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Immunomodulatory activity of zinc peroxide (ZnO_2) and titanium dioxide (TiO_2) nanoparticles and their effects on DNA and protein integrity

Stanely Makumire[a], Venkata S.K. Chakravadhanula[b,c], Gabriele Köllisch[e,f], Engelbert Redel[c,d,**], Addmore Shonhai[a,g,*]

[a] Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, South Africa
[b] Helmholtz Institute Ulm (HIU) Electrochemical Energy Storage, Albert-Einstein-Allee 11, 89081 Ulm, Germany
[c] Karlsruhe Institute of Technology (KIT), Institute of Nanotechnology (INT), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[d] Karlsruhe Institute of Technology (KIT), Institute of Functional Surfaces (IFG), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[e] Department of Parasitology, Faculty of Biology, Philipps University, Marburg, Germany
[f] Institute of Immunology, Philipps University, Hans-Meerwein-Str. 2, 35043 Marburg, Germany
[g] Current address: Department of Biochemistry, School of Mathematics & Natural Sciences, University of Venda, South Africa

HIGHLIGHTS

- We synthesized charge stabilized ZnO_2 and TiO_2 nanoparticles.
- We investigated the effects of the nanoparticles on human PBMCs, DNA and protein.
- 1 μg/mL of ZnO_2 nanoparticles modulated expression of cytokines by PBMCs.
- TiO_2 nanoparticles did not stimulate cytokine production by PBMCs.
- Only ZnO_2 nanoparticles caused damage to both protein and DNA.

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ABSTRACT

Nanoparticles that are made from zinc and titanium oxide have found widespread applications, including their use in sunscreens. However, there is little information regarding their effects on immune cells. In the current study, we synthesized charge stabilized and "ligand free" colloid stable ZnO_2 and TiO_2 nanoparticles. Most previous published studies commonly used ZnO and TiO_2 nanoparticles. In the current study we investigated the comparative toxicity of ZnO_2 and TiO_2 nanoparticles. Therefore, our results based on ZnO_2 which is more oxidative than ZnO provides novel data on the possible toxicity of this species of nanoparticles. First, we investigated the immunomodulatory action of these nanoparticles on human peripheral blood mononuclear cells and their effects on DNA and protein integrity. A minimum concentration of ZnO_2 nanoparticles of 1 μg/mL inhibited the production of two inflammatory cytokines: interleukin-1-β and interleukin 6 by peripheral blood mononuclear cells in the presence of lipopolysaccharides. On the other hand, TiO_2 nanoparticles at a concentration range of 0.1–100 μg/mL did not present apparent toxicity to the peripheral blood mononuclear cells. ZnO_2 nanoparticles at a minimum concentration of 2 μg/mL caused DNA damage in vitro. TiO_2 nanoparticles at a concentration range of 25–100 μg/mL only

* Corresponding author. Present address: Department of Biochemistry, University of Venda, P. Bag X5050, Thohoyandou 0950, South Africa. Tel.: +27 15 9628531.
** Corresponding author at: Institut für Funktionelle Grenzflächen (IFG), Hermann von Helmholtz Platz 1, 76344 Eggenstein-Leopoldshafen, Baden-Württemberg, Germany. Tel.: +49 721 608 2 4102; fax: +49 721 608 2 3478.
E-mail addresses: engelbert.redel@kit.edu (E. Redel), addmore.shonhai@univen.ac.za, adshon@gmail.com (A. Shonhai).

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caused marginal DNA damage. ZnO_2 nanoparticles at a minimum concentration of 5 μg/mL were capable of promoting aggregation of malate dehydrogenase, and facilitated its degradation at higher concentrations. Exposure of malate dehydrogenase to TiO_2 at a concentration range of 2.5–15 μg/mL did not alter the solubility of malate dehydrogenase. Altogether, our findings suggest that charge stabilized ZnO_2 nanoparticles are nascent and interact with DNA and protein and may be harmful to immune cells. In addition, the propensity of ZnO_2 nanoparticles to promote protein aggregation could facilitate the production of protein complexes that may interfere with normal immune functions.

1. Introduction

The use of nanoparticles (NPs) to make various consumer and industrial products has been on the increase lately. Metal oxides such as ZnO and TiO_2 NPs have gained popularity as candidates in the production of sunscreens because of their ability to protect the skin from Ultraviolet light A (UVA) and Ultraviolet light B, respectively (Smijs and Pavel, 2011). However, the application of these NPs has raised safety concerns as these materials have the capability to access the body via dermal, ingestion and inhalation routes (Li et al., 2010). They may also end up accessing several body organs such as the liver, kidney and spleen via the blood circulatory system (Wang et al., 2007).

Apart from its application in sunscreens, TiO_2 is used in the paint, paper and coatings industries (Zhang et al., 2009). TiO_2 has been implicated in the mortality of industry workers in Europe through exposure to the dust from the metal oxide (Boffetta et al., 2004). However, evidence for a direct correlation of exposure to TiO_2 with incidences of lung cancer was not established (Boffetta et al., 2004). In addition, a study that was conducted in the United States of America could not establish a link between exposure to industrial TiO_2 dust and mortality (Ellis et al., 2010). However, TiO_2 has been linked with DNA damage (Li et al., 2010) and tumour induction in rats (Dankovic et al., 2007).

Although minimal uptake of ZnO NPs through the skin has been reported (Cross et al., 2007), the cumulative effect of these NPs in the body remains of concern. Consequently, it is important to evaluate the effect of metal oxide NPs such as those made from TiO_2 and ZnO_2 on the functional integrity of immune cells. In addition, it is also important to understand the effect of these NPs on the structure and function of biomolecules. Based on an Amest test that was conducted previously, there was no evidence to suggest that ZnO NPs are mutagenic (Yoshida et al., 2009). Similarly, another, more recent study based on a chromotest could not establish evidence for the genotoxicity of several nanoparticles, including ZnO and TiO_2 (Nam et al., 2013). On the other hand, another study showed that pregnant rats that were treated with ZnO NPs gave birth to reduced number of pups and the progeny exhibited poor health indicators (Jo et al., 2013). Altogether, this suggests that the effect of these NPs on human health and their specific action on biological processes is yet to be fully understood.

