

RESEARCH ARTICLE

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Kinetic effects of TiO₂ fine particles and nanoparticles aggregates on the nanomechanical properties of human neutrophils assessed by force spectroscopy

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Abstract

Background: Increasing applications of titanium dioxide (TiO₂) fine particles (FPs) and nanoparticles (NPs) require coupled knowledge improvement concerning their biokinetic effects. Neutrophils are quickly recruited to titanium implantation areas. Neutrophils mechanical properties display a crucial role on cell physiology and immune responsive functions. Then, micro and nanomechanical characterization assessed by force spectroscopy (FS) technique has been largely applied in this field.

Results: Scanning electron microscopy (SEM) images highlighted neutrophils morphological changes along TiO₂ FPs and NPs aggregates exposure time (1, 5, and 30 min) compared to controls. FS approaches showed an increasing on attraction forces to TiO₂ FPs and NPs treated neutrophils. This group depicted stronger stiffness features than controls just at 1 min of exposure. Treated neutrophils showed a tendency to increase adhesive properties after 1 and 5 min of exposure. These cells maintained comparatively higher elasticity behavior for a longer time possibly due to intense phagocytosis and cell stiffness opposing to the tip indentation. Neutrophils activation caused by FPs and NPs uptake could be related to increasing dissipated energy results.

Conclusions: Mechanical modifications resulted from TiO₂ FPs and NPs aggregates interaction with neutrophils showed increasing stiffness and also cell morphology alteration. Cells treatment by this metal FPs and NPs caused an increase in attractive forces. This event was mainly observed on the initial exposure times probably regarding to the interaction of neutrophils membrane and phagocytosis. Similar results were found to adhesion forces and dissipated energy outcomes. Treated cells presented comparatively higher elasticity behavior for a longer time. SEM images clearly suggested cell morphology alteration along time course probably related to activation, cytoskeleton rearrangement and phagocytosis. This scenario with increase in stiffness strongly suggests a direct relationship over neutrophil rolling, arrest, and transmigration. Scrutinizing these interactions represents an essential step to clarify the mechanisms involved on treatments containing micro and nanomaterials and their fates on the organisms.

Keywords: Force spectroscopy, Neutrophil nanomechanics, Titanium dioxide microparticles, Titanium dioxide nanoparticles

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Background

Titanium dioxide (TiO₂) has been widely used in automotive and aerospace industry, personal care, food, and even in pharmaceutical and biomedical products [1,2]. Increasingly, this metal and its alloys are used in the biomedical field for medicine and dentistry [3,4]. Pure titanium and some of its alloys are non-toxic and generally have been described as being biocompatible with human tissues [5]. Despite its biocompatibility, toxicological concerns are depicted in the literature related to ions, microparticles (MPs), fine particles and nanoparticles [6-10]. Despite being inert [11], titanium may promote inflammatory reactions by recruiting inflammatory cells [12], such as neutrophils.

Neutrophils represent the first line of innate defense, comprising about 50-70% of all human leukocytes [13]. They are quickly summoned to areas containing titanium micro or nanoparticles [14]. It is well-known that MPs and NPs can be internalized by phagocytic and even non-phagocytic cells [15-17], and can interact with cell membranes, subcellular organelles, proteins, and nucleic acids [18,19]. Neutrophils internalize TiO₂ MPs and NPs, leading to a change in shape and motility; these events are modulated by cytoskeleton proteins, and enhance the production of superoxide anion, cytokines, and chemokines due to cell activation [20-23]. There are different pathways for the uptake of MPs and NPs: micro-sized particles are internalized via phagocytosis by monocytes, macrophages, and neutrophils [17], while NPs (ranging from 1 to 100 nm) seem to use other pathways in spite of the fact that, in some situations, NPs can aggregate or agglomerate, resulting in MPs behaviour, being an important factor in understanding cytotoxicity. However, the effect of the aggregate size of nanoparticles on cells is unclear [24]. Agglomeration (particles are linked by weak forces, eg van der Waals forces, capillary) and aggregation (particles held together by chemical bonds-strong forces) of TiO₂ MPs and NPs are known mechanisms [25] and may occur on the surface of the cells membrane affecting communication with the external environment [26]. Furthermore it is known that the length and chemical nature of the aggregates of TiO₂ are related to the toxicity [27]. In addition to phagocytosis, uptake pathways include clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and pathways independent of clathrin/caveolin. The passive pathway should also be considered for NPs uptake [28].

Neutrophil recruitment into areas with MPs or NPs proceeds in a cascade process. Rolling of neutrophils inside vessels is mediated by selectins (PSGL-1) and their counter-receptors (L and P selectins) and integrin-mediated (LFA-1, Mac-1) arrest [29]. These adhesion mechanisms activate different signaling pathways in the cell, leading to neutrophil extravasation, promoting

cytoskeletal rearrangement, and inducing superoxide production and degranulation [30,31]. The behavior of neutrophils and other cells following contact with macro or micro-sized particles are different from following contact with NPs, due to specific surfaces, energy, and physicochemical properties [25,32,33]. When circulating neutrophils encounter a macro-sized TiO₂ surface, the adhesion process starts, mediated by selectins, FcγIII receptor (CD16) and finally integrins (CD11b); cells are not activated to undergo a respiratory burst by TiO₂ surfaces [34]. On the other hand, neutrophils phagocytose TiO₂ NPs and MPs aggregates smaller than the cell [15,23]. Furthermore, TiO₂ MPs and NPs may activate and induce alterations in cell morphology [20,35] and inhibit apoptosis (at 50–100 μg/mL). They also induce the production of several different cytokines/chemokines, mainly IL-8 and Gro-α [20]. Therefore, when this interaction occurs, MPs and NPs play a crucial role in cell physiology and mechanical properties. Moreover, the mechanical properties of cells are important when aiming to understand mechanisms such as adhesion, endothelial transmigration, and diapedesis [36].

