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Short Communication

# Effect of detonation nanodiamonds on phagocyte activity

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## Abstract

Detonation ND (nanodiamond) holds much promise for biological studies and medical applications. Properties like size of particles, inclination for modification of their surface and unambiguous biocompatibility are crucial. Of prime importance is interaction between ND and immune cells, which supervise foreign intrusion into an organism and eliminate it. Neutrophils are more reactive in inflammatory response implementing cytotoxical arsenal including ROS (reactive oxygen species). The aim of the work was to estimate the ability of two ND samples (produced by Diamond Center and PlasmaChem) to keep the vitality of neutrophils from the inflammatory site. The ability of cells to generate ROS in the presence of ND particles is considered as indicating their biocompatibility. IR spectra and size of particles in the samples were characterized. Acid modification of ND was carried out to get the luminescent form. In the biological aspect, ND demonstrated up or down action, depending on the concentration, time and conditions of activation of cells. Weak action of ND in whole blood was obtained possibly owing to the ND adsorbed plasma proteins, which mask active functional groups to interact with the cell membrane. ND did not influence the viability of isolated inflammatory neutrophils in low and moderate concentrations and suppressed it in high concentrations ($\geqslant$1 g/l). Addition of ND to the cell suspension initiated concentration-dependent reaction to produce ROS similar to respiratory burst. ND up-regulated response to bacterial formylpeptide, but up- and down-modified (low or high concentrations, accordingly) response to such bacterial agents as OZ (opsonized zymosan), which neutrophils swallow up by oxygen-dependent phagocytosis. Localization of the particles on the cell surface as into the cells was identified by monitoring the intrinsic fluorescence of oxidized ND. The various mechanisms that could account for penetration of ND particles into the cell are discussed. Common conclusion concerns compatibility of ND with living neutrophils from inflammatory site and their normal functioning for infection safeguard.

Keywords: biocompatibility; luminescence; nanodiamond; neutrophil; phagocytosis; reactive oxygen species

## 1. Introduction

Nanoscale objects of non-biological nature are presently attracting intense interest of researchers prompted by their potential in biological and future medical applications (Kulakova, 2004; Lisichkin et al., 2009; Ho, 2010;). Ultradisperse detonation diamond [ND (nanodiamond)] is one of the most promising objects in this area. It was shown to be non-toxic for the organism (Dolmatov, 2008; Schrand et al., 2009). This feature discriminates it from other carbon nanoparticles, nanotubes and fullerenes, which were found to be toxic in organisms of various taxonomic groups (Kolosnjaj et al., 2007; Murray et al., 2009). ND belongs only to the fourth group of toxic substances, which makes it a very weak toxic agent (Schrand et al., 2007; Dolmatov, 2008).

The various functional groups present on the surface of ND particles (Dolmatov, 2008) offer a considerable potential for chemical modification aimed at conferring to the new properties. This ability differentiates it favourably from carbon nanotubes, in which functional groups are located at their open ends only. As a consequence, in order to graft a functional group on to the side surface of a tube, one has to disrupt the system of aromatic C–C bonds. The same applies to the fullerene. Thus, if one confers to the ND certain properties which might be attractive to medicine or experimental biology, it could be successfully employed, for instance, for biolabelling and imaging techniques (Chao et al., 2007) or directed delivery of drugs.

There are publications in the literature exploring possible ND bioapplications (Schrand et al., 2009), as well as reports announcing successful introduction of this substance into cells by inserting it into micelles and liposomes (Smith et al., 2007). Direct interaction of a cell with ND particles is, however, still awaiting a dedicated investigation. Cells of an innate immune system are of particular significance in this context because they provide the fastest defensive response of the organism to external invasion and damage. Our previous publication has demonstrated the concentration-dependent effect, the ND produce on the ability of granulocytes from human peripheral blood to generate ROS (reactive oxygen species) for cytotoxic purposes. The goal of the present study is to investigate *in vitro* the effect of ND on the part played by phagocytes in non-fractionated peripheral blood and cells from a site of acute inflammation in animals, as well as visualization of the interaction of ND particles with cells.