There is only little information on the effects of ZnO and TiO_2 NPs on human immune cells. A recent study proposed that TiO_2 and ZnO NPs may be toxic to immune cells and that the effect of these nanoparticles varies across different immune cells (Andersson-Willman et al., 2012). The study conducted by Andersson-Willman et al. (2012) demonstrated that TiO_2 and ZnO NPs within the 1–100 μg/mL concentration range did not induce toxicity in human peripheral blood mononuclear cells (PBMCs). However, it was observed that monocyte-derived dendritic cells were sensitive to ZnO and not to TiO_2 NPs. In addition, ZnO but not TiO_2 NPs also appeared to change the phenotype of natural killer cells by decreasing the CD16 expression. TiO_2- and SiO_2-NPs, as well as crystalline silica led to the expression of MHC-II, CD80, and CD86 in murine dendritic cells, suggesting that these nanomaterials may be toxic (Winter et al., 2011).

It has been proposed that the toxicity of ZnO NPs is largely through two ways: generation of free radicals and induction of apoptosis (Premanathan et al., 2011). However, the specific mechanism in which nanoparticles influence biological events remains to be fully understood. The interaction of nanoparticles with DNA could result in a variety of biological and biochemical defects, leading to mutations and genomic instability (Li et al., 2010). In addition, it is known that most NPs have a propensity to interact with proteins and because of this they occur under physiological conditions as protein coated entities (Cedervall et al., 2007; Luthuli et al., 2013). Because of these features, NPs have potential applications in biosensors and drug delivery. Conversely, however, the adsorptive function of NPs is thought to promote their toxicity (Horie et al., 2009). ZnO and TiO_2 are reported to possess strong absorptive properties (Horie et al., 2009) and ZnO NPs are thought to interact with proteins, leading to their denaturation (Chatterjee et al., 2010). In addition, TiO_2 and ZnO have been found to promote the aggregation of platelets and erythrocytes, suggesting that they could be prothrombogenic (Šimundić et al., 2013). Furthermore, TiO_2 has been implicated in promoting haemolysis in vitro (Aisaka et al., 2008).

In contrast to a recent upsurge in the production of commercial nanoparticle metal-oxides that contain polymer or ligand capping agents to stabilize them, in the current study, we synthesized surface charge stabilized and "*ligand free*" TiO_2 and ZnO_2 NPs as stable colloidal dispersions using a previously described approach (Redel et al., 2012). Furthermore, to the best of our knowledge no study has been conducted to investigate the biotoxicity of ZnO_2 NPs. The size of the NPs whose biotoxicity we investigated is much smaller compared to TiO_2 and ZnO NPs whose toxicity on human PBMCs were recently reported (Andersson-Willman et al., 2012). The TiO_2 and ZnO NPs described in the previous study possessed a negative charge and their diameters were around 30 nm and 17 nm, respectively. In contrast, our NPs were positively charged and possessed lower polydispersity (PD), compared to those that were previously described (Andersson-Willman et al., 2012).

Given the unique features of the TiO_2 and ZnO NPs, and their potential application in commercial products such as sunscreens we sought to evaluate their toxicity by studying their effects on DNA and protein structure. We exposed human PBMCs to varying concentrations of TiO_2 and ZnO_2 NPs, either in the absence or in the presence of LPS which is known to be a potent immune stimulant. The effect of the NPs on the immune cells was determined by quantification of two cytokines (interleukin-1 beta [IL-1-β], and interleukin 6 [IL-6]). It has been reported that protein aggregates may modulate the function of immune cells (Hermeling et al., 2005; Joubert et al., 2012). TiO_2 and ZnO NPs have been shown to adsorb protein (Horie et al., 2009) and thus modulate protein structure. Thus by promoting protein misfolding and aggregation, NPs may interact with proteins to generate potent immune modulatory aggregates. We therefore sought to investigate the effect of TiO_2 and ZnO_2 NPs on the integrity of an aggregation prone protein, MDH. We further explored the effect of TiO_2 and ZnO_2 NPs on the

structural integrity of plasmid DNA in vitro. In general, we found ZnO_2 NPs to have more deleterious effects on the human PBMCs as well as on protein and DNA integrity. Furthermore, our findings suggest that, in contrast to ZnO NPs that were used in previous studies, the ZnO_2 described here appear more toxic, possibly because of their stronger oxidizing function.

2. Materials and methods

2.1. Synthesis and characterization of metal oxide nanoparticles

TiO_2 nanoparticle dispersions were prepared according to a previously described protocol (Puzzo et al., 2009). The TiO_2 NPs were prepared by adding drop by drop, 17.5 mL of titanium iso-propoxide (98%, for synthesis) to 108 mL of a 0.1 M HNO_3 solution and the mixture was constantly stirred strongly at room temperature (RT). After the addition of the Ti-isopropoxide precursor (98%, for synthesis), the mixture was left to hydrolyze by stirring the suspension at 80 °C for an additional 8 h (Puzzo et al., 2009). The reaction was stopped by cooling the dispersion down to RT. A white-milky dispersion which was easily purified by filtration through a simple paper-filter was obtained. The final step also facilitated the removal of possible agglomerates and/or unreacted Ti-precursor (Puzzo et al., 2009).

ZnO_2 dispersions were produced using an established protocol (Redel et al., 2012). ZnO_2 NPs were prepared by firstly dispersing 1–3 g of elemental Zn metal powder (200 or 325 mesh), in 10–15 mL of deionized water. To the resulting dispersion 1–3 mL of AcOH (Glacial Acid) and 10–35 mL of H_2O_2 (30% p.a.) were added slowly (drop wise). The ratio of H_2O_2 and AcOH (Glacial) was strictly maintained at 10:1, respectively (Redel et al., 2012). Furthermore, as recommended this reaction has to be performed at 0 °C (by cooling the exothermic oxidative/dissolution process with an ice-bath). Under this reaction condition the intermediate formed ZnO NPs that were directly transformed into ZnO2 NPs as the final synthesis product. The reaction can proceed under normal conditions (no additional N_2 or Ar atmosphere is required). To ensure safety, this reaction must be conducted using high volume reaction vessels (250 or 500 mL) and it is important never to close the reaction or storage vessel too tightly (Redel et al., 2012).

> "**Danger:** Due to the exothermic character of this process, an instant ice cooling bath is required throughout the reaction. It is important to be properly equipped and clothed when performing the ZnO_2 synthesis. This reaction must be conducted in a well-ventilated fume-hood and in a well-ventilated laboratory. We also recommended that only a very well trained and experienced scientist or lab technician should carry out the above described synthesis procedures" (Redel et al., 2012).

