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### ► **To cite this version:**

Rawand Masoud, Tania Bizouarn, Sylvain Trépout, Frank Wien, Laura Baciou, et al.. Titanium Dioxide Nanoparticles Increase Superoxide Anion Production by Acting on NADPH Oxidase. PLoS ONE, 2015, 10 (12), <10.1371/journal.pone.0144829>. <hal-01707694>

**HAL Id: hal-01707694**

**<https://hal.science/hal-01707694v1>**

Submitted on 13 Feb 2018

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RESEARCH ARTICLE

# Titanium Dioxide Nanoparticles Increase Superoxide Anion Production by Acting on NADPH Oxidase

Rawand Masoud<sup>1,2☉</sup>, Tania Bizouarn<sup>1,2☉</sup>, Sylvain Trepout<sup>3,4,5‡</sup>, Frank Wien<sup>6‡</sup>, Laura Baciou<sup>1,2☉</sup>, Sergio Marco<sup>3,4,5‡</sup>, Chantal Houée Levin<sup>1,2☉\*</sup>

**1** Laboratoire de Chimie Physique, UMR 8000, Université Paris Sud Orsay France, **2** CNRS, UMR 8000, Orsay, France, **3** Institut Curie, Centre de Recherche, Centre Universitaire, Orsay, France, **4** INSERM U1196, Centre Universitaire, Orsay, France, **5** CNRS UMR9187, Centre Universitaire, Orsay, France, **6** Synchrotron SOLEIL, L'Orme des Merisiers, Gif-sur-Yvette, France

☉ These authors contributed equally to this work.

‡ These authors also contributed equally to this work.

\* [Chantal.houee@u-psud.fr](mailto:Chantal.houee@u-psud.fr)



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**Citation:** Masoud R, Bizouarn T, Trepout S, Wien F, Baciou L, Marco S, et al. (2015) Titanium Dioxide Nanoparticles Increase Superoxide Anion Production by Acting on NADPH Oxidase. PLoS ONE 10(12): e0144829. doi:10.1371/journal.pone.0144829

**Editor:** Elena A. Rozhkova, Argonne National Laboratory, UNITED STATES

**Received:** July 22, 2015

**Accepted:** November 23, 2015

**Published:** December 29, 2015

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**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** The financial support of ANR 2010-blanc-1536-01 is acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Titanium dioxide (TiO<sub>2</sub>) anatase nanoparticles (NPs) are metal oxide NPs commercialized for several uses of everyday life. However their toxicity has been poorly investigated. Cellular internalization of NPs has been shown to activate macrophages and neutrophils that contribute to superoxide anion production by the NADPH oxidase complex. Transmission electron microscopy images showed that the membrane fractions were close to the NPs while fluorescence indicated an interaction between NPs and cytosolic proteins. Using a cell-free system, we have investigated the influence of TiO<sub>2</sub> NPs on the behavior of the NADPH oxidase. In the absence of the classical activator molecules of the enzyme (arachidonic acid) but in the presence of TiO<sub>2</sub> NPs, no production of superoxide ions could be detected indicating that TiO<sub>2</sub> NPs were unable to activate by themselves the complex. However once the NADPH oxidase was activated (i.e., by arachidonic acid), the rate of superoxide anion production went up to 140% of its value without NPs, this effect being dependent on their concentration. In the presence of TiO<sub>2</sub> nanoparticles, the NADPH oxidase produces more superoxide ions, hence induces higher oxidative stress. This hyper-activation and the subsequent increase in ROS production by TiO<sub>2</sub> NPs could participate to the oxidative stress development.

## Introduction

Titanium dioxide (TiO<sub>2</sub>) nanoparticles (NPs) are metal oxides NPs manufactured in large quantities and commercialized for several uses because of their high stability, anticorrosive and photocatalytic properties [1]. For example, they are present in household products, plastics industry, electronics, pharmaceutical additives and food colorants [2,3]. In nanomedicine, TiO<sub>2</sub> NPs are under investigation as useful tools in advanced imaging and nanotherapeutics [4]. TiO<sub>2</sub> NPs are being explored in cancer diagnosis. They bring many benefits in cancer

therapy by absorbing near infrared light [5], and thus being considered as potential photosensitizers for photodynamic therapy [6]. Very promising is the finding that photo-activated nanostructured TiO<sub>2</sub> exhibited selective cytotoxicity against breast epithelial cancer cells [7]. Furthermore, the physical properties of TiO<sub>2</sub> NPs make them very interesting products for a use in various skin care and cosmetic products such as sunscreens [8]. TiO<sub>2</sub> NPs are under investigation as novel treatments for acne vulgaris, atopic dermatitis, hyperpigmented skin lesions, and other non-dermatologic diseases [2,3].

Despite their omnipresence in everyday life, modest research effort has been made in studying their potential adverse effects on living bodies and environment. TiO<sub>2</sub> NPs can be absorbed into the human body by inhalation, ingestion, and dermal penetration, then they can be distributed to vital organs, including lymph, brain, lung, liver, and kidney [9–11]. TiO<sub>2</sub> NPs can enter not only in cells, but also mitochondria and nuclei [12]. Most work to date has shown that TiO<sub>2</sub> NPs toxicity is strongly related to reactive oxygen species (ROS) generation and consequent oxidative stress [12–16].

TiO<sub>2</sub> NP-mediated ROS responses have been reported to orchestrate a series of pathological events leading to genotoxicity, immunotoxicity, neurotoxicity and carcinogenicity [17,18]. Neutrophils have been shown to be quickly recruited to titanium dioxide areas [19]. Moreover, cellular internalization of TiO<sub>2</sub> and ZnO NPs has been shown to activate immune cells including macrophages and neutrophils that contribute to ROS production [20–24]. TiO<sub>2</sub> NPs increased respiratory burst when fish neutrophils were incubated with these NPs [25]. Moreover, they lead to the activation of human ones [23,24]. Recently the same group showed that these nanoparticles enhance the ability of human neutrophils to exert phagocytosis by acting on Syk-dependent signaling pathway [26]. ROS production involves the activation of NADPH oxidase enzymes [22,27], a key player of oxidative stress in immune system cells but also in many other cell types (thyroid, kidney, neurons, and skin) [28–32].

NADPH-oxidase is the only enzyme whose function is to generate superoxide free radicals, which are transformed subsequently into other ROS [33–36]. It is a multi-subunit enzyme complex composed of membrane-bound flavocytochrome *b558* (cyt *b558*), comprising two subunits (Nox2 also known as gp91<sup>phox</sup>, and p22<sup>phox</sup>), present in the membranes phagocytes, and four cytosolic components. Nox2 harbors all the redox carriers (bound FAD, two hemes and the NADPH binding site) that transfer electrons from one side of the membrane cell to the other. The cytosolic components include p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and a small GTPase Rac1 or Rac2 [37].

In resting phagocytes, the components of the complex exist as separated entities but upon cell activation by pro-inflammatory mediators, the cytosolic subunits undergo posttranslational modifications such as phosphorylation and migrate to the membrane bound cyt *b558* to constitute the activated NADPH-oxidase complex, the only form able to produced superoxide ions [38].

