering similar number of cells. Of course, using this ICP-MS methodology can not be differentiated from NPs or Ti metal ions inside the cells. In this regard, analytical methodologies that permit species identification within cell cytosol should be designed and evaluated for a deeper insight into the obtained toxicological data.

On the other hand, the same type of cells were then exposed to solubilised Ti (IV) metal ions in order to compare the possible differential cytotoxicity of species. A challenging part is the required synthesis of a stable Ti(IV) solution that could be added to the cell culture medium for toxicity studies. It is worth mentioning that in the absence of complexing molecules (for instance serum proteins), Ti (IV) tends to hydrolise and precipitate even at low pH values. In order to stabilize the ion in solution, complexation with biologically present anions, e.g. citrate has been recommended by other authors.[18] Thus, Ti(IV) citrate was prepared according to the literature to obtain $[Ti(cit)_3]^{8-}$ (which seems to be stable at a pH range from 5.5 to 7.5) using Ti(IV) tetrachloride as Ti source

and the appropriate excess of citrate and then added to the cell medium. Since 10% heat-inactivated foetal calf serum is present in the medium (which contains high concentration of transferrin), it is very likely that Ti is then forming the Ti-transferrin complex previously detected in human serum. As a negative control, the same concentration of citrate was also added to the cell medium without Ti(IV). The obtained results are summarized in Figure 4 (4A, MTT assay, 4B, Trypan blue and 4C Hoechst assay) for MC3T3 osteoblasts (even if similar results were observed using HT29 enterocytes as well, data not shown here).

The MTT experiments in MC3T3 osteoblasts revealed that the cell viability does not seem to be compromised at the concentrations assayed for TiO_2 NPs. However, there is a significant decrease in the number of viable cells in the case of using $[Ti(cit)_3]^{8-}$ at 50 μg/mL (see Fig. 4A). These results were confirmed by the trypan blue experiments since some authors have claimed that maybe the MTT assay was not suitable to address the cytotoxicity of TiO_2 NPs[26]. Trypan blue results can be seen in Figure 4B for both, $[Ti(cit)_3]^{8-}$ and TiO_2 NPs where we can observe a significant decrease in cell viability at 50 μg/mL (statistically significant, $p<0.001$). In addition, in the case of the trypan blue assay, this effect can be observed already at 10 μg/mL. These results agree well with previous experiments reported by others where cell toxicity was detected also at 5 and 50 μg/mL.[27] It is important to note that Ti concentrations under evaluation were selected here to be physiologically relevant considering the existing literature about the metal released by metallic implant into the peri-implant environment.[28]

Finally, cellular proliferation was assessed by the Hoechst assay and results (Fig 4C) indicate two different effects for TiO_2 NPs: a significant decrease on cell proliferation seems apparent at higher concentrations (10 and 20 μg/mL) for both Ti species while at the lowest concentrations (0.01 μg/mL) a significant increase in cell proliferation, above

the control levels ($p<0.001$), was noticed. Some authors have ascribed these results to the incorporation in the cells, together with TiO_2 NPs, of a protein shell (protein corona) which, in the acidic media of the endosomes might induce the release the adsorbed proteins with a final improvement of cell growing and mitochondrial activities.[22] When the concentration of TiO_2 NPs is higher, this effect can not counterpart the toxicity effect due to the NPs themselves and the cellular proliferation decreases slowly with the concentration.

In the case of the ionic form for Ti, the effects start to be observed at 10 μg/mL but this concentration generates a decrease on cell proliferation of about 40% with respect to the control and goes above 50% at 50 μg/mL. In any case, our cell viability and proliferation experiments indicate that both, dissolved ions and NPs may pose an important toxicity risk at Ti concentration levels of μg/mL, levels that have been reported in some peri-implant tissues.[29] It is important to notice that Ti ions show, in our experiments, higher toxicity to cells than the NPs at similar Ti levels. Thus, the electrochemical corrosion of Ti implants in the human body seems to pose a higher toxicity stress than the mechanical wear products originated by frictions (which are expected to be the main source of Ti NPs).