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**Abbreviations:** fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; ICB RAS, Institute of Cell Biophysics of the Russian Academy of Sciences; ND, nanodiamond; OZ, opsonized zymosan; ROS, reactive oxygen species.

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## 2. Materials and methods

We used the following ND samples: dry powder, trademark UDA-TAN (Diamond Center); aqueous suspension of ND (1 mass %, PlasmaChem). The size of ND particles in the powder was determined by transmission (LEO912 AB OMEGA, Carl Zeiss) and scanning (Hitachi S520) electron microscopes and, in the suspension, by dynamic laser light scattering with a Malvern ZetaSizer (Germany). Characterization of the chemical state of ND surface was effected by measuring IR absorption spectra of samples in pellets with KBr, with a Fourier-transform IR spectrometer (IR200 Thermonicolet) providing a resolution of 4/cm. Raman scattering spectra were recorded with a Ramanor U1000 spectrometer (Jobin Yvon).

The effect of ND was studied on whole peripheral blood and isolated neutrophils. Samples of peripheral blood were taken from laboratory male mice of NMRI outbred line. Isolated neutrophils were extracted from a site of acute inflammation in the peritoneal cavity of mice 5 h after injection of 150 μl of zymosan suspension (5 mg/ml). The abdominal cavity was washed with 3 ml of cooled $Ca^{2+}$-free Hanks' solution. The exudate was collected, centrifuged for 5 min at 600 ***g***, and the sediment was resuspended in Hanks' $Ca^{2+}$-free solution. Prior to measurements, the cell suspension ($10^7$ cells/ml) was kept for 1 h at 4°C. We worked in full compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimentation and other Scientific Purposes, 1986, 86/609/EEC. ICB RAS (Institute of Cell Biophysics of the Russian Academy of Sciences) was granted Permission no. 12306).

Human cells were isolated from peripheral blood of healthy volunteers by the standard technique (Safronova et al., 2008). Informed consent to participation in experiment was obtained from all donors.

The intensity of ROS production by cells of mice was estimated from luminol-dependent chemiluminescence with the use of CHEMILUM-12 device developed at ICB RAS, which was capable of chemiluminescence measurements on 12 samples simultaneously. One first measured in the blood samples the level of spontaneous (non-activated) chemiluminescence, after which commercial ND suspension diluted 10–$10^6$ times was added, and incubation was continued for 6 min, followed by addition of 0.25 mg/ml of OZ (opsonized zymosan) to activate phagocytosis. The operation with isolated cells was conducted in the same order, the only exclusion being that the cells were activated by OZ and chemotactic peptide fMLF (*N*-formyl-methionyl-leucyl-phenylalanine) to estimate the effect of ND on the receptor-dependent activation of NADPH oxidase, an enzyme responsible for ROS generation by phagocytes.

The survival of mice cells was derived from Trypan Blue staining after incubation with ND in dilutions of $10^5$ for a preset time at 37°C. The number of living (unstained) cells in a sample was counted in the Goryaev chamber and expressed as a percentage of the total number of cells. Localization of ND particles relative to cells was studied with a Leica TCS SP5 confocal microscope (Leica). Preparation of cell samples for confocal microscopy included incubation of cell suspensions ($10^6$ cells/ml) with ND ($2 \times 10^{-3}$ mass %) for 10 min at 37°C and their fixation with a 4% formaldehyde solution. Statistical analysis: the