The aim of our study was to investigate the enzymatic behavior of NADPH oxidase in the presence of TiO<sub>2</sub> NPs and to check if NADPH oxidase could be a pathway involved in ROS generation by TiO<sub>2</sub> NPs as it has been suggested [27]. We have developed a cell-free system [39–41] that allows controlling the environment, testing and identifying the potential effects of different molecules in various steps of oxidase activation [42,43]. In such cell-free systems, activation is obtained by mixing all proteins with an activator, arachidonic acid (AA). In this study, we have used a construction called trimera, which consisted of the following domains Nter-p47<sup>phox</sup> (amino acids 1–286) linked to the N-ter p67<sup>phox</sup> (amino acids 1–212), and the full length Rac1 Q61L [44]. In a previous paper, we have verified that the rates of production of superoxide anions were similar when the classic cytosolic proteins are replaced by trimera protein to activate the cyt *b558* and also that the dependences of the activity in function of the

enzyme activator AA concentration were also found comparable with the cytosolic fractions and the trimera [45]. Thus, the trimera was chosen in order to avoid complications due to some assembly steps and to activate the cyt *b558* in a reproducible manner. We have constantly validated our main conclusions with the separated subunits. We studied not only the effect of TiO<sub>2</sub> NPs on the function of NADPH oxidase but we also examined their effects on proteins conformations by different methods (fluorescence, synchrotron radiation circular dichroism (SRCD), transmission electron microscope (TEM), dynamic light scattering (DLS)). The use of these combined methods has provided a broad view of how TiO<sub>2</sub> NPs influence NADPH oxidase functioning and hypotheses about the origin of oxidative stress TiO<sub>2</sub> NPs dependent.

## Material and Methods

### Materials

Equine heart cytochrome *c* (cyt *c*), superoxide dismutase from bovine erythrocyte, arachidonic acid (AA), phenylmethanesulfonyl fluoride (PMSF) and Dulbecco phosphate buffer saline (PBS) and standard PBS were from Sigma (Saint Quentin Fallavier, France). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was from Acros. Ni-sepharose, superdex 75 and Ficoll-Paque Plus were from GE Healthcare, France. Anatase TiO<sub>2</sub> NPs were a generous gift of Dr Hynd Remita. They were suspended in deionized water (1 mg/mL) and sonicated in an ultra-sound bath for 10 min before use. The experiments were performed in phosphate buffer saline (PBS buffer). It is known that TiO<sub>2</sub> NPs are affected by the buffer and especially by phosphate [46]. We chose this buffer to be as close as possible to living medium.

### Neutrophil membrane preparation

The neutrophils were prepared from human blood from healthy donors (ESF Paris, France) as described in [47]. Briefly, 500 mL of blood was sedimented in 2% dextran solution for 40 min and centrifuged 400 x g 8 min. Dulbecco PBS was added to the pellets, and then the neutrophils were separated from lymphocytes and the red cells by centrifugation for 30 min at 400 x g on Ficoll solution. The red cells were further eliminated after their lysis by centrifugation for 8 min, 400 g, 4°C. The pellet resuspended in PBS pH 7.4 containing 340 mM sucrose, 7 mM magnesium sulphate, 1 mM PMSF, 0.5 mM leupeptin was sonicated in the 30% pulse mode at power pulses (6) in an ice-cooled beaker 6 times during 10 s with resting time of 1 min between the sonications (sonicator XL, Misonix inc.). Neutrophil membranes and cytosol were separated by centrifugation for 1h30 at 200 000 g at 4°C. The membrane fractions were resuspended, aliquoted and stored at -80°C for further experiments.

### Trimera preparation

The plasmid coding for the trimera was kindly provided by Prof. E. Pick (University of Tel Aviv, Israel). It codes for the Nter-p47*phox* (amino acids 1-286) linked to the N-ter p67*phox* (amino acids 1-212), and the full length Rac1 Q61L [44]. The trimera was expressed and isolated from *E.coli* BL21-(DE)3-*plysS*. Purification of trimera was performed mainly as previously described [45]. Briefly, after a first step through a nickel affinity chromatography, the protein was further purified by size exclusion chromatography; the protein was then dialyzed overnight against a phosphate buffer (100 mM NaCl and 30 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.5) and stored at -80°C. The protein concentration was estimated using a NanoDrop2000 spectrophotometer (Thermo scientific, France) and the extinction coefficient of 124,000 mol<sup>-1</sup> L cm<sup>-1</sup> at 280 nm (Expasy, Protparam). The purity of all proteins were checked by migration on 10% BisTris-NuPAGE

SDS gels (Invitrogen), stained with Coomassie Brilliant Blue and quantified by the ImageJ software.

### Dynamic light scattering measurements

Dynamic light scattering (DLS) experiments were performed to estimate the NPs size. DLS measurements were performed at room temperature on a Malvern NanoZS equipped with a 633 nm laser. Data were collected with a scattering angle of 173°. A range between 2 and 60 µg/mL of TiO<sub>2</sub> NPs suspensions prepared in PBS or water was tested.

### Transmission electron microscopy measurement

The morphology and size of NPs were also determined by transmission electron microscopy (TEM). The solutions contained 0.5 mg/mL TiO<sub>2</sub> NPs +/- 50 µg/mL trimera and +/- 1 mg/mL membrane proteins containing 25 µg/mL *cyt b558*. 4 µL of the suspension was deposited onto glow-discharged carbon-coated copper grids and after 1 minute of interaction, the excess of solution was removed with a filter paper (Whatman). As a result, the sample is dried onto the support. Zero-loss (20 eV window) images of TiO<sub>2</sub> NPs were acquired on field emission gun transmission electron microscope operating at 200 kV (JEOL 2200FS, JEOL LTD<sup>®</sup>).

### Intrinsic fluorescence Assays

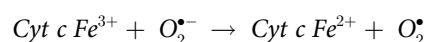
Steady-state fluorescence spectra were performed on Fluorolog3- Horiba spectrofluorimeter at 25°C. Various concentrations of TiO<sub>2</sub> NP suspensions (10- 100 µg/mL) were added as indicated to a final volume of 3 mL of buffer (PBS supplemented with 10 mM MgSO<sub>4</sub>) containing trimera (5 µg/mL, 60 nM) in a quartz cuvette. The tryptophan fluorescence spectra of trimera were obtained by exciting the samples at 290 nm (2 nm bandwidth) and recorded between 300 to 550 nm (5 nm bandwidth). The excitation wavelength was chosen at 290 nm to optimize the signal to noise ratio and to reduce the contribution of tyrosine residues to the signal [48]. 3 mL of buffer was used as baseline.

### Circular dichroism spectroscopy

Synchrotron radiation circular dichroism (SRCD) spectra were measured on the DISCO beam Line at the synchrotron radiation SOLEIL, Gif/Yvette, France. The calibration was made using a solution of camphorsulphonic acid (CSA). Spectra were measured over the wavelength range from 170 to 260 nm. Three scans were measured and averaged for the samples and the baseline. The averaged baseline was subtracted from the samples and the curves obtained smoothed. SRCD spectra were recorded at 25°C. The solutions contained 1.5 mg/mL (18 µM) trimera +/- 60 µg/mL TiO<sub>2</sub> NPs, +/- 300 µM AA prepared in 100 mM sodium fluoride; 10 mM sodium phosphate pH 7.0. Spectra are expressed in delta epsilon units, calculated using mean residue weights of 82,681 Da for the trimera. They were fitted using the free software BestSel [49]. Control spectra were recorded with TiO<sub>2</sub> NPs.

### Measurement of superoxide ion production rates

Superoxide anion production rates were indirectly quantified by the initial rate of cytochrome *c* (*cyt c*) reduction, as previously described [50]. The reaction is the following:



Unless indicated, the components of the cell-free system were added as follows: membrane fractions (MF; 2-5 nM *cyt b558*), trimera (100-200 nM) and arachidonic acid (40 µM) in 500 µl

PBS supplemented with 10 mM MgSO<sub>4</sub> and incubated for 4 minutes at 25°C in order to allow the NADPH oxidase complex to assemble. The production was initiated by addition of NADPH (250 μM) and the rate of O<sub>2</sub><sup>•-</sup> was quantified by the reduction of cyt *c* (50 μM). The rate was measured at 550 nm in a Thermo evolution 500 spectrophotometer, using a molar extinction coefficient ( $\Delta\epsilon$  of the reduced *minus* oxidized form of cyt *c*) of 21 mM<sup>-1</sup> cm<sup>-1</sup>. Control of the production of the O<sub>2</sub><sup>•-</sup> species was performed by addition of 50 μg/mL superoxide dismutase (SOD).