Concerning the mechanical properties of cells, a variety of methods and techniques have been used to probe cells such as micropipette aspiration [37], magnetic twisting cytometry (MTC) [38], and atomic force microscopy (AFM). Conceptually, AFM is a type of scanning probe microscopy (SPM) and is perceived as a tool central to the goals of the burgeoning field known as nanotechnology [39]. It represents an effective tool to probe the viscoelastic properties of cells, to study the membrane and subcellular structures, and plays a key role in determining cell function in response to inflammatory stimuli [40,41]. AFM is mainly used for surface topography analysis by using attractive (short-range chemical, van der Waals and electrostatic forces) and repulsive interaction forces among a few atoms attached to the tip of a cantilever and the sample surface (nanoindentation). This technique has increasingly been applied for the characterization of single molecules under tensile or torsional load, cell-to-cell interactions, interactions of surfaces with molecules, and investigations into the mechanical properties of cells [42]. Further details concerning AFM are presented elsewhere [36,43,44]. The nanoindentation technique using force spectroscopy enables researchers to assess living or fixed neutrophils, in a passive or activated state, pointing out the micro-rheology of the entire cell surface (e.g. the cell body, lamellipodia, uropodia, and projections of the cytoskeleton), allowing the analysis of viscoelastic properties [45,46].

Force spectroscopy is the force-versus-distance measurement taken when using AFM in vertical mode only [47]. Cantilever deflection is recorded as it indents the cell, by reflecting a laser off the cantilever into a split photodiode.

Hertzian mechanics gives a linear cell-based elastic model on AFM deflection data to determine cell elasticity, among other mechanical parameters [42,48]. Focusing on neutrophil rheology, Lee and coworkers stated that the neutrophil body is significantly stiffer than the regions closer to the leading edge, while leading edge and tail regions were mechanically indistinguishable [45]. On the other hand, Rosenbluth and coworkers, when comparing neutrophils, myeloid (HL60) and lymphoid (Jurkat) cells, reported that there is a significant cell type effect on stiffness. Neutrophils were significantly softer than HL60 cells and significantly stiffer than Jurkat cells [48].

Increasing applications of FPs and NPs in the dental and medical field (dental implants, orthopedic prostheses, and antineoplastic therapies) have led to concerns regarding the biokinetic effects of micro and nanomaterials and their fate *in vivo*. Therefore, the present study aimed to analyze some mechanical properties related to the effects of TiO₂ FPs and NPs aggregates exposure on human neutrophils by a force spectroscopy approach over time (1, 5, and 30 min). Parameters like snap-in force, maximum load force, detachment force, Young's modulus, and dissipated energy were depicted by approaching and retracting a non-functionalized tip over adherent cells on local nano-domains in Z-direction. Cell features like stiffness and elasticity were investigated.

Methods

Exposure times

The methodology of human neutrophils interaction with titanium dioxide FPs and NPs was adjusted and various exposure times were tested. Once the literature has few information concerning neutrophils interaction with TiO₂ micro, submicron and nanoparticles by AFM, only the first hour were assessed by FS. However during experiments for this study, cellular analysis intervals tested above thirty minutes, resulted in locking the cantilever tip, by preventing to achieve the force curves measurements. Hence the exposition times used were 1, 5 and 30 minutes.

TiO₂ FPs and NPs characterization

Samples of 99.9% pure titanium oxide FPs and NPs (Sigma-Aldrich, TiO₂ reference number 14027, titanium (IV) oxide puriss) were immersed in 1 mL of Milli-Q water using a 5 mL sterilized disposable syringe. Particle hydrodynamic diameter was analyzed by dynamic light scattering (DLS) in triplicate using a Nanotrak U2131I (Microtec Inc., USA, at Instrutécnica, SP). Moreover, TiO₂ FPs and NPs were sized by Transmission Electron Microscopy (TEM) analysis of the dry powder (JEM- 2011, JEOL Ltd.) and also by AFM. Particles were sterilized in an autoclave at 121°C for 20 min [49]. Suspensions containing

200 µg/mL TiO₂ FPs and NPs were submitted to ultrasound bath for 30 min before use, aiming to avoid particles aggregation.