Stability measurements were performed by zeta-potential measurements, see additional material in Supplementary Material Information section. Before performing the bio-tests the resulting aqueous metal oxide dispersions were sonicated for 10 min to avoid aggregation of the NPs. Additional characterizations and measurement details/data on the XRD, zeta-potential, and IR measurements can be found in the Supporting Material Information, for details see also Figs. S1–S6.

A Titan 80-300 (FEI) was used to study the structure and morphology of the synthesized NPs. Bright-field transmission electron microscopic images, selected area electron diffraction (SAED) patterns and high resolution transmission electron microscopic micrographs were obtained by diluting the nanoparticle solution with deionized water, sonication, followed by dispersion onto carbon coated Au grids (Quantifoil GmbH).

2.2. Cell culture and stimulation assays

Human PBMCs were isolated from samples of heparinized blood taken from adult healthy subjects with their informed consent. Ethical approval was sought and granted by the ethics committee of the University Hospital of Gießen and Marburg. The PBMC were isolated from buffy coats using Ficoll density gradient centrifugation with LSM 1077 lymphocyte separation solution (PAA, Pasching, Austria). Cell cultures were performed in RPMI-1640 medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), 1 mM sodium pyruvate, 1% non-essential amino acids and 2% AB-Serum at 5% CO_2 and 37 °C. The in vitro stimulation of PBMC started immediately after cell isolation or from previously frozen cells that were retrieved and cultured at a concentration of 3×10^6 cells/mL.

For the induction of cytokine release, the cells were cultured for 24 h in the presence of TiO_2 or ZnO_2 at the indicated concentrations. As the NPs themselves did not induce cytokine release, cell viability was monitored by costimulation with the potent stimulus LPS (100 ng/mL) (Invivogen, Toulouse, France). After incubation, the cell-free supernatants were collected and stored at −20 °C until analysis by hIL-6 (BD Bioscience, San Jose, USA), hIL-1-β ELISA (R & D, MI, USA). Potential interference of the NPs with the ELISA reaction was only observed at much higher concentrations (>100 μg/mL) than indicated.

2.3. Investigation of the effect of ZnO_2 and TiO_2 nanoparticles on the solubility of malate dehydrogenase

Previously, Luthuli et al. exposed the protein MDH to cysteine-coated gold NPs and observed that the NPs protected MDH from heat-induced aggregation (Luthuli et al., 2013). In the current study, we sought to investigate the effect of TiO_2 and ZnO_2 on the structural integrity of MDH in vitro. TiO_2 or ZnO_2 NPs at a final concentration of 2.5, 5, 10, 15, 25, 50, 75 and 100 μg/mL, respectively, were added to 20 μL of assay buffer (20 mM Tris, pH 7.4; 100 mM NaCl) containing 1 μM of pig heart MDH (Sigma Aldrich, St. Louis, MO). As control, untreated MDH (without any NPs added) was used. The reaction mix was allowed to stand at 37 °C for 20 min. To separate soluble protein from aggregated, samples were centrifuged for 10 min at 14000 rpm. A total of 15 μL of the supernatant was removed (soluble protein) and mixed with 5 μL of sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) loading buffer. The remaining pellet was re-suspended in SDS-PAGE loading buffer. The samples were analyzed by SDS-PAGE. The analysis was repeated three times.

2.4. Assessment of the effects of ZnO_2 and TiO_2 nanoparticles on plasmid DNA

Plasmid pQE60/DnaK also known as pBB46 is 5566 base pairs (bp) in size and originated in Dr. W. Burkholder's lab (Stanford University, USA). We have previously used this plasmid in a separate study (Shonhai et al., 2005). The plasmid possesses

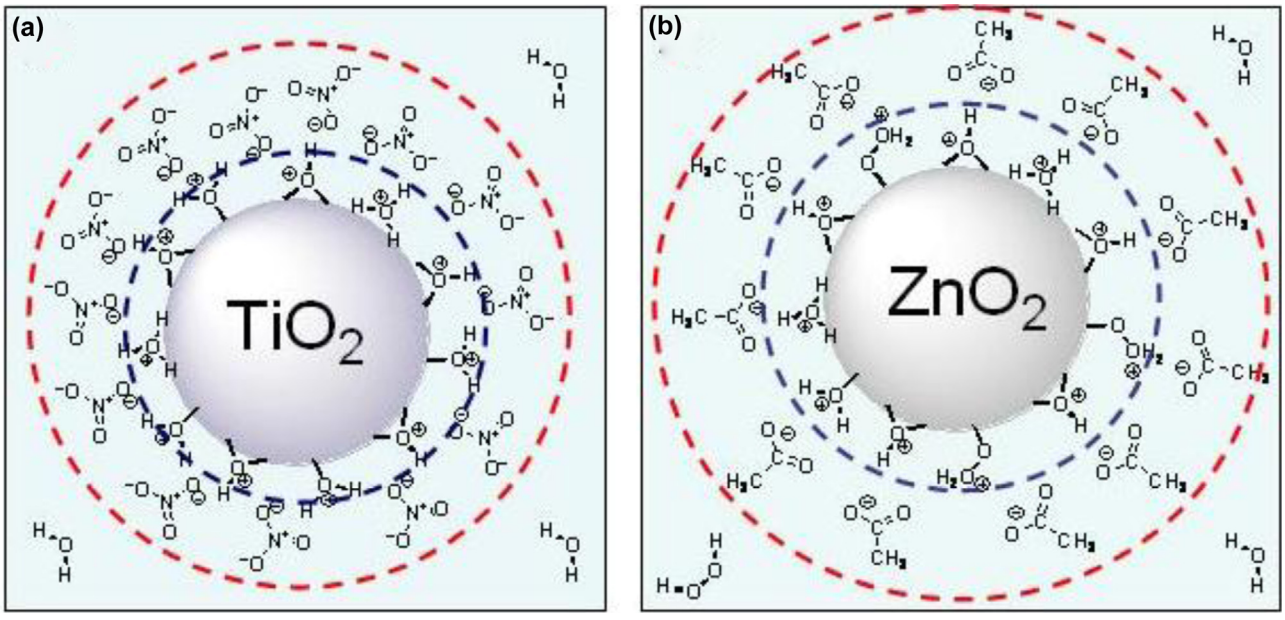

Fig. 1. Schematic drawing of the charge stabilization in (a) TiO_2 NPs and (b) ZnO_2 NPs.