## Results

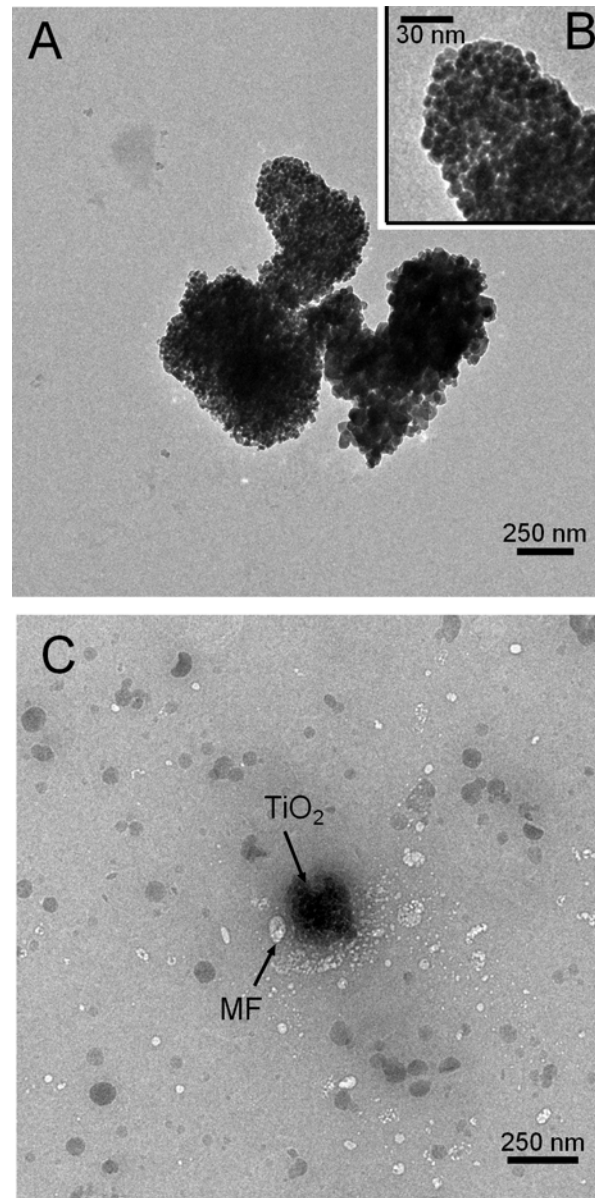
### 3.1 TiO<sub>2</sub> NPs size characterization

The hydrodynamic size of TiO<sub>2</sub> NPs in water and PBS was estimated by DLS. The average size of the NPs aggregates in water was about 350 ± 50 nm for the concentration range of 2–80 μg/mL of TiO<sub>2</sub>. This NP aggregate population was predominant (100%) for TiO<sub>2</sub> NPs concentration lower than 20 μg/mL but another population of larger agglomerates whose sizes were estimated to about ~2000 nm (5%) appeared when TiO<sub>2</sub> NPs concentration was higher than 20 μg/mL. We noticed also that the size of the NP aggregates in a physiological medium, such as PBS, is similar (460 ± 50 nm) to that in water. By TEM we can also observe particle aggregation (Fig 1A). The aggregates are constituted by particles of about 30 ± 5 nm (Fig 1B). These results are in accordance with those in the literature where it was shown that TiO<sub>2</sub> NPs tend to associate to form relatively strongly bonded aggregates or soft agglomerates [17]. TEM images also showed that TiO<sub>2</sub> NPs are in contact with the membrane fractions (Fig 1C). Moreover, aggregation of TiO<sub>2</sub> NPs is similar when they were together with the proteins, (Fig 1C). Similarly, DLS measurements showed that the size of TiO<sub>2</sub> NPs aggregates did not change when MF (0.5 μg/mL cyt *b558*) and trimera 18 μg/mL were added to 20 μg/mL TiO<sub>2</sub> NPs. The concentrations were similar to what we have in the cell free system assays for the measurements of NADPH oxidase activities.

### 3.2 Tryptophan fluorescence of trimera in the presence of TiO<sub>2</sub> NPs

The conformation changes of the trimera were evaluated by measuring the intrinsic fluorescence spectra of tryptophan residues, before and after addition of TiO<sub>2</sub> NPs. Trimera contains a total of thirteen tryptophan residues (seven, four and two in the p47*phox*, p67*phox* and Rac portions, respectively). The amplitude of the emission spectrum decreased linearly by the addition of TiO<sub>2</sub> NPs without any change of the wavelength at the maximum (340 nm) (Fig 2, S1 Fig). The decrease of fluorescence intensity might indicate a quenching due to proximity of TiO<sub>2</sub> NPs and some tryptophan residues without change in the surrounding of these residues. The intensity of the shoulder at around 440 nm increased concomitantly with the decrease of the intensity of the 340 nm band and is due to emission from TiO<sub>2</sub> NPs (inset of Fig 2). A similar quenching happens with Trp amino acid in solution with a bathochromic effect on the maximum. This indicates some affinity between Trp and TiO<sub>2</sub> NPs (S2 Fig)

The eventual changes of the secondary structure due to the NPs were investigated by SRCD spectroscopy. We have recorded the SRCD spectra of 1.5 mg/mL (18 μM) trimera in the absence and in the presence of 60 μg/mL TiO<sub>2</sub> NPs and 300 μM AA (Fig 3). In S1 Table are gathered the percentages of  $\alpha$ -helices and  $\beta$ -sheets obtained by fitting the spectra with the Bestsel software [49]. Analysis of the SRCD spectra of the trimera indicates that this chimeric protein is mostly in random coil (ca. 40%) and that the content of helices is very low (3–4%) (S1 Table). Although it was supposed that the addition of an amphiphile like AA would induce larger changes in the structure of the cytosolic proteins, [48,51] we observe only slight modifications of trimera secondary structure upon addition of AA. Similarly, only slight changes in



**Fig 1.** TEM images of (A) 0.5 mg/mL TiO<sub>2</sub> NPs alone; (B): enlarged view of the cluster; (C) 0.5 mg/mL TiO<sub>2</sub> NPs with membrane fraction (MF) (25 μg/mL *cyt b558*) and 50 μg/mL aggregated trimera;. (The black bar gives the scale: 250 nm for Fig 1A and 1C and 30 nm for Fig 1B). The samples are dried (see [materials and methods](#)).