Human neutrophil preparation

Neutrophils were isolated from human whole blood using a density gradient technique as described in a previous study [50]. Briefly, 12 mL of peripheral blood were drawn from three healthy individuals with sterile heparinized syringes and not pooled. Collected blood was added to a Percoll density gradient (60%/70%) and centrifuged. After centrifugation, polymorphonuclear cells were harvested from the interface of the two gradients using a Pasteur pipette and re-suspended in 500 µL of Ca-/Mg-free blood buffer in Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, Missouri Sigma-Aldrich Corp.). The suspension containing 7×10^5 cells was divided into six aliquots: three were incubated (1, 5, and 30 min) with 20 µL of the TiO₂ FPs and NPs suspension at 37°C and three were kept as controls (1, 5, and 30 min). After incubation, cells were fixed with 100 µL of Karnovsky solution (12% paraformaldehyde + 8% glutaraldehyde + 0.2 M cacodylate buffer). Once fixed, cells were washed with PBS for 5 min and centrifuged for 2 minutes, three times (4500 g at 4°C). Each cells aliquot (1, 5, and 30 min) was then placed on separated pre-sterilized circular coverslip. For force spectroscopy measurements, only well-adhered cells were selected.

Ethics and consent statement

All the experiments were performed with the approval of the Research Ethics Committee of Medical Faculty, University of Brasilia (registration number: CEP-CFM-45/2010), in compliance with the Helsinki Declaration. Likewise, adult participants in the study (all human subjects) provided informed consent for blood donation.

Scanning electron microscopy (SEM) analyses

Fixed neutrophils were buffered in 0.1 M sodium cacodylate buffer and 0.1 M osmium tetroxide for 1 h, dehydrated and sputter-coated with gold. A SEM microscope (Jeol 840A operating at an accelerating voltage of 20 kV) was used to depict possible morphological changes in neutrophils.

Force spectroscopy analyses

Commercial AFM equipment (SPM-9600, Shimadzu, Japan) operated in air contact mode was used to analyze surface interaction forces between the tip atoms and cell membranes. Circular coverslips (10 mm) containing adhered neutrophils of each sample were fixed on a metallic support using a double-sided adhesive tape. A pyramidal silicon nitride tip (curvature radius < 20 nm)

integrated with a cantilever with length of 200 μm (spring constant 0.15 N/m and approximately 24 kHz resonance frequency) was used in this technique. The acquisition rate was 1 Hz with 20 V of amplitude and operating point 3 V. At each incubation time point (1, 5, and 30 min) 10 cells from each group (control and TiO_2) were analyzed and five force curves per cell were measured, resulting in 300 events, taking into account the control and experimental groups.

Data analysis

SPIP v. 5.1.5 was used to force curve analysis set at 23°C and 0.15 N/m spring constant, cone indentation (Sneddon), with five fitting points and the approach curve as the baseline correction curve. FS data were further analyzed using Origin 8.6 software by ANOVA and the Tukey test ($P < 0.05$).

Results and discussion

Particle size is a primary parameter when considering the effect of MPs, FPs and NPs in cellular interactions. According to Watari et al. [35], increasing the specific surface area usually implies a micro or nanosizing effect, which enhances chemical reactivity and consequently leads to serious toxicity for soluble and suspended materials. Watari et al. [51] considered particle size biointeractive below cell size, namely approximately 10 μm or smaller. These authors also point out critical sizes related bioreactivity; the micro or nanomaterial critical size means that, below 200 nm, a particle becomes invasive to the organism's structures, leading to a lack of recognition of this material by the immune system [35,51]. Furthermore, TiO_2 particles and nanoparticles aggregation is related to understanding cytotoxicity. It is known that the surface area, length and chemical nature of the TiO_2 MPs and NPs aggregates are related to the cell toxicity [24,27]. It has been shown that nanoparticles are able to promote a more pronounced toxicity effect than microparticles [52]. Once the MPs and NPs have great potential for agglomeration and aggregation it is unlikely to occur cellular exposure to isolated form of this particles [53]. Okuda-Shimazaki et al. [24] demonstrated large (596 nm) TiO_2 aggregates showed a larger effect on cell viability and gene expression when compared with the small aggregates (166 nm). This suggests that particle aggregate size is related to cellular effects. Bermudez et al. [54] exposed rats to inhalation of TiO_2 NPs (21 nm) for the evaluation of pulmonary toxicity. Even with the aerosols formed was possible to detect the presence of particles aggregates with 1.37 μm , which were able to promote cytotoxicity.