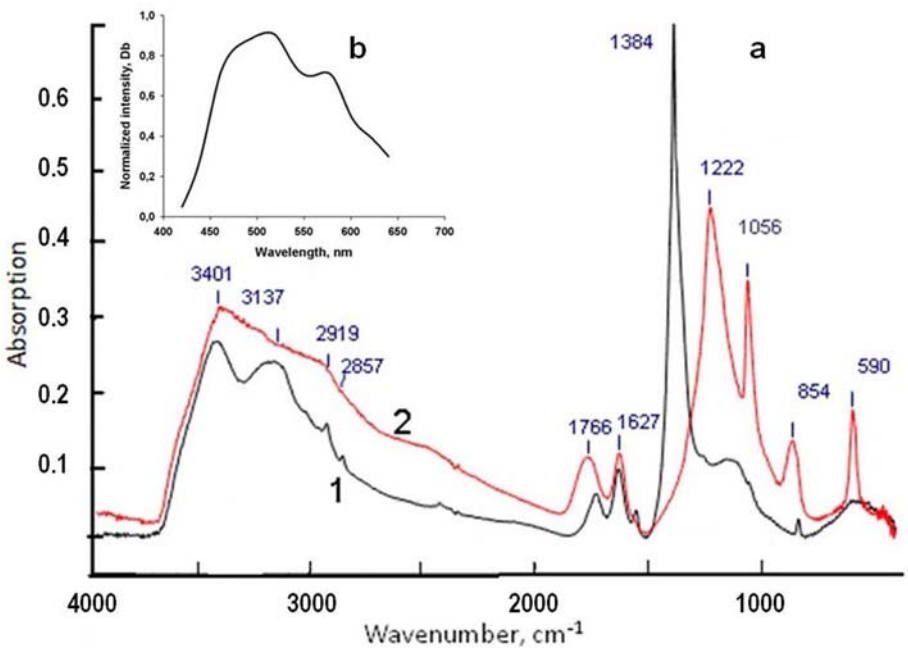


**Figure 1** **Spectral characteristics of detonation NDs**
(**a**) IR spectra of UDA-TAN, original (1) and modified by the acid mixture (2). The intensities of the bands deriving from vibrations of the C–H groups have become weaker. Growth in intensity of the 1766/cm band originating from vibrations of the carboxyl group is apparent. These facts suggest oxidation of the diamond surface. (**b**) Luminescence spectrum of oxidized UDA-TAN excited with argon laser at 488 nm. Oxidized ND produces luminescence in the 450–650-nm interval peaking at 520 nm. Oxidation-modified ND reveals a specific feature in that its luminescence retains a stable intensity throughout the period of observation, which discriminates it from less stable luminescent probes used in biology for visualization of intracellular structures.


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influence of ND was calculated as the ratio of ROS production in cells treated with ND to that in control cells taken as 100%. The data presented below are actually average values of the effect with an S.E.M., which were derived from a preset number of independent experiments, all performed on cells of the same animal. The deviations from the control value were found by the Student criterion.

# 3. Results and discussion

Commercial UDA-TAN is a powder containing ND aggregates 200 nm to 60 μm in size. We have not succeeded in obtaining a stable aqueous suspension of this ND even after ultrasonic treatment. Therefore, to facilitate dispersion, the ND powder was oxidized with a boiling mixture of concentrated nitric and sulfuric acids in the 1:4 ratio.

The changes in the chemical state of the surface of an ND sample are seen readily from IR spectra (Figure 1). In the spectrum of the original ND we observe a very strong band at 1384/cm, which was assigned to bond vibrations in the $NO_3^-$ group (Lisichkin and Korol'kov, 2006). The band at 3154/cm derives possibly from vibrations of secondary amino groups on the diamond surface; they can be traced to the ammonolysis employed in industrial purification of UDA-TAN. One can see also weak absorption bands at 2919 and 2857/cm, which can be associated with valence vibrations of the C–H groups in the $sp^3$

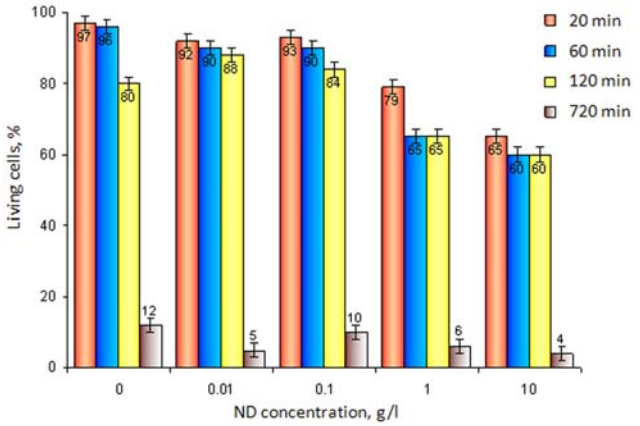


**Figure 2 Survival of isolated neutrophils of mice following incubation with ND (produced by PlasmaChem)**

The cells were incubated in a physiological solution with ND of different concentrations for 20, 60, 120 and 720 min at 37°C. Survival was estimated as portion of the cells without Trypan Blue dye inside. Numbers indicate average portions of living cells in suspension. $P<0.05$ in comparison with control cells.