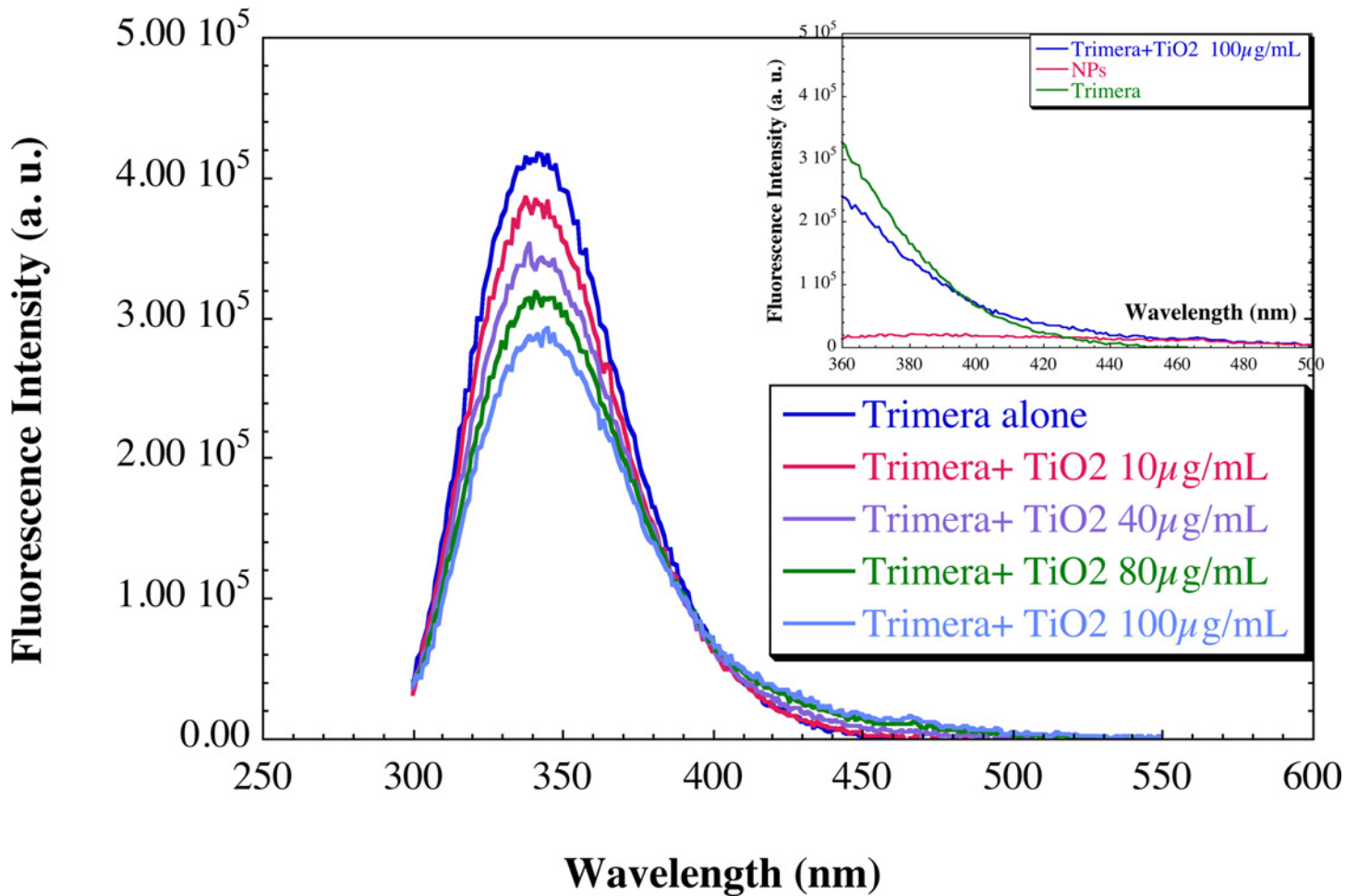
doi:10.1371/journal.pone.0144829.g001

the structure are observed with NPs. There is a loss of  $\alpha$ -helices and an increase of the disorder ([S1 Table](#)).

Altogether these results show that the interaction between NPs and trimera, indicated by fluorescence quenching, have no big consequence on the secondary structure of trimera.

### 3.3 Effects on the functionality

**3.3.1 Effects of TiO<sub>2</sub> NPs on the NADPH oxidase activity.** First, we have checked that TiO<sub>2</sub> NPs alone did not reduce *cyt c* (data not shown), which means that in these conditions,



**Fig 2. Fluorescence emission spectra of the trimera-TiO<sub>2</sub> NPs suspensions.** The solution contains 5 μg/mL (60 nM) trimera and TiO<sub>2</sub> NPs at the concentrations of 0, 10, 40, 80 and 100 μg/mL in a final volume of 3 mL of buffer (PBS supplemented with 10 mM MgSO<sub>4</sub>). The emission spectra were measured using an excitation wavelength of 290 nm as described in the Materials and Methods section. Results are representative of at least three independent experiments. In inset: enlargement of the fluorescence spectrum in the region 360-500 nm for three solutions. Fluorescence spectra of 5 μg/mL trimera alone (green), 100 μg/mL TiO<sub>2</sub> NPs alone (red) 5 μg/mL trimera in the presence of 100 μg/mL TiO<sub>2</sub> NPs (blue).

doi:10.1371/journal.pone.0144829.g002

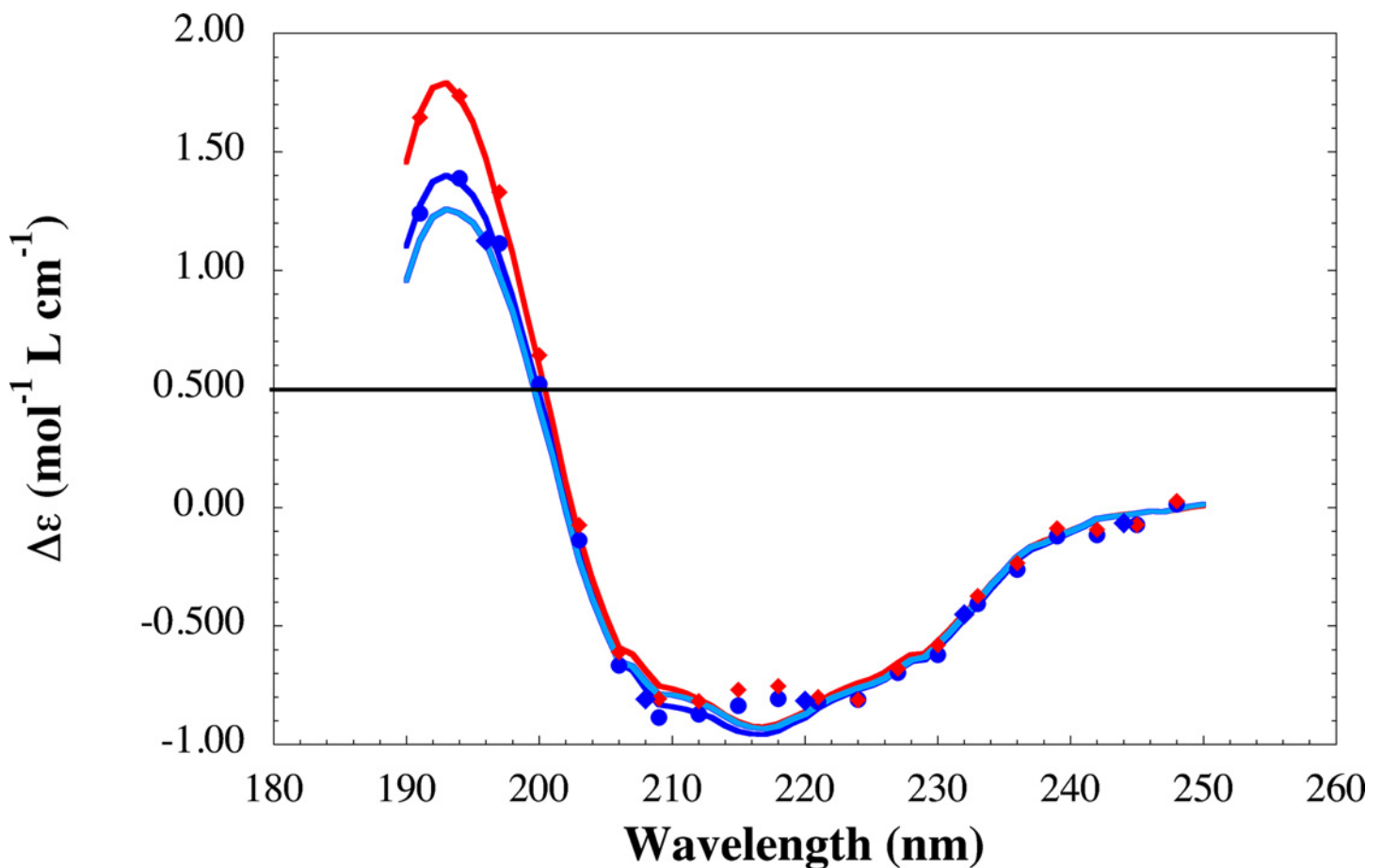
NPs by themselves do not produce superoxide ions. In order to investigate the effect of NPs on the NADPH oxidase, the rate of superoxide anion production was measured upon addition of TiO<sub>2</sub> NPs in the cell free assay conditions previously optimized with trimera [45] (Fig 4). The initial slope of the kinetic curve is equal to the rate of superoxide anion formation. This rate was faster in the presence of TiO<sub>2</sub> NP. The identification of O<sub>2</sub><sup>•-</sup> was performed by addition of 50 μg/mL SOD.