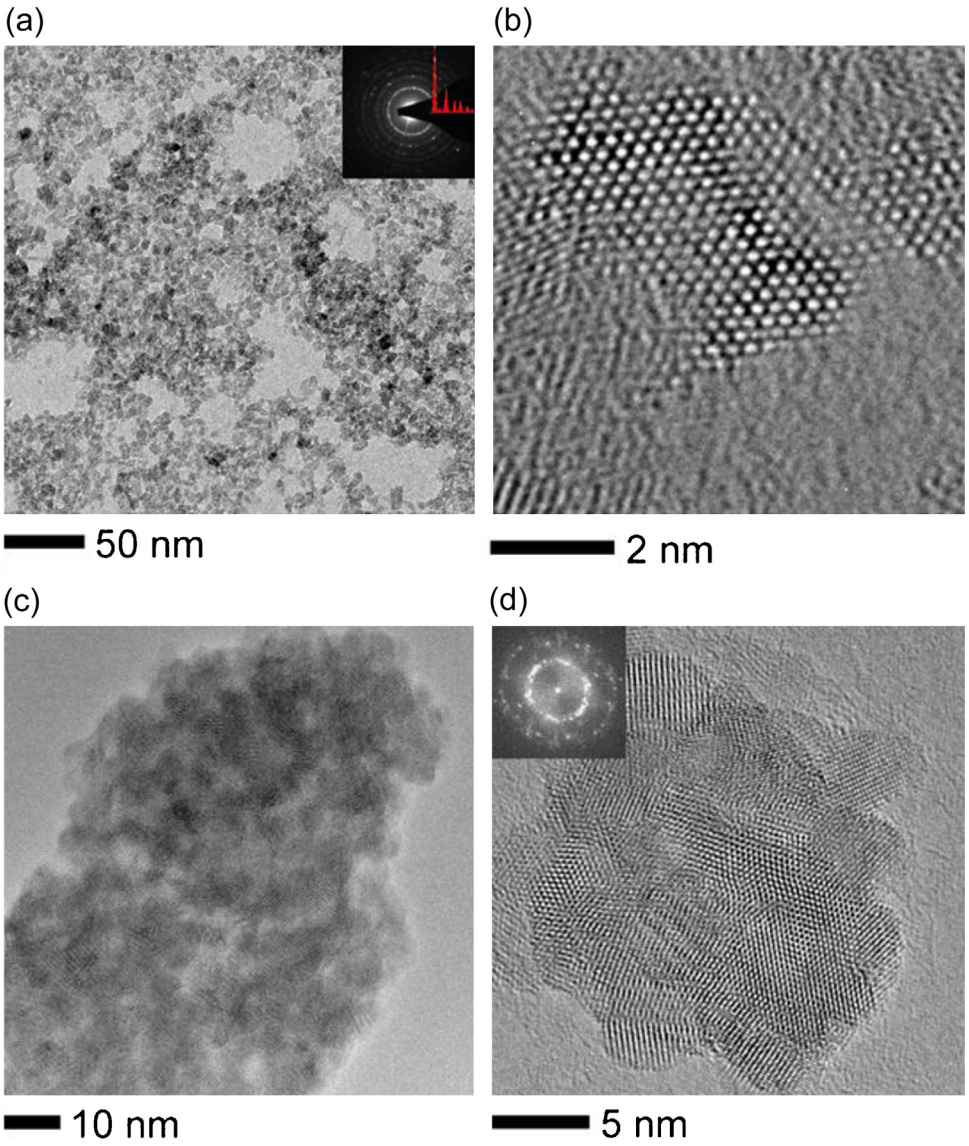

Fig. 2. (a) Bright-field TEM overview image of TiO_2 nanoparticles with an SAED pattern as inset. (b) HRTEM micrograph of the TiO_2 nanoparticles. (c) Bright-field TEM overview image of ZnO_2 nanoparticles. (d) HRTEM micrograph of agglomerated ZnO_2 nanoparticles with the associated FFT pattern.

ampicillin resistance. A preparation of the plasmid DNA was extracted, quantified and confirmed through restriction digestion. The DNA was exposed to varying concentrations of charge stabilized ZnO_2 and TiO_2 NPs as per method by Dunpall et al. (Dunpall et al., 2012) with slight modifications. Untreated plasmid DNA was used as a negative control. These treatments were incubated at 37 °C for 24 h. Agarose gel electrophoresis was conducted to evaluate DNA damage. 15 μL of each sample were mixed with 5 μL of DNA loading dye; and resolved by agarose gel electrophoresis at 1.0% gel density.

3. Results

3.1. Synthesis of charge stabilized ZnO_2 and TiO_2 nanoparticles

The colloidal stability of the TiO_2 and ZnO_2 dispersions we produced originated primarily from charged surface-species on the NPs surface. Furthermore the NPs possessed a layer of counter-ions which gave rise to a double layer of electrically charged repulsive forces between the NPs (according to the DVLO – Derjaguin and Landau, Verwey and Overbeek theory, (Redel et al., 2012; Verwey and Overbeek, 1999); see also Fig. 1). It has been shown that colloidally formed ZnO NPs are directly transformed into ZnO_2 as the final product when suspended under aqueous and acidic H_2O_2 conditions (21). Both dispersions were stable under acidic conditions, pH 1.3 (undiluted) and pH 1.8 (1:10 diluted with deionized water) for the TiO_2/HNO_3 dispersion and pH 4.4 (undiluted) and pH 5.1 (1:10 diluted with deionized water) for the $ZnO_2/AcOH/H_2O_2$ dispersion, respectively. Because the particles were synthesized under acidic conditions, thus their surfaces were 'positively charged'. This was because they possessed a protonated surface oxide and/or hydroxide groups (Fig. 1a and b; (Redel et al., 2012; Verwey and Overbeek, 1999)). The synthesized NPs are colloidal stable in aqueous solutions, no aggregation or further precipitations could be observed.

Zeta-potential measurements of the nanoparticles were determined and found to be $\varphi = 10.19$ mV for TiO_2/HNO_3 at pH = 1.8 (1:10 dilution) as well as with $\varphi = 14.24$ mV for $ZnO/AcOH/H_2O_2$ dispersion at pH 5.1 (1:10 dilution). The particles we synthesized possess double charge stabilized surfaces (Fig. 1). The size of the TiO2 and ZnO2 NPs we produced was in the range of 4–8 nm in diameter (Fig. 2) and are colloid stable. Additional characterization data for ZnO_2, TiO_2 NPs used in this study, including XRD, Zeta-potential and IR measurements/data can be found in the Supporting Material Information, for details see Figs. S1–S6.