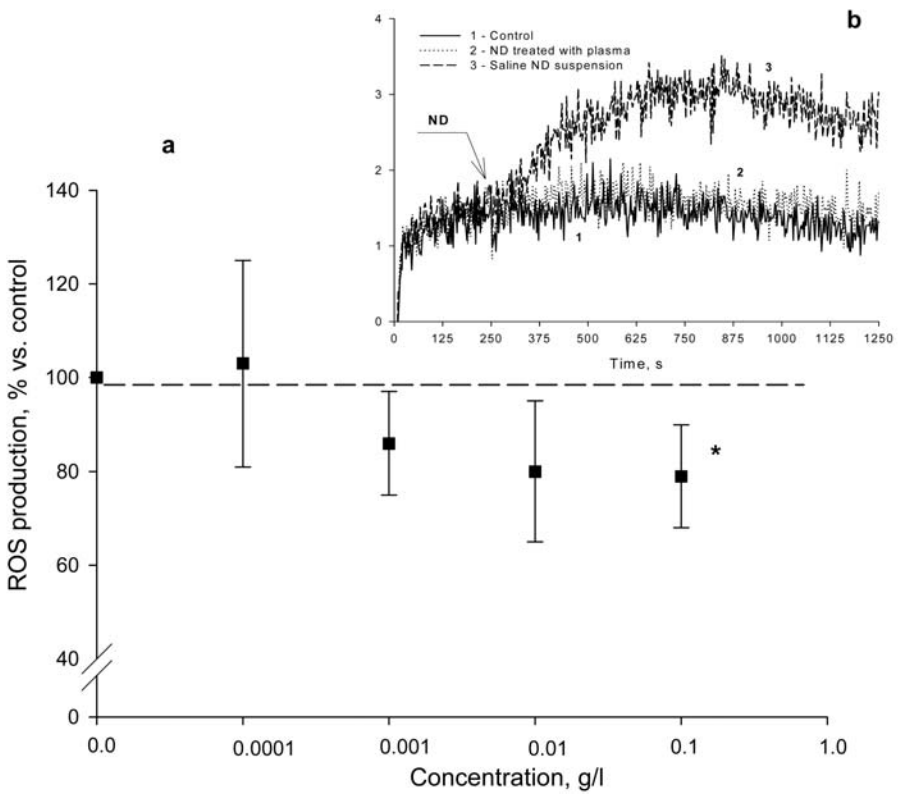


**Figure 3 Interaction between ND and components of peripheral blood**

(**a**) Effect of ND (produced by PlasmaChem) on ROS production activated in phagocytes of whole blood of mice by OZ. OZ (0.25 mg/ml) was added to the blood samples incubated with ND taken in different concentrations for 5 min at 37°C. *$P<0.02$, $n=4$. (**b**) The top inset demonstrates that ND treated with blood plasma does not influence ROS generation in isolated cells (2) in contrast with saline ND suspension (3). Also, ND only does not change phagocyte activity in the whole blood (not shown).