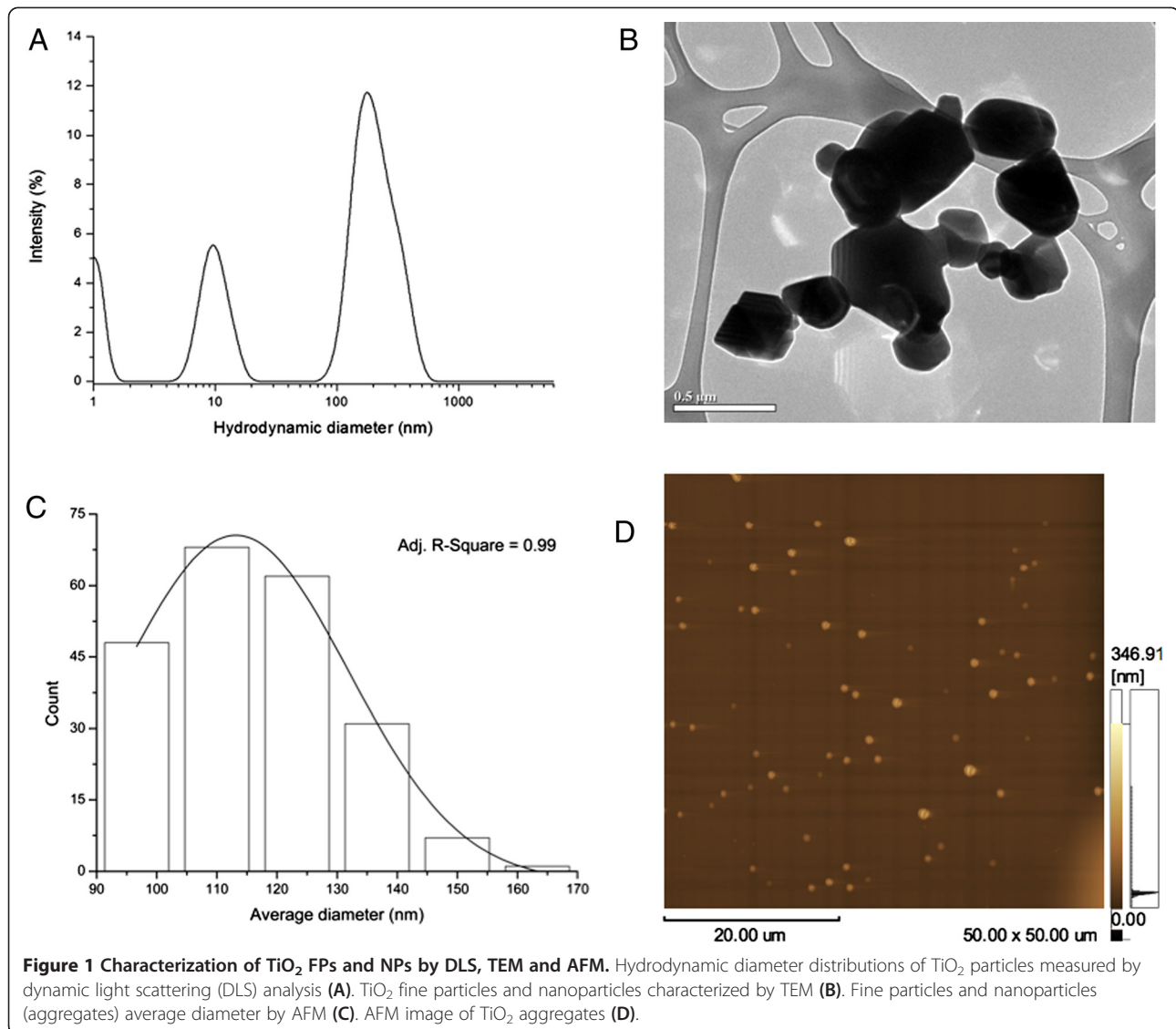
Figure 1 shows the TiO_2 FPs and NPs aggregates hydrodynamic diameter distributions (A), where approximately 67% of the particles were about 180 nm in hydrodynamic

diameter (see Additional file 1: Table S1- Peak Summary). The TEM image showed TiO_2 aggregates with size from NPs up to FPs and MPs (B). Also AFM demonstrated an average diameter below 170 nm with a peak at 115 nm (C). AFM image of TiO_2 aggregates (D).

Figure 2 shows SEM images comparing non-exposed neutrophils (A, B, and C) and TiO_2 particles aggregates exposed neutrophils (D, E, and F) after 1 min (A and D), 5 min (B and E), and 30 min (C and F), respectively. TiO_2 FPs and NPs aggregates are seen close to neutrophils (Figure 2E, white arrow).

It is possible to observe neutrophil morphological changes with increasing exposure time due to the influence of TiO_2 aggregates. Neutrophils left the quiescent state (Figure 2A,B, and C) and transitioned to an activated form, extending their pseudopodia in order to phagocytose TiO_2 aggregates (Figure 2D,E, and F). Several other studies [15,23,55] have demonstrated similar morphological alterations resulting from TiO_2 FPs and NPs treatment, characterized by increasing cytotoxic biomarker expression (lactate dehydrogenase, superoxide anion, and proinflammatory cytokines), and inversely related to particle size. However, there is a lack of knowledge concerning the nanomechanical modifications resulting from TiO_2 FPs and NPs aggregates exposure. The next step in this study was to evaluate the nanomechanical effects caused by the presence of TiO_2 aggregates on the neutrophil surface at the same time points, using the FS technique.

The FS results were derived from force-distance curves consisting of an AFM tip approximation and detachment from the sample surface. This event was monitored by an optical laser signal, which reflects from the upper reflective surface of the cantilever. Attractive, repulsive, and/or adhesive forces can manifest while this process occurs. The first event related to tip approximation involves attractive forces (such as van der Waals forces and capillarity interactions) and it is known as snap-in, whereupon the tip "jumps" to establish close contact with the sample surface. Figure 3 shows statistically significant differences in snap-in measurements between control and TiO_2 aggregates treated neutrophils after 1 and 5 min of exposure. The treatments caused an increase in snap-in force measurements when compared to the control. These results could be related to morphological changes caused by neutrophil stimulation, as observed in Figure 2. The control cells showed a time-dependent increase in snap-in force values from 1 to 30 min, while TiO_2 aggregates exposed cells showed increased force values from 1 to 5 min. Moreover, statistically significant differences ($P < 0.05$) were also found between aggregates exposed cells and their respective controls for the 1 and 5 min incubation times, but not at 30 min (Figure 3). These data may demonstrate that attractive forces like van der



Waals and electrostatic forces on the cell membrane are more intense with longer incubation times. The interaction between FPs and NPs aggregates and the neutrophil membrane and uptake by phagocytosis increase the snap-in forces. Such forces seem to plateau at some point near 30 min, where the interaction with particles aggregates no longer enhances the values. Additional experiments with longer exposure times could demonstrate the behaviour of snap in forces along time.