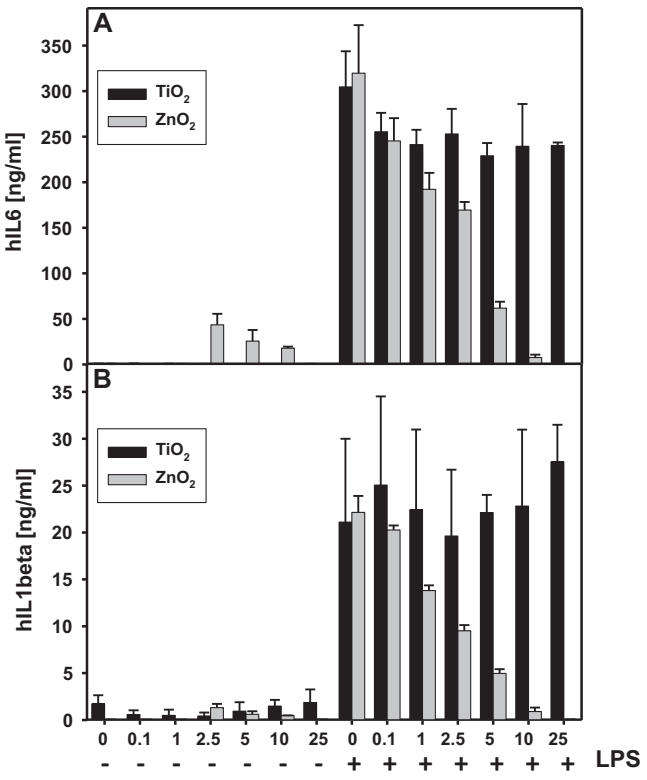

Fig. 3. ZnO_2 and not TiO_2 suppress LPS-induced production of cytokines by human PBMCs. Human PBMCs were cultured in vitro in the presence of variable concentrations (μg/mL) of ZnO_2 or TiO_2 NPs. The cells were either cultured in the absence (−) or in the presence (+) of LPS (100 ng/mL). After incubation, the cell-free supernatant was collected and analyzed for the presence of hIL-6 and IL-1β using ELISA. Three independent experiments were performed in triplicates each time; statistical significances of the various NP concentrations compared to the 0 μg/mL control were analyzed by paired t-test with $^*p < 0.01$.

The Bright-field TEM image in Fig. 2(a) shows the typical morphology of the TiO_2 nanoparticles dispersed on the TEM grid. From the HRTEM micrograph in Fig. 2(b) and the SAED pattern as an insert of Fig. 2(a) we can conclude that the TiO_2 NPs nanoparticles are crystalline in nature. The measured d-spacing from the SAED pattern and from the fast Fourier transformation (FFT) of the HRTEM micrographs confirm the results from the XRD (Fig. 2a and b) analysis (Anatase metrics). Fig. 2(c) shows a TEM Bright-field image with the typical morphology of the ZnO_2 NPs. The HRTEM micrographs of Fig. 2(d) show that the ZnO_2 NPs are also crystalline. The lattice spacing obtained from the associated FFT pattern (inset of Fig. 2d) confirms the results from the XRD analysis, for details see Supporting Material Information.

3.2. *Zno_2 nanoparticles interfere with LPS-stimulation of human PBMCs*

We explored the effects of various concentrations of the NPs on human PBMCs cultured in vitro. Cells were stimulated with NPs and after 24 h the cytokines in the supernatant were quantified by IL1-β and IL-6 ELISA. The exposure of the PBMCs to varying concentrations of TiO_2 and ZnO_2 did not result in stimulation of the immune cells (Fig. 3). To test the viability of the cells in the presence of NPs, we performed costimulation with the potent stimulus LPS. The treatment of the PBMCs with LPS resulted in significant production of both IL-6 and IL-1-β. The exposure of the PBMCs to varying concentrations of TiO_2 NPs did not interfere with the LPS-stimulated production of the two cytokines (Fig. 3A and B). We did not observe stimulation of the cells even at higher concentrations of TiO_2, up to 100 μg/mL (data not shown). Therefore, based on IL-6 and IL1-β production, the cells were not affected by TiO_2 NPs. However, the treatment of the cells with increasing concentrations of ZnO_2 NPs in the presence of LPS resulted in a proportional decrease in the production of both cytokines. At a concentration of 10 μg/mL of ZnO_2 NPs, the production of the two cytokines was nearly completely suppressed. The immune cells were affected by ZnO_2 particles starting from sub-minimal concentrations of 0.1-1 μg/mL ($p < 0.01$) and were completely unresponsive at a concentration of 25 μg/mL. ZnO_2 NPs suppressed the production of the two cytokines, whereas TiO_2 did not appear to affect the cells under the experimental settings we used.

3.3. *Effects of ZnO_2 and TiO_2 nanoparticles on the solubility of malate dehydrogenase*

NPs are known to interact with proteins (Luthuli et al., 2013) and this may lead to the misfolding of the latter and possible aggregation. It has been reported that the formation of protein aggregates is sometimes related to undesirable immunomodulatory reactions as the protein aggregates may activate or suppress immune responses (Hermeling et al., 2005; Joubert et al., 2012). It is therefore possible that TiO_2 and ZnO_2 NPs may interact with proteins, leading to the production of immune modulating complexes. For this reason, we sought to investigate the effect of TiO_2 and ZnO_2 on the structural integrity of protein in vitro.

MDH is an aggregation prone protein which is ideal for studying the effect of nanoparticles and other entities such as molecular chaperones on proteins in vitro (Luthuli et al., 2013; Shonhai et al., 2008). In the current study, we investigated the effect of ZnO_2 and TiO_2 NPs on the aggregation of MDH at 37 °C in vitro. MDH was exposed to varying concentrations of the NPs followed by the determination of its solubility (Fig. 4). Most of the MDH that had not been exposed to the NPs, occurred as soluble product (lanes S1, Fig. 4A–D) and only a small amount of the protein occurred in the pellet fraction (lanes P1, Fig. 4A–D). We sought to explore the effect of TiO_2 and ZnO_2 NPs on the solubility of MDH by exposing the protein to low nanoparticle concentration range (2.5–15 μg/mL) and much higher concentration range (25–100 μg/mL). At the lower concentration range, TiO_2 NPs marginally promoted the occurrence of more MDH in the pellet fraction, compared to the negative control (Fig. 4A). At the higher concentrations used, TiO_2 NPs facilitated the appearance of 100% of the MDH as a pellet (Fig. 4B). This suggests that TiO_2 nanoparticles promote aggregation or simply bind to MDH, resulting in a complex that easily sediments during centrifugation.