The activity of the complex was investigated in parallel with either the trimera or the mix of cytosolic proteins p47<sup>phox</sup>, p67<sup>phox</sup> and Rac. All components were incubated together with TiO<sub>2</sub> NPs (2-60) μg/mL and 40 μM AA. The rate of superoxide anion production in the absence of NPs was considered as 100% of NADPH oxidase activity. No major difference was noticed between the trimera and the cytosolic proteins (Fig 5). In both cases we clearly observed an increase in the NADPH oxidase activity in the presence of NPs. The curves of Fig 5 exhibit a bell shape profile with a maximum (140% of the reference) at around 20 μg/mL of TiO<sub>2</sub> NPs. For higher concentrations of TiO<sub>2</sub> NPs (> 20 μg/mL), the rate returned close to the activation level of the control. This result indicates that TiO<sub>2</sub> NPs potentiate the NADPH

oxidase activity. The activity remains constant for concentrations higher than 40 μg/mL probably due to some aggregations of NPs at higher concentrations.

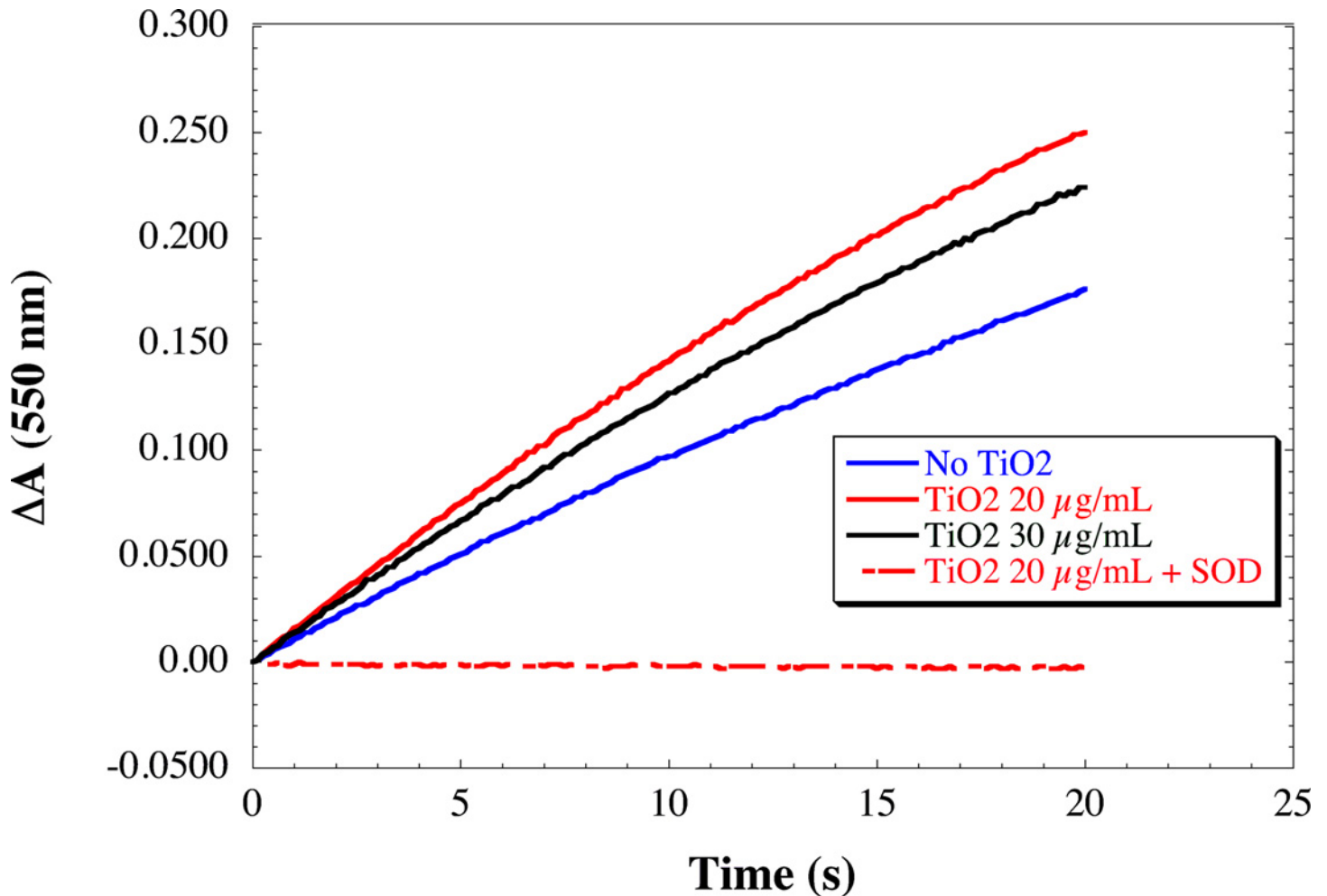
Thus, we further questioned whether TiO<sub>2</sub> NPs alone (20 or 40 μg/mL) could activate the NADPH oxidase complex and thus replace AA as activator (Fig 6). Almost no NADPH oxidase activity (5 ± 2%) was detected with NPs instead of AA (control). Comparable results were obtained using the separated subunits where a maximum activity of 4 ± 2% of AA-dependent activity was reached (data not shown).

Since TiO<sub>2</sub> NPs cannot be considered as activating molecules, the significant increase in the rate of O<sub>2</sub><sup>•-</sup> production with NPs might be due to an indirect effect on the optimized oxidase condition by disturbing the optimal AA concentration. We therefore investigated the effect of TiO<sub>2</sub> NPs on the AA activation profile. To probe this effect, we performed titrations of the oxidase activity vs. AA concentration in the absence and in the presence of 20 μg/mL TiO<sub>2</sub> NPs added after arachidonic acid (Fig 7). The rate of production with 40 μM AA alone (the concentration used as reference in this paper), was considered as 100%. In agreement with the above-mentioned results, in the presence of TiO<sub>2</sub> NPs, the O<sub>2</sub><sup>•-</sup> production rate was higher on the full range of AA concentrations. Both curves exhibited bell-shapes as usual but the optimal concentration of AA was lower (ca. 62 μM) in the presence than in the absence (ca. 90 μM) of NPs. Similar results were obtained when NPs were added before AA (data not shown).