CONCLUSIONS

The use of combined elemental mass spectrometry (ICP-MS, in particular), spectrophotometry (for cell toxicity) and microscopy (TEM) has permitted to dig a little deeper on the effect of Ti generated debris from metal implants in biological systems. First, the ICP-MS quantitative measurement of the incubated TiO_2 NPs with human serum samples has revealed some solubilisation of the Ti, partially in the form of metal ions and part as small size NPs that remain disaggregated in solution. The released metallic ions

show high affinity for serum transferrin, binding the protein in one of the metal binding sites (the N-lobe) as observed using HPLC-ICP-MS. Regarding cellular toxicity, our studies indicate that in terms of reducing cell viability and proliferation, titanium present as TiO_2 NPs or as Ti (IV) ions did not show differential toxicity risk. Both Ti species cytotoxic effect was concentration dependent and was pronounced at concentrations greater than 5 μg/mL. However, it can not be neglected that the release of ions from NPs produced during wear of implants might contribute to the toxicity associated to the TiO_2 NPs and that both situations are difficult to be discriminated so far.

Transmission electron microscopy experiments have also shown that best solubilisation conditions for TiO_2 NPs includes the addition of protein rich media such as foetal serum with following ultrasonication. In any case, partial aggregation of the NPs in solution can be still detected. Regarding the cellular incorporation of TiO_2 NPs, the complementary use of ICP-MS and TEM permitted to address the rate of incorporation of Ti into the cells (<5%) and also the shape (a coated vesicle) indicating that the uptake of TiO_2 NPs occurred most likely via receptor-mediated endocytosis.

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REFERENCES

[1] J. J. Jacobs, J.L. Gilbert, R. M. Urban. Current concepts review-Corrosion of metal orthopaedic implants. *J Bone Joint Surg (Am)* 1998a, *80*, 268-82.

[2] R.M. Urban, J. J. Jacobs, M. J. Tomlinson, J. Gavrilovic, J. Black, M. Peoc'h . Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip or knee replacement. *J Bone Joint Surg (Am)* 2000, **82**, 457-77.

[3] T. D. Richardson, S. J. Pineda, K. B. Strenge, T. A. Van Fleet, M. MacGregor, J. C. Milbrandt, J. A. Espinosa, P. Freitag. Serum titanium levels after instrumented spinal arthrodesis. *Spine*, 2008, **33**, 792-796.

[4] R. Ramachandran, S. B. Goodman, R. L. Smith. The effects of titanium and polymethylmethacrylate particles on osteoblast phenotypic stability. *J. Biomed. Mater. Res. A*, 2006, **77**, 512–517.

[5] G.J. Atkins, K.J. Welldon, C.A. Holding, D.R. Haynes, D.W. Howie, D.M. Findlay. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. *Biomaterials*, 2009, **30**, 3672–3681.

[6] Y. Zhang , W. Yu, X. Jiang, K. Lv, S. Sun, F. Zhang, Analysis of the cytotoxicity of differentially sized titanium dioxide nanoparticles in murine MC3T3-E1 preosteoblasts. *J. Mater. Sci.: Mater. Med.*, 2011, **22**, 1933–1945.

[7] N. J. Hallab. A review of the biologic effects of spine implant debris: Fact from fiction. *SAS Journal*, 2009, **3**, 143-160.

[8] A. Sarmiento-González, J. M. Marchante-Gayón, J. M. Tejerina-Lobo, J. Paz-Jiménez, A. Sanz-Medel. High-resolution ICP-MS determination of Ti, V, Cr, Co, Ni, and Mo in human blood and urine of patients implanted with a hip or knee prosthesis. *Anal. Bioanal. Chem*., 2008, **391**, 2583-2589.

[9] Y. Nuevo Ordoñez, M. Montes Bayón, E. Blanco González, J. Paz Aparicio, J. D. Raimundez, J. M. Tejerina Lobo, M. A. Peña, A. Sanz Medel. Titanium release in serum of patients with different bone fixation implants and its interaction with serum biomolecules at physiological levels. *Anal. Bioanal. Chem*., 2011, **401**, 1-8.