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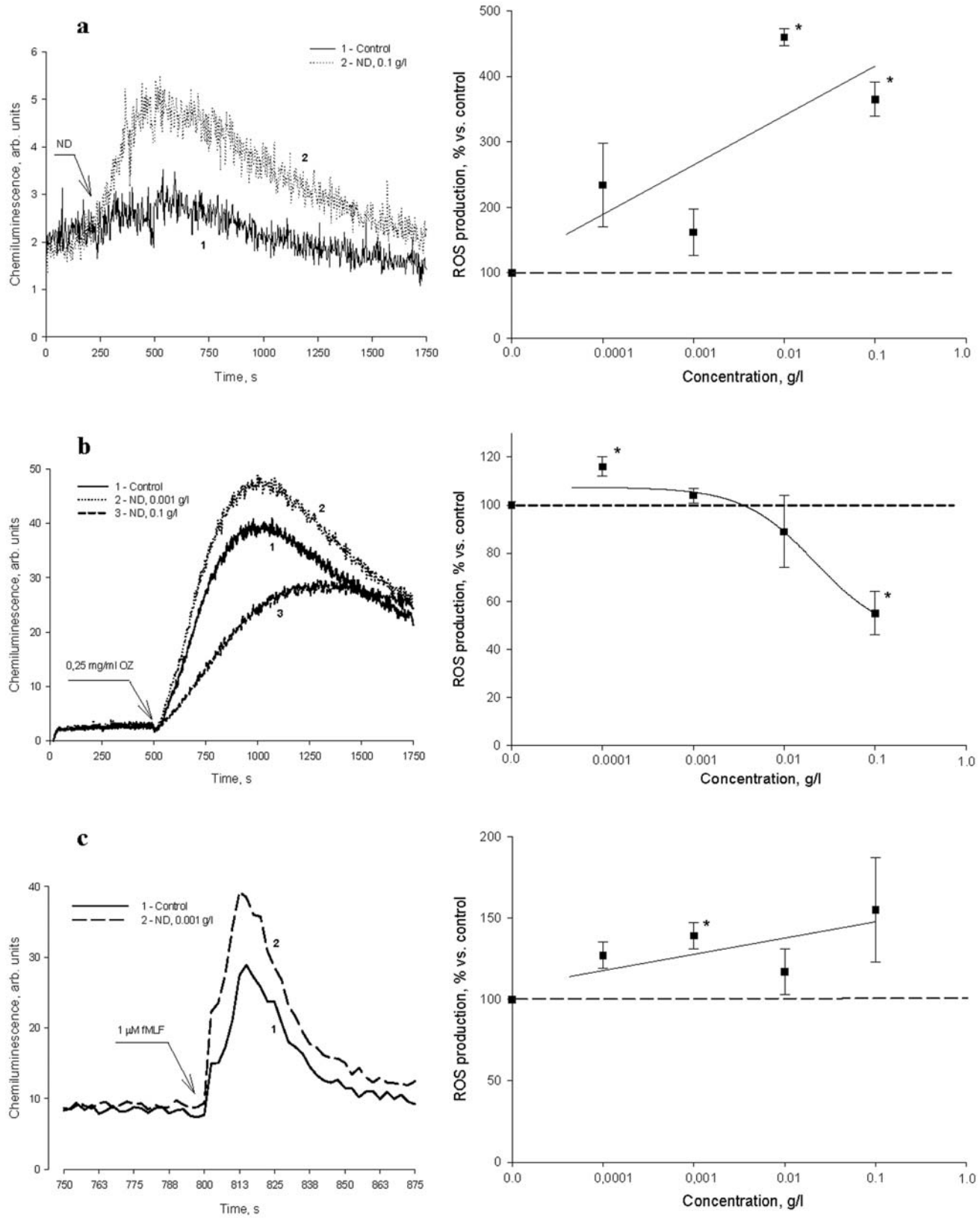


**Figure 4** **NDs modify production of ROS in isolated neutrophils from acute inflammatory centre in peritoneal cavity of mice**

The intensity of ROS generation (in arbitrary units) was estimated by luminol-dependent chemiluminescence technique. Cells ($10^6$ cells/ml) were incubated for attaching and adaptation in Hanks' solution with $Ca^{2+}$ during nearly 250 s without any additions (base level of ROS production), then ND suspension in required concentration was added (**a**). Necessary concentrations of ND suspension were derived by consecutive 10-fold dilution from the stock ND