Variations in sample surface mechanical properties such as viscoelasticity and adhesion forces, as well as fixation solutions, also influence cantilever oscillations [56,57]. On the other hand, one should consider that FPs and NP interference with neutrophil membranes is related to surrounded or attached TiO₂ particles. This event could promote interactions with the tip, considering the ability of FPs and NPs to aggregate into numerous

large clusters, as seen in Figure 2D and E [58]. For example, a study Guduru [56] demonstrated that bare polylactic-co-glycolic acid (b-PLGA) NPs interacted with ovarian cancer cells (SKOV-3) and after 24 h of incubation, and most of the particles remained on the cell surface. NPs in close contact with the membrane surface may promote a lack of communication with the external environment [26].

The next event occurring in a force curve experiment is associated with the maximum force applied that relates to sample surface micro and nanostiffness. Maximum loading force measurements comparing control and TiO₂ aggregates treated neutrophils showed statistically significant differences, but only at 1 min of exposure (Figure 4). Interestingly, mechanical assays showed that activated neutrophils were over two-fold stiffer than quiescent cells [59]. Moreover, under load, the

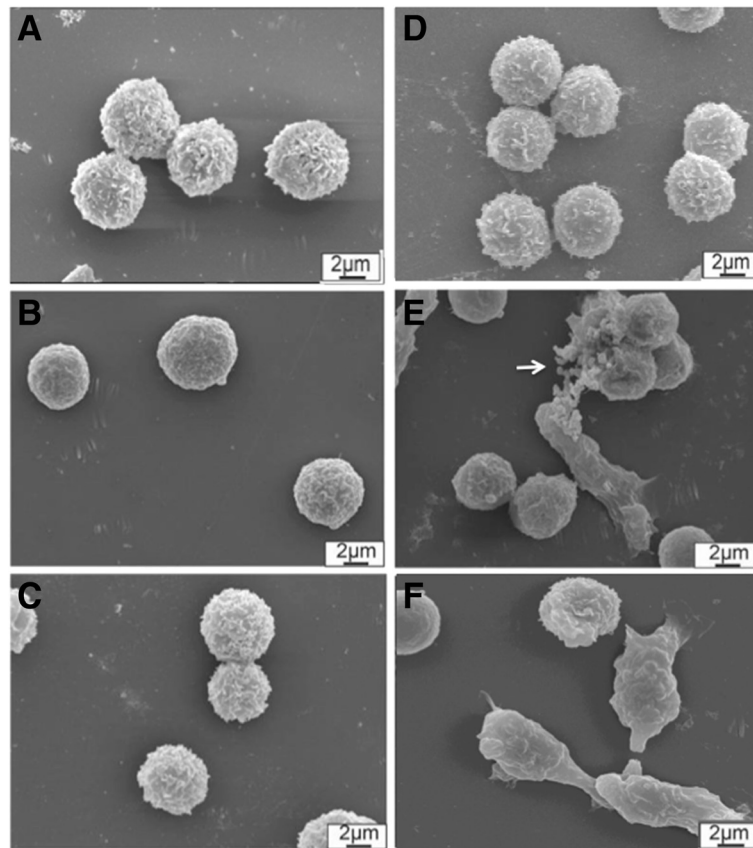


Figure 2 SEM images of controls and TiO₂ FPs and NPs aggregates exposed neutrophils. Images of untreated neutrophils or control (A, B, and C) and TiO₂ fine particles and nanoparticles clusters- exposed neutrophils (D, E, and F) obtained by scanning electron microscopy (SEM) analyses. A and D refer to cells collected after 1 min of exposure, B and E after 5 min, and C and F after 30 min.

membrane deformed in a non-linear elastic manner and demonstrated binary behavior of stiff and slack conditions [60]. Control cells at 30 min had a statistically significant difference to the respective control at 5 min ($P < 0.05$), suggesting a possible increase in membrane softness in quiescent neutrophils as time passes. Furthermore, when compared to control cells, TiO₂ treated neutrophils at 1 min depicted stronger forces, which could represent increased cell stiffness due to phagocytosis or TiO₂ aggregates adhesion to the cell membrane. It must be considered that, during phagocytosis, activated neutrophils might undergo increasing membrane cortical tension together with progressive cell surface and cytoskeletal structure hardening [61].

Detachment force measurement represents a relationship with adhesion forces (i.e. hydrogen bonds, ionic attractions, van der Waals forces, hydrophobic interactions, and/or chemical adhesion) [62]. This FS parameter occurs last on a retraction curve, corresponding to the point where the tip and surface lose contact, and it is related to adhesive interactions between them [63]. Figure 5 shows statistically significant differences ($P < 0.05$) between control and

TiO₂ treated neutrophils after 1 and 5 min of exposure, evidencing a tendency to increase this mechanical feature after stimulation. Once stimulated, cells acquire a polarized morphology, with F-actin polymerization in which integrins connect the cytoskeleton with the extracellular matrix [56]. Control cells at 1, 5, and 30 min demonstrated a statistically significant difference from the silica coverslip ($P < 0.05$). Stronger adhesion forces were seen with the glass than with quiescent neutrophil membrane surfaces, which showed that the adhesion forces were not due to the cell support. Similarly, TiO₂ FPs and NPs aggregates treated neutrophils at 1 and 5 min had lower detachment forces than the silica coverslip.