[10] J. Soto Alvaredo, E. Blanco González, J. Paz Aparicio, A. Sanz-Medel, M. Montes-Bayón, Comparative Ti release from different spinal implants. *Submitted.*

[11] E. P. H. Chan, A. Mhawi, P. Clode, M. Saunders and L. Filgueira. Effects of titanium(IV) ions on human monocyte-derived dendritic cells. *Metallomics*, 2009, **1**, 166–174.

[12] N. J. Hallab, S. Anderson, M. Caicedo, A. Brasher, K. Mikecz, J. J. Jacobs. Effects of soluble metals on human peri-implant cells. *J. Biomed. Mat. Res., 2005*, **74A**, 124–140.

[13] S. Kittler, C. Greulich, J. Diendorf, M. Köller and M. Epple. Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions. *Chem. Mater*., 2010, **22**, 4548–4554.

[14] M. Horie, K. Nishio, K. Fujita, H. Kato, A. Nakamura, S. Kinugasa, S. Endoh, A. Miyauchi, K. Yamamoto, H. Murayama, E. Niki, H. Iwahashi, Y. Yoshida, J. Nakanishi. Ultrafine NiO particles

induce cytotoxicity in vitro by cellular uptake and subsequent Ni(II) release. *Chem. Res. Toxicol*., 2009, **22**, 1415−1426.

[15] T. M. Benn , P. Westerhoff. Nanoparticle Silver Released into Water from Commercially Available Sock Fabrics. *Environ. Sci. Technol.*, 2008, **42**, 4133–4139.

[16] O. Addison, A. J. Davenport, R. J. Newport, S. Kalra, M. Monir, J. F. W. Mosselmans, D. Proops and R. A. Martin. Do 'passive' medical titanium surfaces deteriorate in service in the absence of wear? *J. R. Soc. Interface*, 2012, **9**, 3161-3164.

[17] M. Horie, H. Kato, K. Fujita, S. Endoh, H. Iwahashi. In vitro evaluation of cellular response induced by manufactured nanoparticles. *Chem. Res. Toxicol.*, 2012, **25**, 605-6019.

[18] A.D. Tinoco, A.M. Valentine. Ti(IV) binds to human serum transferrin more tightly than does Fe(III). *J. Am. Chem. Soc*., 2005, **127**, 11218-11219.

[19] J. Renau, L. Megías Eds. Manual de Técnicas de Microscopía Electrónica (M. E.T.). Aplicaciones Biológicas, Editoral Universidad de Granada. ISBN84-338-2470-8.1998.

[20] K. Davda, F. V. Lali, B. Sampson, J. A. Skinner, A. J. Hart. An analysis of metal ion levels in the joint fluid of symptomatic patients with metal-on metal hip replacements *J. Bone. Joint. Surg.* 2011, **93**, 738-745.

[21] Z. Ji, X. Jin, S. George, T. Xia, H. Meng, X. Wang, E. Suarez, H. Zhang, E. M. V. Hoek, H. Godwin, A. E. Nel, J. I. Zink. Dispersion and Stability Optimization of TiO_2 Nanoparticles in Cell Culture Media**.** *Environ. Sci. Technol*., 2010, **44**, 7309-7314

[22] R. Tedja, M. Lim, R. Amal, C. Marquis. Effects of serum adsorption on cellular uptake profile and consequent impact of titanium dioxide nanoparticles on human lung cell lines. *ACS Nano*, 2012, **6**, 4083-4093.

[23] Y. Nuevo-Ordoñez, M. Montes-Bayón, E. Blanco González, A. Sanz-Medel. Titanium preferential binding sites in human serum tranferrin at physiological concentrations. *Metallomics*, 2011, **3**, 1297-1303.

[24] A.L. Di Virgilio, M. Reigosa, P.M. Arnal, M. F.L. de Mele. Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. *J. Hazard. Mater*. 2009, **177**, 711–8.

[25] G. Huajian, S. Wendong, L. B. Freund LB. Mechanics of receptormediated endocytosis. *Proc Natl Acad Sci USA*. 2005, **102**, 9469–74.

[26] I. Pujalté, I. Passagne, B. Brouillaud, M. Tréguer, E. Durand, C. Ohayon-Courtès, and B. L'Azou. Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. *Part. Fibre Toxicol*., 2011, **8**:10.