On the other hand, a minimum of 2.5 μg/mL of ZnO_2 NPs promoted the appearance of MDH in the pellet fraction and this trend increased in the presence of higher concentrations of the metal oxide (Fig. 4C). Furthermore, the ZnO_2 NPs promoted fragmentation of the MDH which increased with the increase in doses of ZnO_2 NPs used (Fig. 4C). In fact when MDH was exposed to higher concentrations (25–100 μg/mL) of ZnO_2 it was completely degraded (Fig. 4D). Altogether, these data suggest that ZnO_2 NPs exhibit more deleterious effects on protein structure compared to TiO_2 NPs.

3.4. *ZnO_2 nanoparticles are more detrimental to DNA stability than TiO_2 nanopartilces*

Because of their tendency to form free radicals, NPs may induce damage to biological entities such as DNA. We were therefore curious to investigate the effect of the charge stabilized ZnO_2 and TiO_2 NPs on DNA stability in vitro. Plasmid DNA occurs in various conformations (supercoiled, relaxed and linearized) depending on its structural integrity, making it appropriate for the investigation of the effects of NPs on DNA stability (Dunpall et al., 2012). We

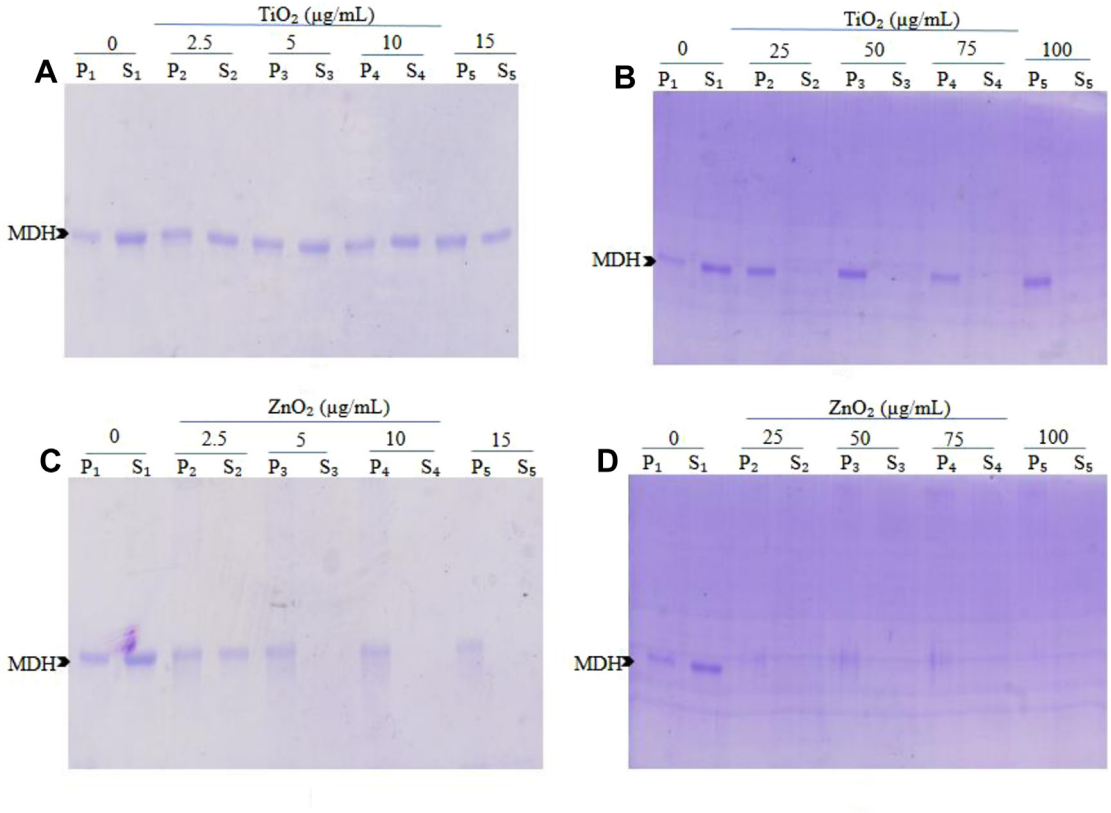

Fig. 4. Effects of ZnO_2 and TiO_2 solubility and stability of MDH in vitro.1 μM of MDH was treated with varying nanoparticle concentrations (2.5, 5, 10, and 15 μg/mL) and (25, 50, 75, and 100 μg/mL) of both metal oxides and the incubated at 37 °C for 20 min. MDH that was suspended in assay buffer in the absence of NPs was used as a negative control. The letters "P" and "S" represent pellet and soluble fraction, respectively.

exposed plasmid DNA to varying concentrations of the NPs and confirmed the integrity of the DNA by agarose gel electrophoresis.

The control plasmid DNA (not pre-treated with NPs), occurred largely in supercoiled form and migrated to an apparent size approximately equivalent to a species of 5000 bp (lane N, Fig. 5 top and lower panels). The exposure of the DNA to a minimum concentration of 2 μg/mL of ZnO NPs resulted in the generation of a linear form of the plasmid (lane 2; Fig. 5; top panel). The DNA that was treated with ZnO_2 NPs at a concentration range of 4–150 μg/mL was extensively degraded as evidenced by the appearance of smears on the gel. Some of the DNA that had been exposed to 25–150 μg/mL of ZnO_2 remained in the loading wells of the agarose gel indicating that it was immobile. This indicates that at high concentrations of the NPs the DNA may have associated with the NPs to form high order complexes which were too large to be resolved by gel electrophoresis. It is also possible that the association of the DNA with the 'positively charged' NPs may have resulted in partial neutralization of the negative charge of the DNA thereby impeding its ability to move towards the positive electrode. However, apart from the formation of complexes between the DNA and NPs, the appearance of smears on the gel signals that some of the DNA was damaged by exposure to high concentrations of ZnO_2 NPs.

At lower concentrations, TiO_2 did not appear to cause DNA damage based on agarose gel electrophoresis analysis (data not shown). However, DNA that was exposed to higher concentrations (25–100 μg/mL) of TiO_2 was partially damaged as we noted formation of faint smears on the agarose gel (Fig. 5; lower panel). However, exposure of the DNA to 125-150 μg/mL of TiO_2 NPs resulted in extensive DNA damage. As was observed for DNA that had been exposed to high concentrations of ZnO_2, we also noted that TiO_2 NPs appeared to complex with some of the DNA to form high order aggregates that were immobile (Fig. 5; lower panel).