**Fig 3. SRCD spectra of trimera alone and in the presence of either TiO<sub>2</sub> NPs or AA.** SRCD spectra of the trimera (18 μM) alone (Blue) and in the presence of TiO<sub>2</sub> nanoparticles (60 μg/mL) (sky blue) and AA (300 μM) (red). The solvent was NaF 100mM /NaPi 10 mM pH 7, 25°C. The points are experimental, the curves are the fits using BeStSel [49].

doi:10.1371/journal.pone.0144829.g003



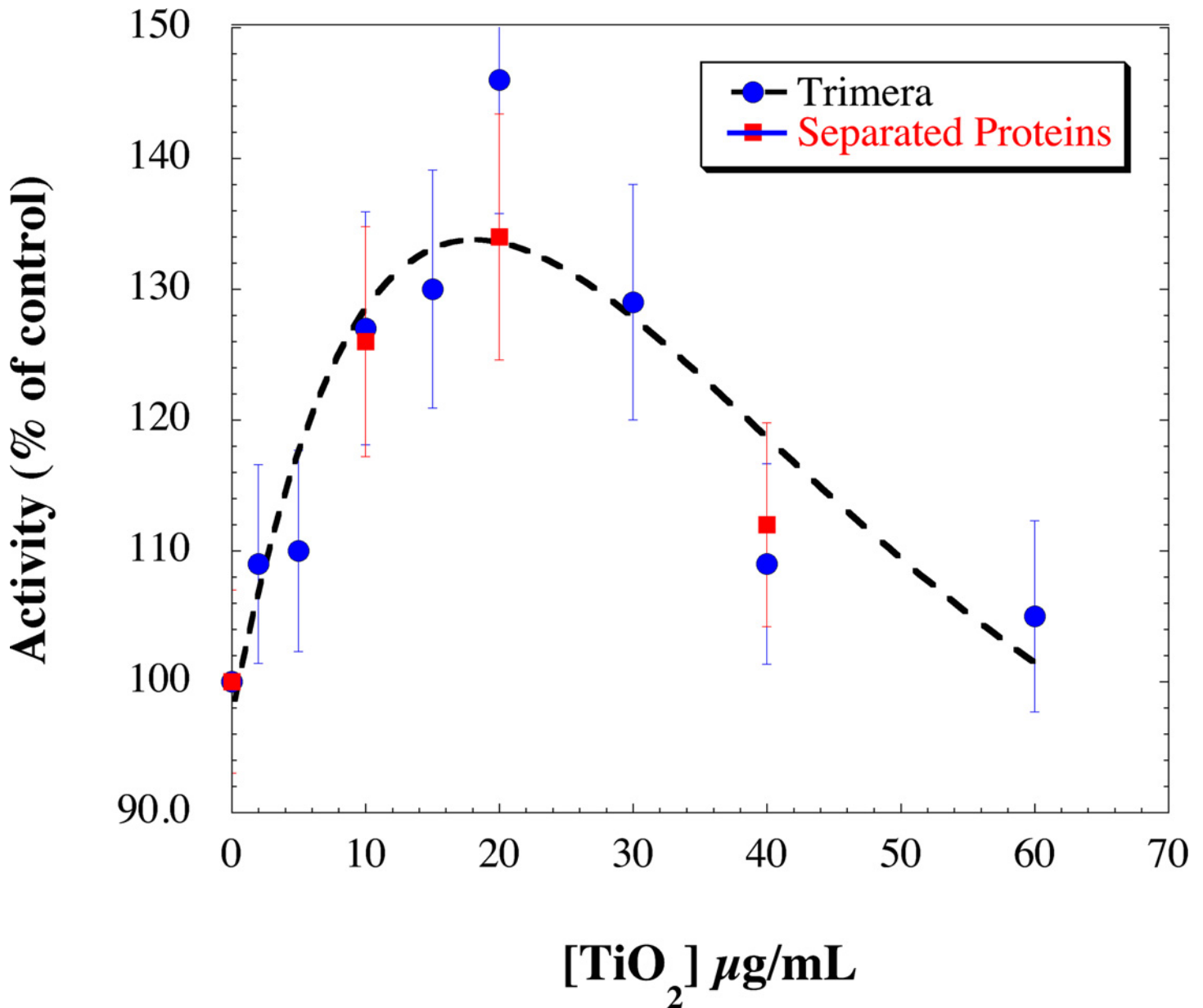
**Fig 4. Kinetics of superoxide anion production in presence of TiO<sub>2</sub> NPs.** Neutrophil membrane fractions (5 nM cyt b<sub>558</sub>) and trimera 200 nM were incubated together in the presence of 40 μM AA and (0, 20, 30 μg/mL) TiO<sub>2</sub> NPs. The production was initiated by addition of NADPH (250 μM) and the rate of O<sub>2</sub><sup>•-</sup> was quantified by the reduction of cyt c (50 μM). Control was performed by the addition of 50 μg/mL SOD. (on the fig: 20 μg/mL TiO<sub>2</sub> in the presence of SOD). The initial rates of production of superoxide are the following: 92.0±0.3, 134.0±0.5, 119.2±0.4 mol O<sub>2</sub><sup>•-</sup>/s/Mol Cyt b<sub>558</sub> for TiO<sub>2</sub> NPs 0, 20, 30 μg/m respectively.

doi:10.1371/journal.pone.0144829.g004

**3.3.2 Effect of TiO<sub>2</sub> NPs addition at different sequences of cell free system assay.** To examine whether TiO<sub>2</sub> NPs have effects on specific steps of the assembly, several concentration of TiO<sub>2</sub> NPs (10, 20, 40 μg/mL) were added at different times: (i) to the membrane fractions alone before mixing to the cytosolic subunits; (ii) to mixed membrane fractions and trimera; (iii) to the membrane fractions plus trimera plus AA (Fig 8). Regardless the stages at which TiO<sub>2</sub> NPs were added, the rates of production of superoxide were the same within uncertainty. The highest O<sub>2</sub><sup>•-</sup> production was still observed when 20 μg/mL TiO<sub>2</sub> NPs were incorporated in the system whatever the sequence of addition of NPs.

## Discussion and Conclusion

Oxide nanoparticles are widely used and their toxicity levels seem to be quite different albeit always related to induction of oxidative stress [27]. Some work has been done on the toxicity of ZnO NPs. A ROS formation enhancement was observed in ZnO-treated liver cells [22,52,53] and on macrophages from wt mice, whereas this formation was impaired in the treated

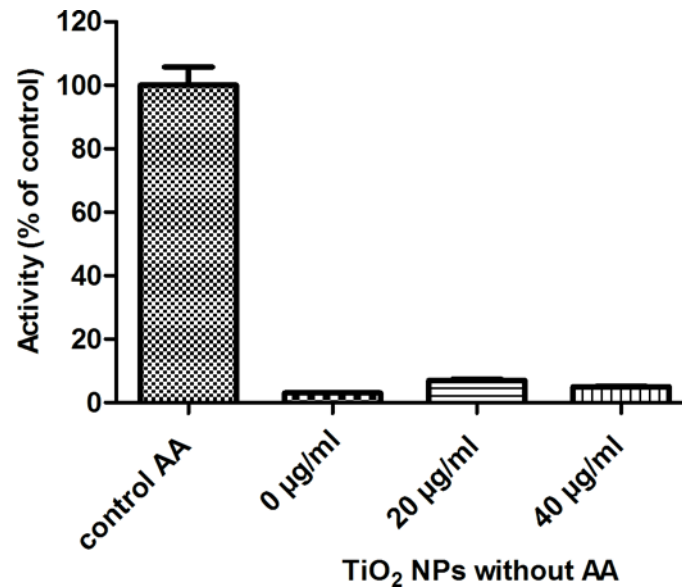


**Fig 5. Dependence of NADPH oxidase activity as a function of TiO<sub>2</sub> NPs concentration.** Neutrophil membrane fractions (5 nM cyt *b558*) and trimera 200 nM (blue dots) or the cytosolic subunits (p67<sup>phox</sup> 200 nM, p47<sup>phox</sup> 260 nM and Rac 580 nM) (red squares) were incubated together in the presence of 40 μM AA and TiO<sub>2</sub> NPs. Oxidase activities were expressed as the percent of activity measured in the absence of TiO<sub>2</sub> NPs (90 mol O<sub>2</sub><sup>-</sup>/s/mol cyt *b558*), and determined as 100%. Points are an average of 3 independent measurements. The dotted curve is a visual fit for both systems.

doi:10.1371/journal.pone.0144829.g005

macrophages from the p47<sup>phox</sup><sup>-/-</sup> animals. To our knowledge, this is the only work involving NADPH oxidase. [22]. The use of TiO<sub>2</sub> NPs has become widespread including in situations where they can be absorbed by living bodies. The photocatalytic activity of TiO<sub>2</sub> is well known [54], however UVA and visible light do not penetrate inside the body. Thus there is no light exposure and no activation of TiO<sub>2</sub> NPs by photo-catalysis.