[27] A.L. Di Virgilio, M. Reigosa, M.F.L. de Mele, *J. Biomed. Mater. Res.* A. Response of UMR 106 cells exposed to titanium oxide and aluminum oxide nanoparticles. 2010, **92A**, 80–86.

[28] C.P. Case, V.G. Langkamer, C. James, M. R. Palmer, A. J. Kemp, P. F. Heap, L. Solomon. Widespread dissemination of metal debris from implants. *J. Bone Joint Surg. [Br]* 1994, **76B**, 701-712.

[29] N.J. Hallab, J.J. Jacobs. Biological effects of implants debris. *Bull. NYU Hosp. Jt .Dis.*, 2009, **67**, 182-188.

LEGENDS OF FIGURES

Figure 1. Transmission electron microscopy (TEM) photograph of solubilised TiO_2 NPs in human serum after sonication.

Figure 2. Titanium speciation in human serum after incubation with TiO_2 NPs and elimination of the remaining particles using anion exchange HPLC-ICP-MS. Sulphur monitoring for protein identification (pale blue trace), titanium (dark blue trace) and iron (pink trace).

Figure 3. Transmission electron microscopy (TEM) photograph of HT29 cells exposed to solubilised TiO_2 NPs (10 μg mL^{-1}). A) Photograph showing the cell cultures and the intracellular space, B and C) magnifications of specific parts of the cell culture.

Figure 4. Studies of cell viability (A and B) and proliferation (C) on MC3T3 murine osteoblasts using different Ti concentrations either as TiO_2 (NPs) and Ti(IV)-citrate complexes. A) MTT assay, B) Trypan Blue and C) Hoechst (*** $p<0.001$).

Table 1. Quantitative analysis of the solubilized Ti (measured by ICP-MS) from TiO_2 nanoparticles (21 nm) incubated in human serum for 7 days at 21 ± 2 ºC.

Ti (incubation solution) (µg/mL)	**Ti (in serum) (ng/mL)**	**% of Ti**
0	1.5 ± 0.3	-
10.7	72 ± 10	0.67
29.8	158 ± 25	0.53
48.6	395 ± 35	0.82