Figure 6 shows a comparison of Young's modulus measurements between control and TiO₂ aggregates treated neutrophils. Generically, this parameter relates to a sample's elasticity/mechanical properties. The Young's modulus of treated samples was smaller than that observed for control cells, demonstrating a comparatively higher elastic behavior over a longer period of time. Control and TiO₂ aggregates treated neutrophils at 1 min demonstrated significant

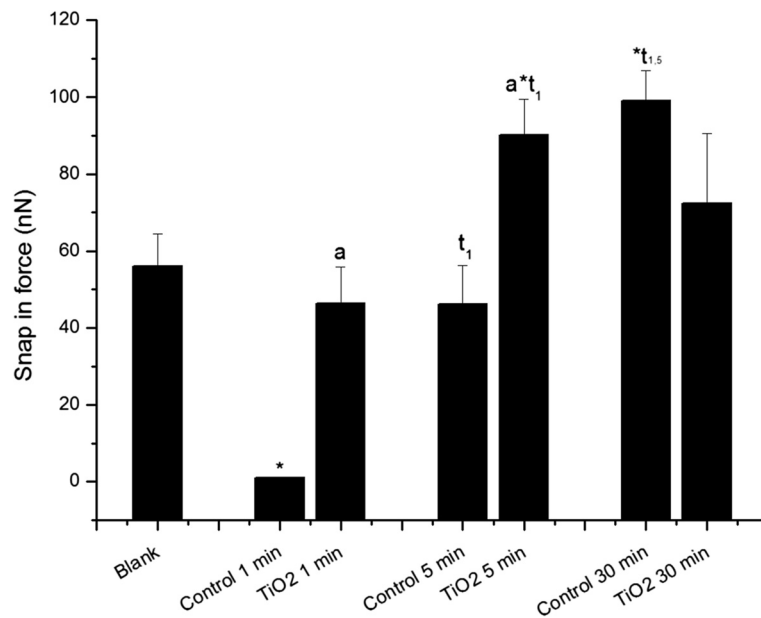


Figure 3 Snap-in force calculated from force spectroscopy curves measured by AFM operating in contact mode. "Blank" refers to bare circular silica coverslip (10 mm) surface analyses, and "TiO₂ 1, 5, and 30 min" refer to force curve analyses obtained from neutrophils adhered to circular silica coverslips (10 mm) and exposed to TiO₂ fine particles and nanoparticles aggregates for 1, 5, and 30 min. "Control 1, 5, and 30 min" refer to neutrophils adhered to circular silica coverslips (10 mm) without any TiO₂ fine particles and nanoparticle treatment, collected at the same time as treated samples (1, 5, and 30 min). The (*) symbol indicates a statistically significant difference between "Blank" and samples (P < 0.05), and (a) indicates a statistically significant difference to the respective control, "t₁" to "Control 1 min", "at₁" to "TiO₂ 1 min" and "t_{1.5}" to "Control 1 min" and "Control 5 min" (P < 0.05).

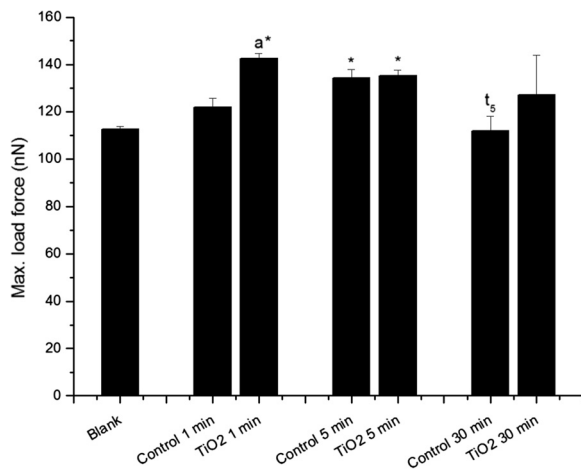


Figure 4 Maximum load force calculated from force spectroscopy curves measured by AFM operating in contact mode. "Blank" refers to bare circular silica coverslip (10 mm) surface analyses, and "TiO₂ 1, 5, and 30 min" refer to force curve analyses obtained from fixed neutrophils adhered to circular silica coverslips (10 mm) and exposed to TiO₂ fine particles and nanoparticles aggregates for 1, 5, and 30 min. "Control 1, 5, and 30 min" refer to fixed neutrophils adhered to circular silica coverslips (10 mm) without any TiO₂ fine particles and nanoparticles treatment, collected at the same time as treated samples (1, 5, and 30 min). The (*) symbol indicates a statistically significant difference between "Blank" and samples (P < 0.05), and (a) indicates a statistically significant difference to the respective control and t₅ to "Control 5 min" (p < 0.05).