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produced by PlasmaChem. After 250 s incubation with ND, the cells were activated by OZ (**b**) or bacterial peptide fMLF (**c**) to initiate respiratory burst. Control cells were activated only by OZ (**b** curve 1) or fMLF (**c** curve 1) without addition of ND. Concentration dependences were obtained from the cell samples prepared from the cells of the same animal in parallel. Duplicate samples were taken for equal conditions. Effect was calculated as ratio of ROS production of the cells treated with ND to ROS production of control cells taken as 100% (dotted lines) and averaged for the cells of four animals. (**a**) Activation of ROS production in isolated mouse neutrophils by NDs: left – chemiluminescence of intact cells (1) and cells treated by 0.1 g/l ND suspension (2); right – effect of ND plotted against concentration. *$P<0.001$ (comparison with control cells). (**b** and **c**) Concentration-dependent effect of ND on ROS production initiated by 0.25 mg/ml OZ or 1 μM fMLF, accordingly: left – original curves (1, control; 2, 3, cells treated by ND of indicated concentrations; right – plots. *$P<0.01$, $n=4$.

carbon atom. In the IR spectrum of oxidized ND (spectrum 2) the 1384/cm band is not present, and the intensities of the bands deriving from vibrations of the C–H groups have become weaker. We witness growth in intensity of the 1766/cm band originating from vibrations of the carboxyl group. All this suggests oxidation of the diamond surface.

We found also that the intensity of the Raman spectrum of oxidized ND is an order of magnitude higher than that of the original material and that the overall spectrum has an unusual shape. This suggests that it can be excited to generate luminescence. Indeed, oxidized ND produces luminescence in the 450–650-nm interval peaking at 520 nm (Figure 1, on an