The toxic effects of TiO<sub>2</sub> NPs seem to be mainly due to indirect production of ROS and therefore to induction of oxidative stress. One of the first studies about interaction between NPs and neutrophils was done in 1988; Hedenborg demonstrated that TiO<sub>2</sub> induced the



**Fig 6. Dependence of NADPH oxidase activity as a function of TiO<sub>2</sub> NPs concentrations in the absence of arachidonic acid.** Membrane fractions (4 nM cyt *b558*) with trimera 200 nM were incubated 4 min in the presence of 0, 20 or 40 µg/mL TiO<sub>2</sub> NPs. Control experiment representing 100% (83 mol O<sub>2</sub><sup>•-</sup>/s/mol cyt *b558*) of the activity was realized in presence of 40 µM AA and in absence of TiO<sub>2</sub> NPs. The rates of superoxide production were measured as described in Materials and Methods. Data are the average of 3 independent measurements.

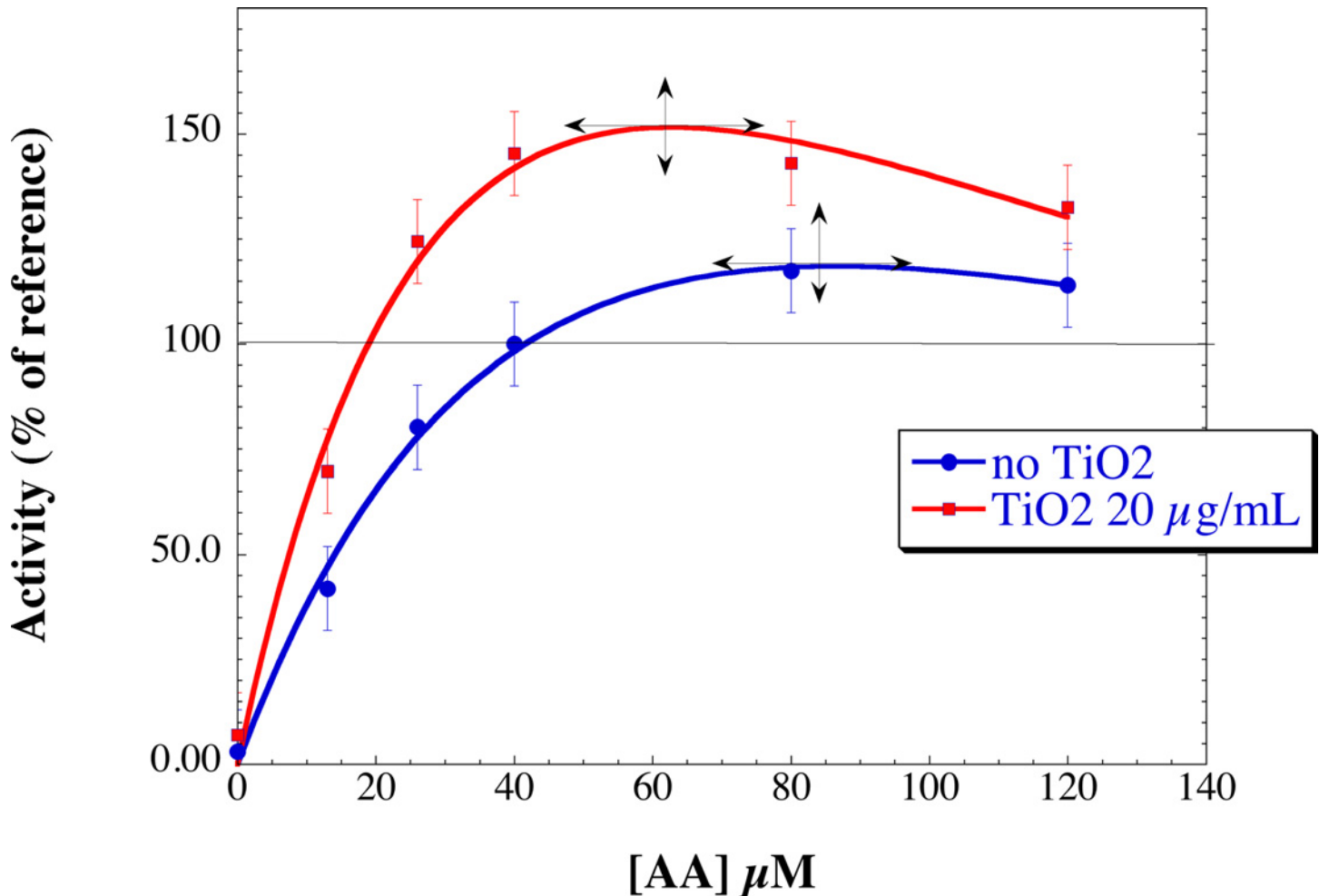
doi:10.1371/journal.pone.0144829.g006

production of ROS by human neutrophils [55]. It has also been shown that TiO<sub>2</sub> NPs can induce oxidative damage to human bronchial epithelial cells in the absence of photoactivation [54,56]. They are known to enhance superoxide production in osteoblasts [16]. TiO<sub>2</sub> NPs were shown to interact with proteins and enzymes in hepatic tissues, interfering with antioxidant defense mechanisms and leading to generation of ROS [57]. Since NADPH oxidase is a major actor of oxidative stress by producing superoxide ions, it was evident that investigating the effect of TiO<sub>2</sub> NPs on this enzyme constitutes a relevant issue.

The aim of this paper was to obtain comprehensive information on the interaction of TiO<sub>2</sub> NPs with the NADPH oxidase. To facilitate such studies, we used a model system that allows performing deeper studies. The different tests performed either with the trimera or with the three separated subunits showed similar results.

It is known that the cytosolic proteins must undergo conformational changes to lead to active enzyme. TiO<sub>2</sub> NPs have no significant effect on the secondary structure, as shown by the CD spectra (Fig 3). However the fluorescence of the Tryptophan residues is affected by the presence of NPs. Both results are similar to those obtained with fibrinogen [58]. The quenching of fluorescence of the endogenous tryptophans of the trimera indicates that the NPs are probably close to one or several Trp residues and implies that a complex may be formed between the NPs and the cytosolic protein. *In vivo*, it was shown that proteins adsorb on TiO<sub>2</sub> NPs. In some cases, these NPs induced conformational changes in proteins and affected their functions [12,59,60].