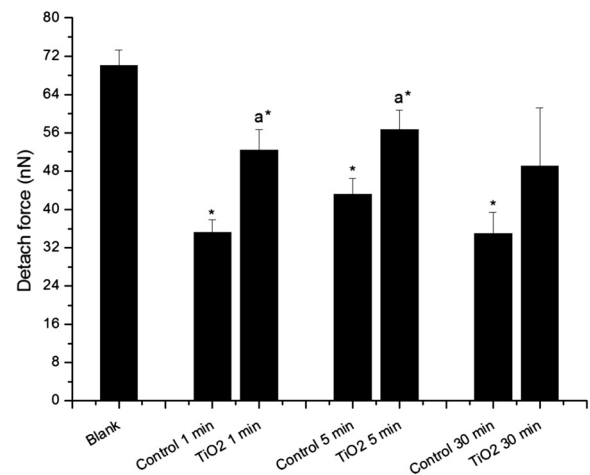


Figure 5 Detach force calculated from force spectroscopy curves measured by AFM operating in contact mode. "Blank" refers to bare circular silica coverslip (10 mm) surface analyses, and "TiO₂ 1, 5, and 30 min" refer to force curve analyses obtained from fixed neutrophils adhered to circular silica coverslips (10 mm) and exposed to TiO₂ fine particles and nanoparticles aggregates for 1, 5, and 30 min. "Control 1, 5, and 30 min" refer to fixed neutrophils adhered to circular silica coverslips (10 mm) without any TiO₂ fine particles and nanoparticles treatment, collected at the same time as treated samples (1, 5, and 30 min). The (*) symbol indicates a statistically significant difference between "Blank" and samples (P < 0.05), and (a) indicates a statistically significant difference to the respective control (P < 0.05).

differences ($P < 0.05$). At the first time point, quiescent cells were much stiffer than treated cells. At 5 min, no statistically significant differences were seen. This may be related to cell activation and assembly of the cytoskeleton in the TiO_2 group, thus increasing cell hardness. Furthermore, a remarkable transition occurred at 30 min. Treated neutrophils showed a statistically significant difference ($P < 0.05$), where it could be seen that intense phagocytosis and cell stiffening opposed the indentation of the cantilever tip. Similar results were found by Roca-Cusachs and coworkers who described adhered/activated neutrophils as being stiffer than quiescent cells [59].

Cell image analyses at 5 and 30 min (Figure 2E and F) showed remarkable changes in morphology, representing possible stimulation, F-actin rearrangement and migration to mediate TiO_2 FPs and NPs aggregates phagocytosis.

Figure 6 shows that quiescent cells and control cells had a strict time-dependent decay pattern. Control cells at 5 and 30 min showed statistically significant differences to 1 min ($P < 0.05$). These features points to a quiescent cell elasticity modulus that decreased throughout the time course [36]. By transposing into a biological environment, cell deformation implies high stiffness, favoring neutrophil arrest at the capillary wall and subsequent adhesion. On the other hand, cell softening and slow deformation would facilitate the neutrophil transmigration progress [64].

Finally, Figure 7 shows cells the dissipated energy behavior over time. Dissipated energy results from tip and sample interactions as a function of either the gap distance or applied bias [65]. Vorden et al. [66] described non-conservative forces as being strongly dependent on these interactions. Energy dissipation can be related to sample viscoelastic properties [67]. A major contributor to energy dissipation is related to AFM tip/cell membrane contact with the lipid bilayer and cytoskeleton. Neutrophils do not have a homogeneous surface due to their complex inner composition, including a nucleus (thicker cell region by $\sim 5 \mu\text{m}$), cytoplasm, and cytoskeleton [57]; thus, different force indentation measurements are expected [44]. The cytoskeleton is known to consist of actin filaments, microtubules, and intermediate filaments with many accessory proteins [68]. In response to TiO_2 stimulation, neutrophils remodel the cytoskeleton and increase cell rigidity, probably contributing to the non-linear and time-dependent mechanical properties. Dissipated energy measurements of TiO_2 FPs and NPs aggregates treated neutrophils showed a strikingly increased tendency over time. A statistically significant difference ($P < 0.05$) between control and TiO_2 aggregates treated neutrophils after 1 and 5 min of exposure was found, which may correspond to the activation of neutrophils and account for the energy dissipation enhancement as MPs and NPs uptake by

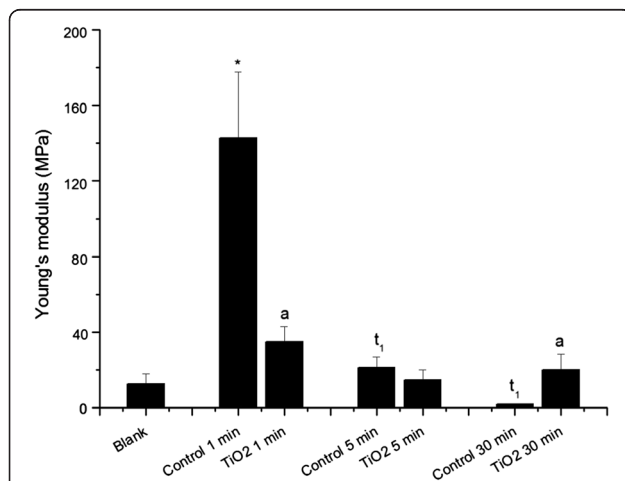


Figure 6 Young's modulus calculated from force spectroscopy analysis measured by AFM operating in contact mode. "Blank" refers to bare circular silica coverslip (10 mm) surface analyses, and "TiO₂ 1, 5, and 30 min" refer to force curve analyses obtained from fixed neutrophils adhered to circular silica coverslips (10 mm) and exposed to TiO₂ nanoparticles for 1, 5, and 30 min. "Control 1, 5, and 30 min" refer to fixed neutrophils adhered to circular silica coverslip (10 mm) without any TiO₂ fine particles and nanoparticles treatment, collected at the same time as treated samples (1, 5, and 30 min). The (*) symbol indicates a statistically significant difference between "Blank" and samples ($P < 0.05$), and (a) indicates a statistically significant difference to the respective control and t₁ to "Control 1 min" ($P < 0.05$).