4. Discussion

Given the unique charge stabilized character and the fairly small sizes (4–8 nm in diameter) of the TiO_2 and ZnO_2 NPs we produced for this and a previous study (Redel et al., 2012), we sought to explore their toxicity in human PBMCs. We further investigated their effect on the integrity of protein and DNA. The charge stabilized ZnO_2 NPs described in this study suppressed the LPS stimulated production of IL-6 and IL-1β by human PBMCs. The ZnO_2 affected cytokine production even when they were at a subminimal level of 1 μg/mL. Not only did we observe possible deleterious effects of ZnO_2 on human PBMCs based on the production of two cytokines (IL6 and IL1-β), but we also showed that the ZnO_2 NPs have deleterious effects on protein and DNA. In addition, our findings show that ZnO_2 NPs were more effective in modulating the function of the PBMCs than TiO_2 NPs. In addition, compared to than TiO_2 NPs, ZnO_2 NPs inflicted more damage on both protein and DNA. To the best of our knowledge, this is the first study that investigated the effects of ZnO2 NPs on the human PBMCs as well as their effects on protein and DNA integrity. Our findings present data on the possible toxicity of ZnO_2 NPs. This far there has been paucity on the toxicity of these NPs as most previous studies largely focused on investigating the toxicity of ZnO species (Andersson-Willman et al., 2012; Horie et al., 2009; Horie and Fujita, 2011).

LPS induces the production of modulators of inflammation, which play a critical role in the immune response of mammals. In the current study, we observed that TiO_2 NPs at varying concentrations (0.1–25 μg/mL) did not interfere with the response of the PBMCs to LPS stimulation (Fig. 3). On the other hand, ZnO_2 NPs suppressed the LPS-stimulated production of both IL6 and IL1-β by PBMCs. This implies that the ZnO_2 we produced could interfere with the human immune response. Sunscreens usually contain 4-30% of

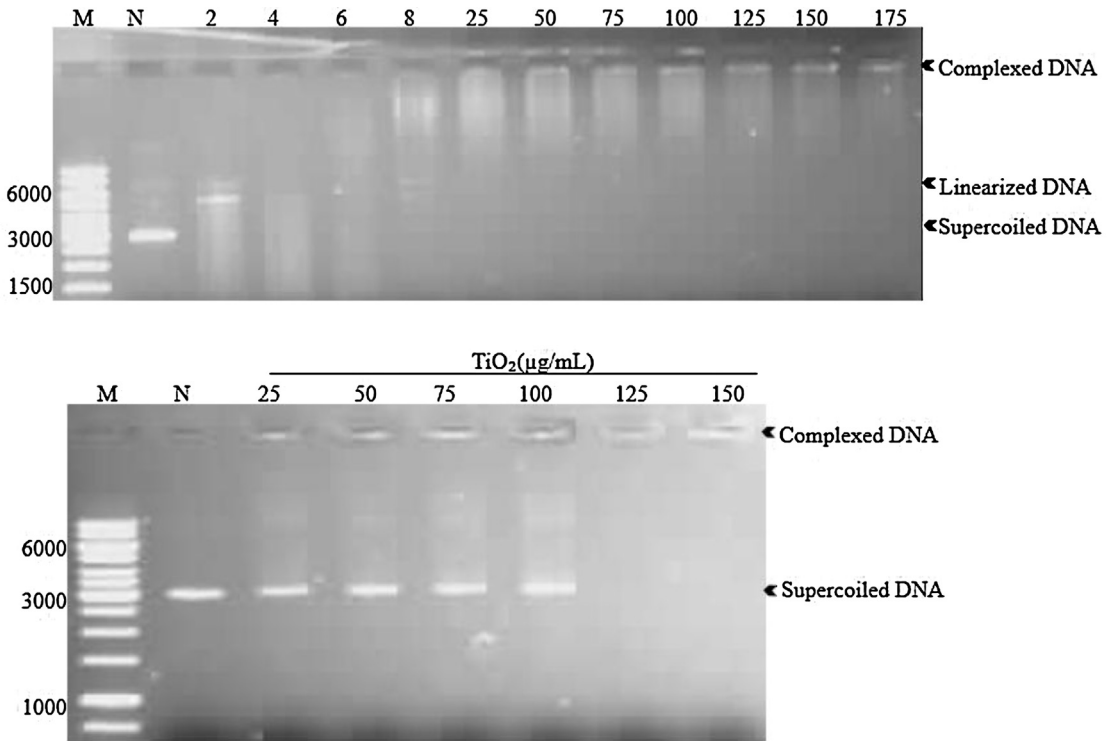

Fig. 5. Assessment of damage induced on plasmid DNA by nanoparticles. The plasmid DNA was exposed to variable concentrations of nanoparticles and DNA damage was assessed by agarose electrophoresis. (top panel) An agarose gel used to resolve DNA pre-treated with ZnO_2 NPs; and (lower panel) An agarose gel used to resolve DNA pre-treated with ZnO_2 NPs; Untreated plasmid DNA was used as a negative control in lane 'N'. Lane 'M' represents molecular markers in bp.

ZnO NPs and only a small fraction of these NPs are thought to penetrate the skin. A previous study suggested that 10 μg/mL of ZnO NPs was possibly above practical exposure risk (Andersson-Willman et al., 2012). Furthermore, it was noted in the same study that exposure of natural killer cells to 10 μg/mL of ZnO resulted in deleterious effects. Findings from the current study reflect the possibility that charge stabilized ZnO_2 NPs may be toxic to PBMCs at much lower concentrations as we saw suppression of cytokine production even in the presence as low as 0.1–1 μg/mL of the metal oxide. In agreement with the study by Andersson-Willman et al. (2012), we observed that TiO_2 NPs did not appear to modulate cytokine production by PBMCs even up to a concentration of 100 μg/mL.