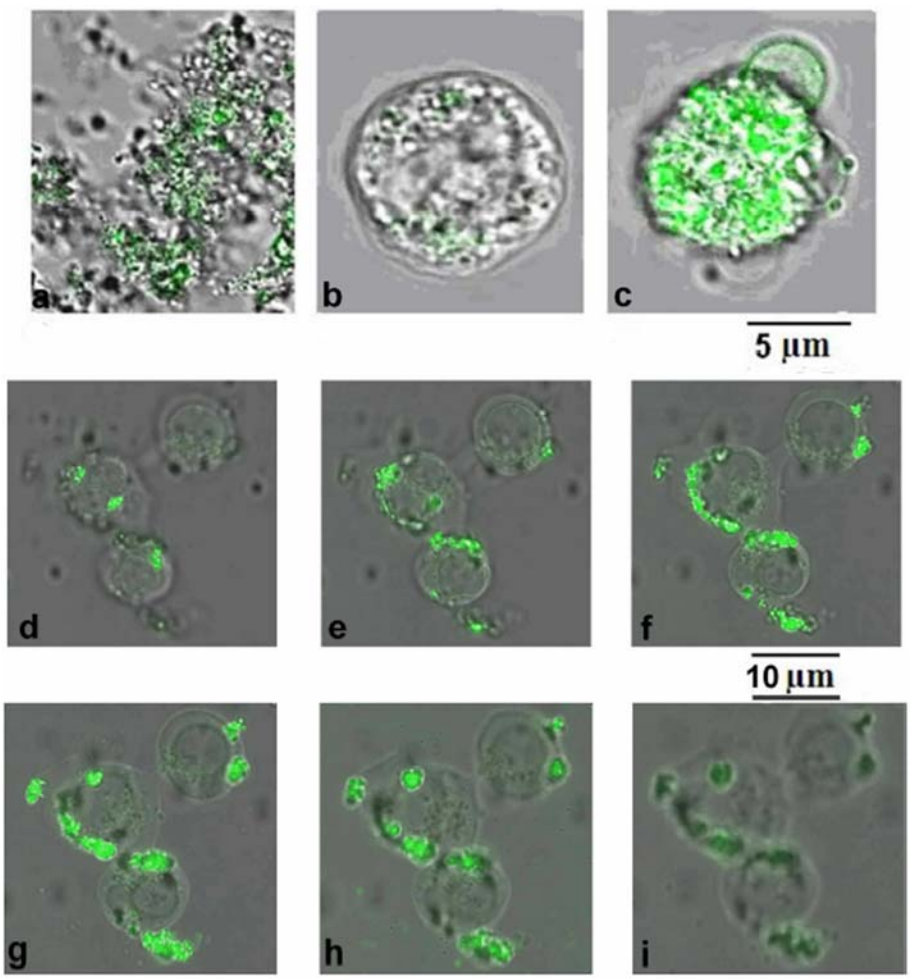


**Figure 5 Fluorescence microscopy of NDs and neutrophils**
(**a**–**c**) Images of ND particles and a living cell: (**a**) luminescent ND particles; (**b**) control cell; (**c**) ND-treated neutrophil. (**d**–**i**) Confocal images visualizing penetration of ND into a cell. Confocal fluorescent images (shown in 20% steps) displayed localization of diamond particles inside the neutrophils; in the micrographs, the bright green spots seen in the successive transverse optical sections of the cell are readily identified with ND particles in the cell. Preparation of cell samples for confocal microscopy included incubation of cell suspensions ($10^6$ cells/ml) with ND ($2 \times 10^{-3}$ mass %) for 10 min at 37°C and their fixation with a 4% formaldehyde solution. Experimental observations were made at excitation wavelengths of 488 and 500–550 nm emission. Preparation of samples for measurements: non-fractionated blood (100 μl) was diluted with modified $Ca^{2+}$-free Hanks' solution in the 1:1 volume ratio and kept for 1 h at 4°C. The samples of total volume 200 μl prepared for measurements in minidishes contained either 20 μl of diluted blood or $10^6$ cells/ml. The physiological solution for measurements had the following composition (in mM): NaCl 138, KCl 6, $CaCl_2$ 1, $NaHCO_3$ 5, $Na_2HPO_4$ 1, glucose 5.5, Hepes 10, luminol 0.35; pH=7.4. The system intended for recording of isolated cells contained also 0.1 mM $NaN_3$ and 3 U/ml of horseradish peroxidase type VI. All substances were purchased from Sigma.

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insert). Oxidation-modified ND reveals a specific feature in that its luminescence retains a stable intensity throughout the period of observation, which discriminates it from less stable luminescent probes used in biology for visualization of intracellular structures. Luminescence of oxidized ND did not typically reveal a decrease in intensity (or bleaching) for a long time with no special measures taken.

The water suspension of oxidized ND contained aggregates with an average size of 600 nm. Ultrasonic treatment reduced the size of the particles, with their size distribution becoming notably narrower (180–250 nm). The ND suspension produced by PlasmaChem exhibits a high stability, with an average nanoparticle size, as deduced from our measurements, of 25 nm. This ND does not produce luminescence.

We demonstrated early that isolated granulocytes (neutrophils included) from human blood remain living and functioning during incubation with ND (Safronova et al., 2008). The cells from inflammatory site are more reactive and sensitive to impact (Wright et al., 2010). Besides, it is important to know reaction of inflammatory cells to ND for medical applications. First, we estimated the survivability of mouse cells in the presence of ND; commercial ND suspension was used. The results obtained are displayed in the form of a diagram (Figure 2). Our data suggest that the viability of cells incubated with ND in concentrations of 0.1 g/l and less for up to 2 h practically does not differ from that of intact cells. With ND present in high concentrations (1 g/l and more), however, nearly 40% of cells perished. Therefore, in all subsequent studies of the influence of ND, we chose concentrations below 1 g/l. Then, we tested the influence of ND on function of neutrophils to produce ROS in whole blood and in suspension of isolated cells.

Adding ND (produced by PlasmaChem) to whole blood of mice did not influence in any way the intensity of spontaneous ROS generation, while the level of ROS production activated by OZ fell off noticeably when the blood was preincubated with 0.1 g/l ND for 5 min (Figure 3a). The observed effect depended only weakly on ND concentration in a sample; moreover, ND treated with blood plasma did not activate the function of neutrophils to generate ROS (Figure 3b). We believe that this could be attributed to adsorption of blood plasma proteins on the surface of ND particles (Schrand et al., 2009), which interacted with active functional groups of ND and abolished interaction with cell membrane.