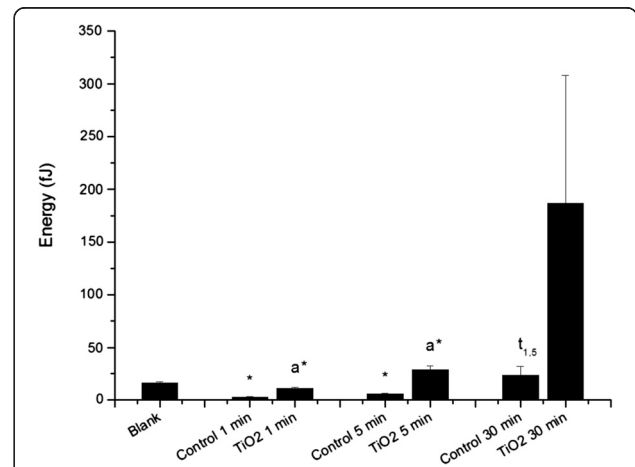


Figure 7 Dissipated energy calculated from force spectroscopy analysis measured by AFM operating in contact mode. "Blank" refers to bare circular silica coverslip (10 mm) surface analyses, and "TiO₂ 1, 5, and 30 min" refer to force curve analyses obtained from fixed neutrophils adhered to circular silica coverslips (10 mm) and exposed to TiO₂ nanoparticles for 1, 5 and 30 min. "Control 1, 5, and 30 min" refer to fixed neutrophils adhered to circular silica coverslips (10 mm) without any TiO₂ fine particles and nanoparticles treatment, collected at the same time as treated samples (1, 5, and 30 min). The (*) symbol indicates a statistically significant difference between "Blank" and samples ($P < 0.05$), and (a) indicates a statistically significant difference to the respective control and t_{1,5} to "Control 1 min" and "Control 5 min" ($P < 0.05$).

cells occurred and rigidity increased. Focusing on the controls, the statistical analysis showed significant differences ($P < 0.05$) between 30 min and both 1 and 5 min control cells. It is possible that quiescent neutrophils could experience some degree of cytoskeletal organization and therefore increasing rigidity along the time course, which would increase the dissipation of energy when the tip is in close contact with the sample.

Conclusions

The present study evaluated the effects of TiO₂ FPs and NPs aggregates on neutrophil nanomechanical properties along a time course. Scanning electron microscopy was used to investigate cell morphological changes. Aiming to correlate the morphological and nanomechanical outcomes, force spectroscopy analyses were performed. To assess the attractive and adhesive interaction patterns between AFM tip and cell surface atoms, parameters like snap-in, detachment, and maximum load force were evaluated, as well as the elasticity modulus (Young's modulus) and dissipated energy. Cells treated with TiO₂ FPs and NPs aggregates showed an increase in attractive force measurements at initial exposure times compared with control cells, which may be related to the AFM tip interaction with activated neutrophils as well as with FPs and NPs aggregates on the cell membrane. Similar results were found for adhesion forces and dissipated energy measurements. Treated neutrophils showed stronger stiffness features than controls at 1 min of exposure and presented comparatively higher elastic behavior for a longer period of time. These results point to increased cellular nanostiffness in response to TiO₂ FPs and NP aggregates treatment. This suggests an intrinsic connection concerning neutrophil morphological alterations with important biological events, such as adhesion and transmigration. A more complete understanding of these interactions will be an indispensable step to elucidate the mechanisms involved in treatments containing nanomaterials, how they interfere with modulation pathways, and the effect on organisms.

Additional file

Additional file 1: Table S1. Peak summary related to the hydrodynamic diameter distributions of TiO₂ fine particles and nanoparticles.

Abbreviations

TiO₂: Titanium dioxide; MPs: Microparticles; FPs: Fine particles; NPs: Nanoparticles; SPM: Scanning probe microscopy; AFM: Atomic force microscopy; SEM: Scanning electron microscopy; PSLG-1: P-selectin glycoprotein ligand-1; LFA-1: Lymphocyte function-associated antigen 1; Mac-1: Macrophage-1 antigen; FcγIII receptor: Low affinity immunoglobulin gamma Fc region receptor III; CD11b: Cluster of differentiation 11b; CD16: Cluster of differentiation 16; ICAM-1: Intercellular adhesion molecule-1; min.: minute; N/m: Newton per meter; nm: nanometer; μL: microliter; DLS: Dynamic light scattering; HBSS: Hank's balanced salt solution; PBS: Phosphate buffered saline solution; MTC: Magnetic twisting cytometry; kHz: Kilohertz; Hz: Hertz; V: Volt.

Competing interests

The author declares that there are no competing interests.

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