It is known that protein aggregates may modulate the immune system, and that this accounts for some of the undesirable outcomes in the clinical application of therapeutic antibodies (Hermeling et al., 2005; Joubert et al., 2012). For this reason, we investigated the effects of TiO_2 and ZnO_2 NPs on the aggregation of protein in vitro. Both ZnO and TiO_2 NPs are known for their propensity to interact with protein (Luthuli et al., 2013; Zaqout et al., 2012). The interaction between NPs and biological molecules are driven by surface charges as well as van der Waals' forces, hydrophobic, hydrophilic, structural and steric forces (Patil et al., 2007). We previously observed that cysteine coated gold NPs interacted with MDH and that these NPs suppressed the heat-induced aggregation of the protein (Luthuli et al., 2013). Gold NPs are fairly stable, and it is possible that their compatibility with biological systems could be partially due to their capability to interact with proteins without adverse effects. Therefore, it seems that the structural-functional fate of proteins upon exposure to NPs is a crucial determinant of cellular toxicity. Our findings suggest that TiO_2 nanoparticles do not seem to exhibit adverse effects on protein structure based on their effect on the solubility of MDH in vitro (Fig. 4). Only at fairly high concentrations (>25 μg/mL) did we find TiO_2 NPs to promote the shift of MDH from solution into a pellet fraction at 37 °C. TiO_2 and ZnO NPs have been shown to exhibit high protein-adsorptive capacity (Luthuli et al., 2013; Zaqout et al., 2012; Patil et al., 2007). The adsorption of proteins on nanoparticle surfaces may result in the formation of insoluble complexes. Alternatively, the presence of the NPs may promote protein misfolding and aggregation (Zhang et al., 2009). The TiO_2 nanoparticles used in this study possess a 'positive charge', and they may have interacted with oppositely charged surfaces on MDH. Altogether, our data suggest that TiO_2 NPs at sub minimal concentrations do not have adverse effects on protein structure.

On the other hand ZnO_2 at concentrations of 2.5–5 μg/mL promoted the precipitation of MDH (Fig. 4C). At higher concentrations, ZnO_2 NPs promoted not only the precipitation of MDH, but facilitated its degradation (Fig. 4C and D). This suggests that ZnO_2 NPs at subminimum levels may have deleterious effects on protein function. On the other hand, the observed function of ZnO_2 NPs in promoting aggregation could account for their role in modulating the LPS-stimulated production of cytokines by human PBMCs (Hermeling et al., 2005; Joubert et al., 2012).

In addition, the degradation of protein in the presence of ZnO_2 NPs could lead to propagation of a myriad of other toxic effects on the cells. Tu et al. speculated that the formation of reactive oxygen species (ROS) may have led to the structural damage of BSA in the presence of ZnO NPs (Tu et al., 2013). However, in their study, Tu et al. exposed the protein-ZnO NPs mix to photocatalytic conditions and heat (Tu et al., 2013), which may have promoted the generation of the reactive oxygen species (ROS). In the current study, we investigated the effect of charge stabilized and ligand-free TiO_2 and ZnO_2 NPs under normal daylight conditions and observed that the ZnO_2 NPs were capable of degrading protein under these conditions. Our findings imply that the charge stabilized ZnO_2 NPs are highly reactive, due to their high oxidative capacity and this may partially account for their toxicity. A study by Šimundić et al. proposed that both ZnO and TiO_2 NPs may promote erythrocyte aggregation

(Hirakawa et al., 2004). The same study further reported that ZnO NPs exhibited more effective toxicity in this regard than TiO_2 NPs. It is therefore possible that the capability of ZnO_2 and TiO_2 NPs to promote protein aggregation may facilitate blood clotting.

Assessment of DNA damage suggested that at least 2 μg/mL of ZnO_2 NPs were required to facilitate DNA damage (Fig. 5; top panel). The capability of these particles to cause DNA damage at such low levels signifies potential risk associated with exposure to ZnO_2 NPs. In contrast to ZnO_2 NPs, TiO_2 NPs only appeared to cause slight damage to DNA at the concentration range of 25–100 μg/mL. It is therefore highly unlikely that TiO_2 NPs would penetrate the skin to be present in tissue at sufficiently dangerous levels. Another study reported TiO_2 at a concentration range of 4–16 μg/mL induced DNA damage in vitro in the presence of H_20_2 (Hirakawa et al., 2004). In yet another study, exposure of plasmid DNA to both ZnO and TiO_2 NPs at a concentration of 0.0125% (w/v) in sunscreen, combined with UV exposure was shown to promote relaxation of the DNA in vitro (Dunford et al., 1997). Based on a chromotest assay, no toxicity of TiO_2 and ZnO at maximum concentration of 100 mg/L was observed (Nam et al., 2013). In our study, we observed deleterious effects on plasmid DNA that was exposed to a minimum concentration of 2 μg/mL of ZnO_2 NPs. Even the TiO_2 NPs at a concentration of 25 μg/mL appeared to promote slight DNA damage. We question the sensitivity of the findings that were obtained using the chromotest assay in the study by Nam et al. (2013), which reported data lying well out of toxicity ranges observed by others. A study by Li et al. reported TiO_2 NPs accumulated in the liver of mice that were fed with TiO_2 NPs (Li et al., 2010). The same study however, only observed possible DNA damage from mice that were exposed to fairly high concentrations of TiO_2 NPs (310 mg/kg body weight of mice). Based on the current study and in agreement with the study by Li et al. (Li et al., 2010), TiO_2 are only toxic at fairly high concentrations. Furthermore, the toxicity of TiO_2 may be potentiated by chemical agents or by UV light. However, we contend that ZnO_2 NPs possesses potential DNA toxicity.

5. Conclusion

This study reports the effects of charge-stabilized and colloidal stable aqueous TiO_2 and ZnO_2 NPs on human PBMCs, protein and DNA. We observed that at a concentration of 1 μg/mL the ZnO_2 NPs modulated the human PBMCs, while TiO_2 NPs had no effect at much higher concentrations of TiO_2 NPs. We further observed that at subminimum level of 2 μg/mL the ZnO_2 NPs manifested to DNA damage, while we could only observe slight DNA damage when much higher levels of TiO_2 NPs were used. While both ZnO_2 and TiO_2 NPs appeared to promote protein aggregation, we observed that ZnO_2 did not only promote protein aggregation, but facilitated protein degradation. Observations from the current study suggest that charge stabilized ZnO_2 NPs are more toxic than their TiO_2 counterparts.

Both nanoparticle dispersions are colloid stable in aqueous solutions. Our findings further indicate that apart from interfering with cytokine production by immune cells, ZnO_2 NPs may also confer toxicity through their interaction of charged surface species as well as the formation of complexes with DNA and proteins. It is also possible that by promoting protein aggregation, ZnO_2 facilitate formation of potent immune modulating protein complexes. The mechanisms by which ZnO_2 NPs modulate immune cells is an aspect that requires further investigation.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2014.02.027.

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