The CD spectra of the trimera in the presence of TiO<sub>2</sub> NPs and in the presence of AA (Fig 1 and S1 Table [61]) do not exhibit much difference. The Nps have no significant effect on the secondary structure of the trimera as suggested by the CD spectra. Yet, we do not observe any ROS production from NADPH complex in the absence of AA suggesting that TiO<sub>2</sub> NPs are unable by themselves to activate the enzyme. TEM images showed that membrane fractions

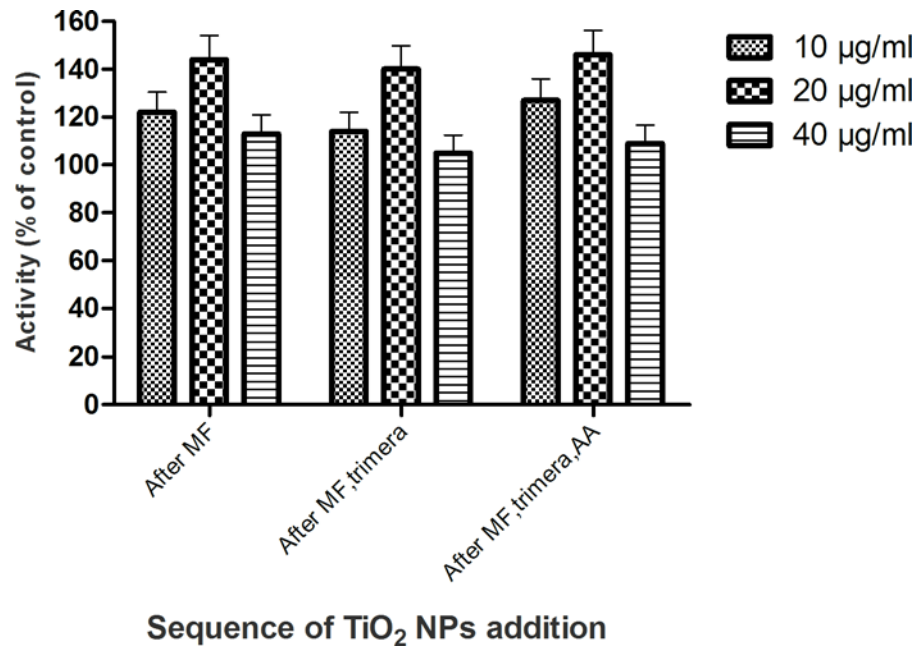


**Fig 7. Effect of TiO<sub>2</sub> NPs on the AA-dependent activation profile.** Neutrophil membrane fractions and trimera were incubated together in the presence of different concentration of AA. The TiO<sub>2</sub> NPs concentration was as follow, blue dots: no TiO<sub>2</sub> NPs; red squares: 20 μg/mL TiO<sub>2</sub> NPs. Oxidase activities were expressed as the percent of activity measured in the presence of 40 μM AA (85 mol O<sub>2</sub><sup>-</sup>/s/mol cyt *b558*) set as 100%. The curves are visual fits of the experimental points and the maxima have been indicated by crosses. The rate of O<sub>2</sub><sup>-</sup> production was measured as described in Materials and Methods.

doi:10.1371/journal.pone.0144829.g007

are close to TiO<sub>2</sub> NPs and suggest also an interaction between TiO<sub>2</sub> NPs and the membranous proteins. Obviously this interaction with the MF does not replace that of the trimera and of AA for activation, and does not prevent AA from having access to the MF, however it might be responsible for the hyperactivation.

Surprisingly, the presence of NPs increases the rate of superoxide anion production (up to 140% of its value without NPs), this effect being dependent on the NPs concentration. They do not interact at a specific step of activation, indicating that their targets are indifferently the membrane fraction as well as the cytosolic proteins and that they can work on the system even when the entire complex is assembled and active. Since the presence of TiO<sub>2</sub> NPs modifies the AA-dependent activation profile of the enzyme shown in Fig 7, we can postulate that more efficient structure of the NADPH oxidase complex is attained in the presence of NPs. We can exclude a consequence of AA availability due to NPs since the higher NADPH oxidase activity is observed at lower concentration of AA in the presence of NPs than in their absence. This phenomenon cannot be attributed only to an interaction with the sole membrane fraction since the sequence of addition of the NP has no effect on it. An effect on the cytosolic fractions



**Fig 8. Effect of TiO<sub>2</sub> NPs as a function of its sequence of addition in the cell free system.** Neutrophil membrane fractions (4 nM cyt *b558*) and 200 nM trimera were incubated together in the presence of 40 µM AA and TiO<sub>2</sub> NPs (10, 20, 40 µg/mL). TiO<sub>2</sub> NPs was added to the solution either after the membrane fractions or after the membrane fractions and trimera or after the membrane fractions, trimera and AA. Oxidase activity was expressed as the percent of activity measured in the absence of TiO<sub>2</sub> NPs (84 mol O<sub>2</sub><sup>-</sup>/s/mol cyt *b558*) set as 100%. Results are presented as the mean±SD of 3 independent experiments.

doi:10.1371/journal.pone.0144829.g008

is also likely. Taken together, these facts indicate that the secondary structure of the cytosolic proteins may be conserved and at the same time modifications must have happened to lead to hyperactivation.

Our TEM images showed that NPs remain in the aggregation state even when they are in contact with the proteins. It was demonstrated that particles bigger than 100 nm, can enter phagocytes [62]. Additionally, it was reported that NPs enhance the ability of human neutrophils to exert phagocytosis by a Syk-dependent mechanism [26]. Thus, the TiO<sub>2</sub> NPs we used can enter cells by phagocytosis and may lead consequently to activation of NADPH oxidase. TiO<sub>2</sub> NPs aggregates are known to interact with neutrophils. Recent work by SEM [19] showed increased stiffness of the membrane and cell morphology alteration. Our present results indicate that this stiffness would not impede the NADPH oxidase functioning. In conclusion, NADPH oxidase hyper-activation and the subsequent increase in ROS production in the presence of TiO<sub>2</sub> NPs could be one of the pathways involved in ROS generation by TiO<sub>2</sub> NPs, thus participate to their toxicity, which is strongly related to oxidative stress development.

### Supporting Information

**S1 Fig. Variation of the fluorescence intensity as a function of TiO<sub>2</sub> concentration.** 340 nm (blue), 440 nm (red). The mixture contained 5 µg/ml (60 nM) trimera and TiO<sub>2</sub> concentrations of 0, 10, 40, 80 and 100 µg/ml in a final volume of 3 mL of buffer (PBS supplemented with 10 mM MgSO<sub>4</sub>). The emission spectra were measured using an excitation wavelength of 290 nm as described in the Materials and Methods section. Results are representative of at least three independent experiments.

(TIF)

**S2 Fig. Fluorescence emission spectra of the tryptophan residues-TiO<sub>2</sub> NPs suspensions.** The solution contains 8 μM L-tryptophan and TiO<sub>2</sub> NPs at the concentrations of 0, 10, 20, 40, 60, 80 and 100 μg/mL in a final volume of 3 mL of buffer (PBS supplemented with 10 mM MgSO<sub>4</sub>). The emission spectra were measured using an excitation wavelength of 290 nm as described in the Materials and Methods section.

(TIF)

**S1 Table. Analysis of the SRCD spectra of the trimera alone or with cis-AA or with TiO<sub>2</sub> NPs.** For definition of helices, sheets and turns, see for instance [60].

(DOCX)

## Acknowledgments

We are grateful to Dr H. Remita for a generous gift of nanoparticles, to Prof. E. Pick for the trimera plasmids and to Dr F. Lederer for the preparation of neutrophils. The financial support of ANR 2010-blan-1536-01 is acknowledged. We thank the COST Action CM1201 (Biometric Radical Chemistry) for very fruitful discussions.

## Author Contributions

Conceived and designed the experiments: CHL TB. Performed the experiments: RM ST TB FW. Analyzed the data: CHL TB LB SM. Contributed reagents/materials/analysis tools: RM TB ST. Wrote the paper: RM CHL LB.

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