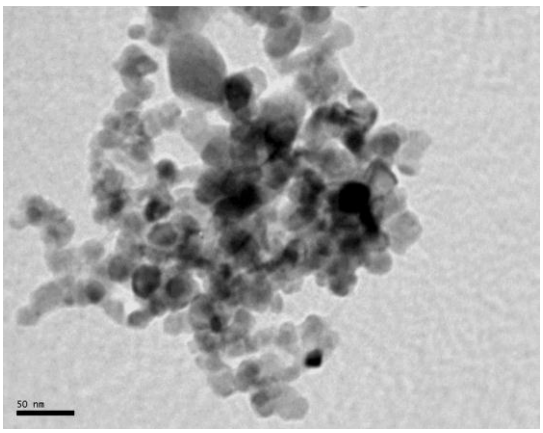

Figure 1

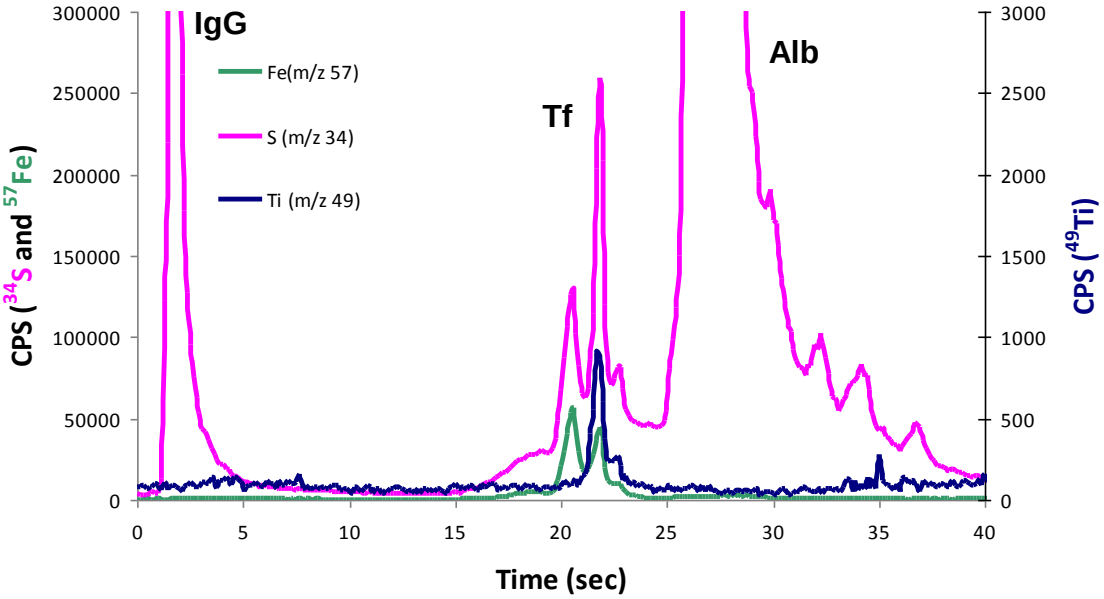

Figure 2