Our earlier study yielded data on the concentration-dependent effect of ND on ROS production by granulocytes of peripheral human blood (Safronova et al., 2008). In the present work, we have estimated the behaviour of cells isolated from a site of acute inflammation in animals. It was found that adding ND to isolated cells activates ROS generation (Figure 4a). This response is similar to the one observed by us in the effect of UDA-TAN commercial suspension on human cells and looked like a respiratory burst reaction depending on the concentration of ND.

We also find here an analogy to the response of neutrophils to OZ whose phagocytosis is accompanied by activation of ROS production (Figure 4b). It is quite possible that phagocytosis occurs also in the interaction of neutrophils with ND particles, which are smaller than those in the UDA-TAN suspension used in our previous work (Safronova et al., 2008). Further experiments provided supportive evidence for this assumption. Successive action of ND in low concentrations (below $10^{-3}$ g/l) and OZ enhanced markedly ROS production (Figure 4b) compared with the response to the OZ only. At high ND concentrations (over $10^{-2}$ g/l), the response of cells to OZ was suppressed. A possible explanation may lie in that ND taken in these concentrations itself activates its ROS production, with the result that the ROS generation systems become desensitized or that we have here formation of products suppressing ROS generation altogether. We have revealed a strong negative correlation ($r=-0.6$) between the effect of the ND alone and the combined impact of ND and OZ. This suggests a non-additive character of the combined ND and OZ action, which, in its turn, argues for a common target.

We have demonstrated also that ND enhances in a concentration-dependent way ROS formation activated by 1 μM fMLF (Figure 4c). Neutrophils of mice have specific receptors for formyl peptide whose binding with fMLF activates signalling to the NADPH oxidase responsible for ROS production. It is possible that interactions between ND and the cell membrane or intracellular signalling messengers transmitting the signal from the receptor to the NADPH oxidase modify the neutrophil state, thus affecting regulation of the activity of the NADPH oxidase; this hypothesis requires, however, experimental verification.

We used intrinsic fluorescence of oxidized UDA-TAN for visualization of the interaction of ND with the cell (Figures 5a–5c). A careful inspection of a variety of preparations and observation of the interaction in time have permitted us to demonstrate convincingly that particles can become localized in different ways, more specifically, on the surface and/or inside a cell (Figure 5d–5i). Confocal fluorescent images reveal localization of diamond particles inside the neutrophils in the cytoplasm, but not in nuclei: the micrographs, the bright green spots seen in the successive transverse optical sections of the cells are readily identified with ND particles in the cell. It had been shown that a primarily endocytic uptake occurs in internalization of ND (the biggest nanoparticles or aggregates), the smallest particles can diffuse through cell membrane and appear free in the cytosol (Faklaris et al., 2009). Phagocytes (including neutrophils) rely on phagocytosis interacting with particles. So, we suggest that ND particles penetrate into neutrophils by at least two mechanisms, via phagocytosis and by diffusion through the cell membrane.

# 4. Conclusion

Compatibility of ND with living neutrophils from the inflammatory site and their normal functioning for safeguarding from infection were demonstrated. ND, when used in low concentrations, modifies the activity of mouse cells taken from a site of acute inflammation in such a way that it increases the activity induced by bactericidal agents (OZ or bacterial formylpeptide). ND, in moderate concentrations, prevents excess cell activation during phagocytosis of OZ. This could prove useful in regulating treatments of inflamed sites. Acid-oxidized ND exhibits intrinsic stable luminescence (with a maximum at 510 nm), without photobleaching, and can be visualized inside a cell.


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## Author contribution

Alexey Karpukhin and Nadezhda Avkhacheva were responsible for the literature review, planning and carrying out of experiments and data processing. Georgiy Lisichkin, Valentina Safronova and Inna Kulakova were responsible for the problem statement and the general coordination of the work. Valeriy Yashin was responsible for the preparation and interpretation of confocal images. Ruslan Yakovlev was responsible for the registration and interpretation of IR-spectra and processing of photographic materials. Valeriy Yashin helped with microscopy procedures.

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