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ASSESSMENT OF NANOPARTICLES TOXICITY BY FLOW CYTOMETRY USING HUMAN ERYTHROCYTES

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ABSTRACT: The use of nanoparticles in biological and medical applications has increased in the last years. Understanding their potential toxicity and effect on the body is crucial before clinical use. The most used cell culture systems to test their cytotoxicity are of nonhuman origin. For assessing cytotoxicity of nanoparticles we developed a new experimental cell system, the RBCs, and we have evaluated the toxic effects by flow cytometric analysis. Recently, we and other researchers have shown that PCD of human erythrocyte is related to an apoptosis mechanism operating in the absence of mitochondria and lacking the terminal effectors of nuclear apoptosis (*erythroptosis* also *called eryptosis*) and to determine human RBC viability, we described a new rapid and sensitive calcein-AM assay by flow cytometric determination. Consequently, in the present study, to evaluate cell-nanoparticles interactions, human RBCs were exposed to different concentrations of nanoparticles and analyzed by flow cytometry, after 3h and 24h incubation endpoints for morphological changes (FSC/SSC) and viability (calcein-AM method). Our results indicate that flow cytometric analysis of RBCs viability using calcein-AM discrimination could provide a rapid and accurate experimental cellular model for effectively screening and evaluating biological responses for *in vitro* nanotoxicology.

Keywords: human erythrocyte, cell viability, flow cytometric analysis, nanotoxicity, nanoparticles.

INTRODUCTION

The term "nano" (derived from Greek *nanos*, signifying-dwarf) is now a popular name for much of modern science, including, nanoscience, nanotechnology, nanotoxicology, nanostructure, etc. Nanoparticles are particles with at least one dimension smaller than 1 micron and potentially as small as atomic and molecular length scales (~0.2 nm). Advances in nanotechnology are driven by rapid commercialization of products containing nanostructures, nanoparticles and nanomaterials with remarkable properties (Buzea *et al.*, 2007).

Nanotechnology has a top research priority in the world for the progress in life science with medical applications for early diagnosis and treatment of diseases, prosthetics and many others areas. Human exposure to nanoparticles from natural and anthropogenic sources is currently very high. Anthropogenic nanoparticles are products of combustion (with sizes down to several nm) and food cooking, chemical manufacturing, welding, mining refining & melting, combustion in vehicle and airplane engines (Rogers *et al.*, 2005), combustion of treated pulverized sewage sludge (Seames *et al.*, 2002), and combustion of coal and fuel oil for power generation (Linak *et al.*, 2000). The production of nanoparticles will increase from 2 300 tons produced today to 58 000 tons by 2020 (Maynard, 2006). Engineered nanoparticles are commonly used in cosmetics, sporting goods, tires, stain-resistant clothing, sunscreens, toothpaste, food additives, etc. Due to their small size, nanoparticles can influence basic cellular processes, such as proliferation, metabolism, and death and probably, they can be associated with neurodegenerative diseases, autoimmune diseases, and cancer (Antonini *et al.*, 2006). Diseases associated with inhaled nanoparticles are asthma, bronchitis, emphysema, lung cancer, and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases. In the gastro-intestinal tract nanoparticles have been linked to colon cancer and Crohn's disease. Nanoparticles that enter in the circulatory system occur to atherosclerosis and blood clots, arrhythmia, heart disease, and ultimately cardiac death. Translocation to other organs, such as liver, spleen, etc, may lead to diseases of these organs. Exposure to some nanoparticles is associated to the occurrence of autoimmune diseases, such as systemic lupus erythematosus, scleroderma, and rheumatoid arthritis. In order to assess the toxicity of various nanomaterials we must compare their toxic effects with those of known toxic particles. Several studies had this initiative (Soto *et al.*, 2005; Muller *et al.*, 2005; Warheit *et al.*, 2004; Lam *et al.*, 2004; Braydich-Stolle *et al.*, 2005; Hussain *et al.*, 2005). However, the database of studied materials is limited. Silver nanoparticle aggregates were found to be more toxic than asbestos, while titanium oxide, alumina, iron oxide, zirconium oxide were found to be less toxic (Soto *et al.*, 2005). On the other hand many nanoparticles have numerous medical applications. Nanofiber scaffolds can be used to regenerate central nervous system cells, the regeneration of axonal tissue initiated by a peptide nanofibers scaffold (Ellis-Behnke *et al.*, 2006) and

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possible other organs. Certain nanopowders, possess antimicrobial properties (Bosi *et al.*, 2003), (Koper *et al.*, 2002). In nanomedicine, nanospheres composed of biodegradable polymers can incorporate drugs, allowing the timed release of the drug as the polymer degrades (Uhrich *et al.*, 1999).

Currently, research on nanotoxicity has concentrated on empirical evaluation of the toxicity of various nanoparticles, with less regard given to the relationship between nanoparticle properties (composition, crystallinity, size, size dispersion, aggregation, ageing) and toxicity. This approach gives very limited information, and should not be considered adequate for developing predictions of toxicity of nanoparticle materials.

Further studies must include, at least, research on nanoparticles accumulation, short- and long-term toxicity and their interactions with cells.

For assessing *in vitro* cytotoxicity of nanoparticles and their ecotoxicity, we developed a new experimental cell system based on the use of nucleated RBCs from fishes and batrachians which are directly exposed to pollutants or to nanoparticles absorbed by different ways or using the unicellular alga *Chlorella* (Bratosin *et al.*, 2011; Petrescu *et al.*, 2013) and we have evaluated the toxic effects by flow cytometric analysis.