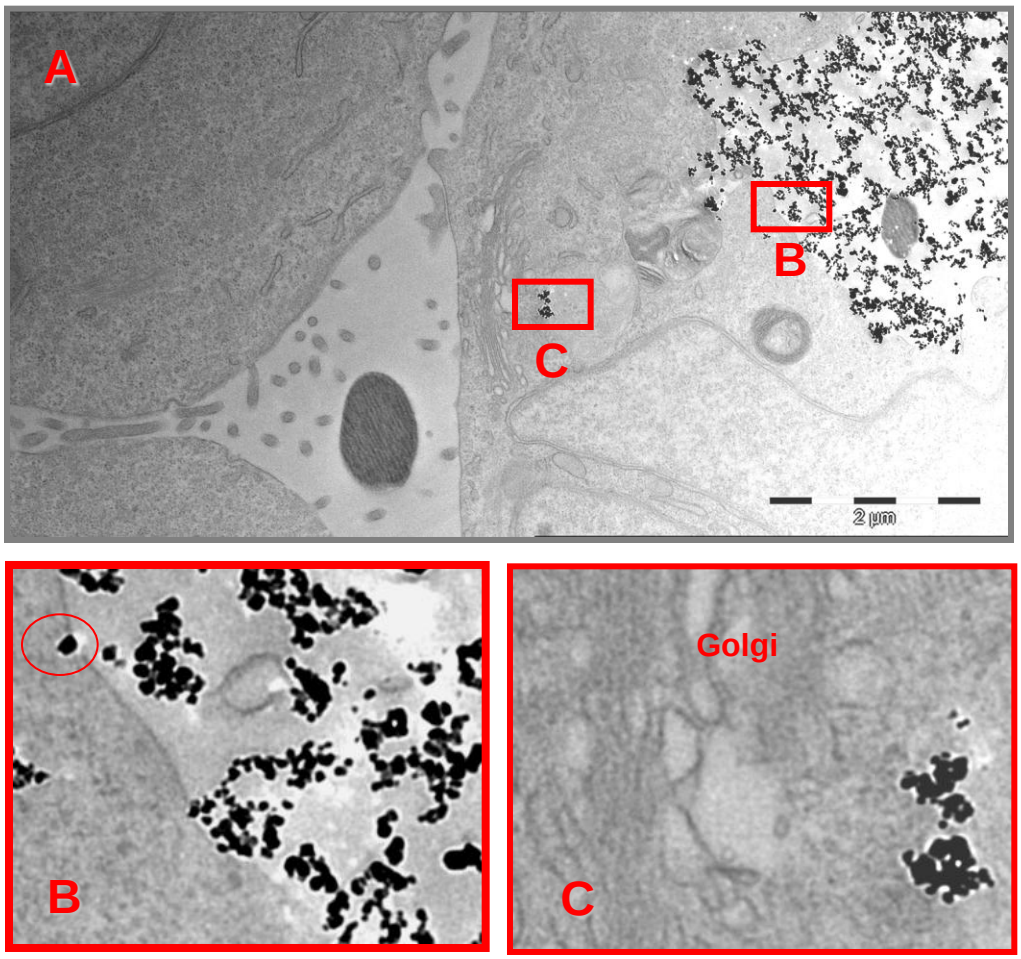

Figure 3

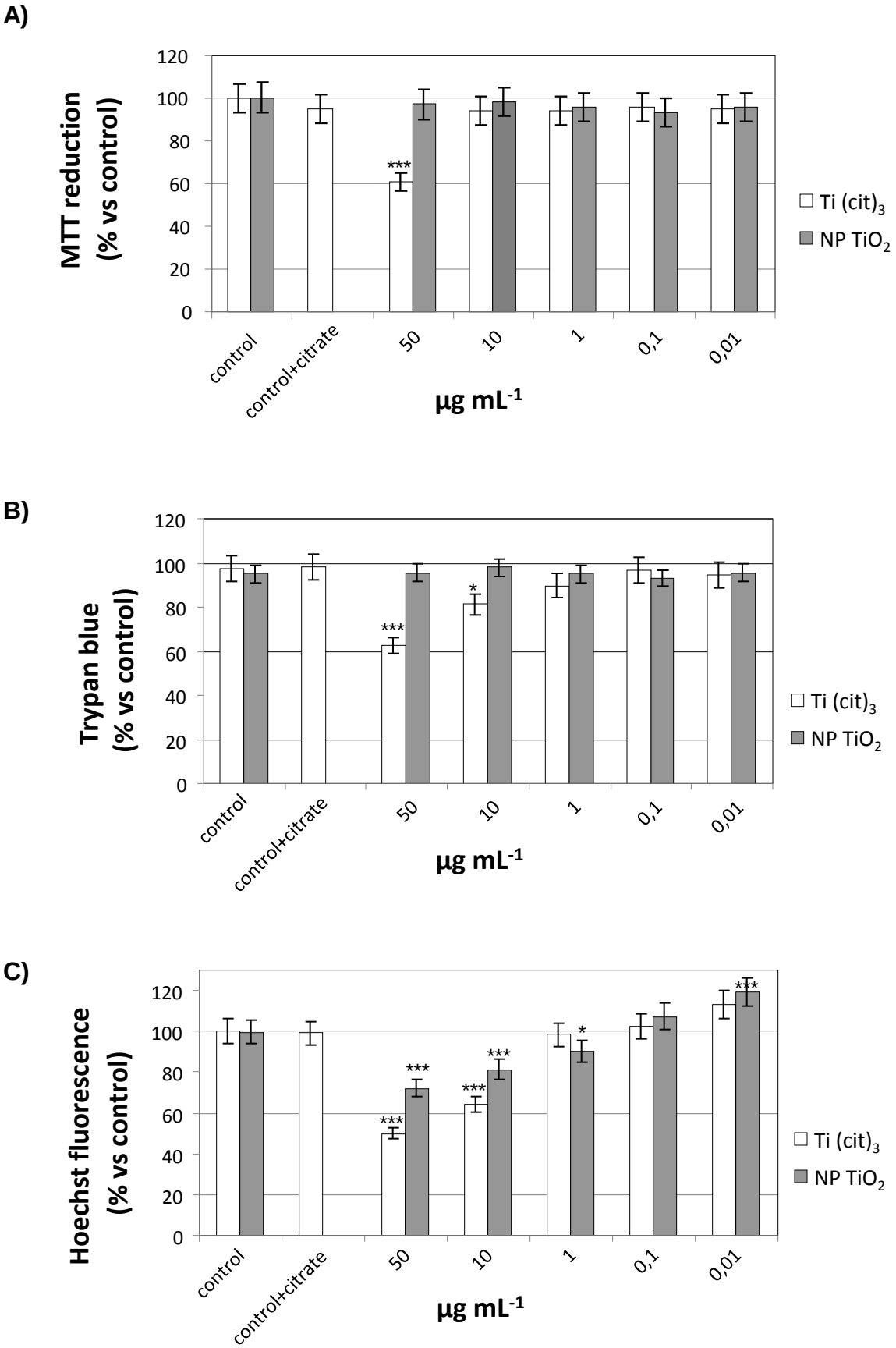

Figure 4