We and others have recently shown that PCD of human erythrocyte is related to an apoptosis mechanism operating in the absence of mitochondria and lacking the terminal effectors of nuclear apoptosis, named *erythroptosis* by Daugas & Kroemer (Bratosin *et al*, 1999; Bratosin *et al*, 2001; Berg *et al.*, 2001; Daugas *et al.*, 2001; Bratosin *et al.*, 2009) and to determine human RBC viability, we described a new rapid and sensitive calcein-AM assay by flow cytometric determination. Consequently, in the present study, to evaluate cell-nanomaterials interactions, human RBCs were exposed to different concentrations of nanoparticles and analyzed by flow cytometry, after 3h and 24h incubation endpoints for morphological changes (FSC/SSC) and viability (calcein-AM method).

MATERIALS AND METHODS

Chemicals

The fluorogenic dye calcein acetoxymethyl ester (calcein-AM), was purchased from Sigma Aldrich (Saint Louis, Mo, USA). In the presented study carbonaceus nanoparticle synthesized by laser pyrolysis were used to test their cytotoxycity. The experimental set up and the most important parameters were described elsewere (Morjan *et al.*, 2003; Morjan *et al.*, 2004) The synthesis mecanism involves ethylene or acetilene as the main carbon source. SF_6 was used in reactive mixture as S and F dopant, NO_2 used as N dopant and $Fe(CO)_5$ vapours as Fe dopant [Dumitrache *et al.*, 2004].

In Table 1 are presented 4 different nanoparticles synthesized by laser pyrolisys and also other two nano-carbon samples commercially available: active graphite and Multiwall carbon nanotubes (MWCNT) both produced by ARKEMA. The raw nanopowder has been dispersed in physiological serum using a biocompatible polyanionic polymer (CMCNa) as stabilized agent at the same concentration for all dispersions (1 g/l) A well stabilized dispersion were realized via a ultrasonic horn procedure (15 min. into an ice cooled bottle). (Lin *et al.*, 2010)

Table 1

Experimental data regarding different carbon based nanoparticles, subject of toxicological tests: Nanoparticle synthesized by laser pyrolysis (P1- P4) or commercially available (P5, P6)

Sample	Reactive Gas mixture	Elemental composition (EDX) at.%
P1	C_2H_4+$Fe(CO)_5$	C 62, Fe 31%, O 7%
P2	C_2H_2+N_2O+SF_6	C 92%, N 0.8%, S 1.5%, F 5 %, O traces
P3	C_2H_2+SF_6	C 91%, S 2%, F 7%
P4	C_2H_2+C_2H_4	C 100%
P5	comercial	Active graphite: C 100%
P6	comercial	MWCNT; C 100%

Erythrocytes collection and cell treatments

Human blood type $0Rh^+$,collected in heparin was centrifugation at 2,000 g; 4°C; 5 min. Plasma, platelets and leukocytes were removed by aspiration and the red blood cells were resuspended (10^7 cells per ml) for experiment in isotonic phosphate-buffered saline (PBS) solution pH 7.4 (Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, NaCl 140 mM and KCl 2.7 mM).

In order to evaluate nanoparticles toxicity, nanoparticles (0.03mg/ml) were diluted in sterile PBS for obtaining 6 serial dilutions and incubating for 3h and 24 hours in presence of 10^7/ml RBCs in culture plates.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer (San Jose, CA, USA) using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition. The results were expressed as MFI value of the logarithmic fluorescence intensity.

Morphological changes analysis by scattered light flow cytometry in the mode FSC/SSC

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with the cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle

scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents (Darzynkiewicz *et al.,* 1997)

Flow cytometric assay of cell viability using calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin *et al.,* 2005. The membrane-permeable dye calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µM in PBS buffer pH 7.4. To 20 µl of a RBC suspension in PBS buffer, pH 7.4 (10^7 cells per ml) were added 170 µl of the same buffer and 10 µl of a 100 µM calcein-AM solution in PBS buffer, pH 7.4. (final concentration in calcein-AM: 5 µM). The mixture was incubated at 37^{o}C in the dark for 45 min and then diluted with 0.3 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells and gated for monoparametric histogram FL1 (calcein fluorescence). Ten thousand cells were analyzed on a Becton Dickinson FACScalibur cytometer using CellQuest software. The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. Experiments were carried out in triplicate.

RESULTS

Detection of altered morphology by light scattering flow cytometry

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides informations about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane.

According to Darzynkiewich (Darzynkiewicz *et al.*, 1997), during apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents.

As shown in Fig. 1, flow cytometric analysis announce significant morphological changes of nucleated RBCs incubated for 3h in saline supernatants of different nanoparticles (P1-P6), at different dilutions, compared to RBCs incubated only in saline isotonic solution (T0 and T24h).

In fact, as demonstrated in Fig. 2, for 3h and 24h, the XGeo Mean values (cell side scatter) the YGeo Mean values (cell density scatter) varies more or less depending on the toxicity of nanoparticles compared to the statistical value of normal RBCs. Great P2 sample toxicity has led to substantial changes at 3h, and especially after 24 when there was an almost complete lysis for all sample dilutions.

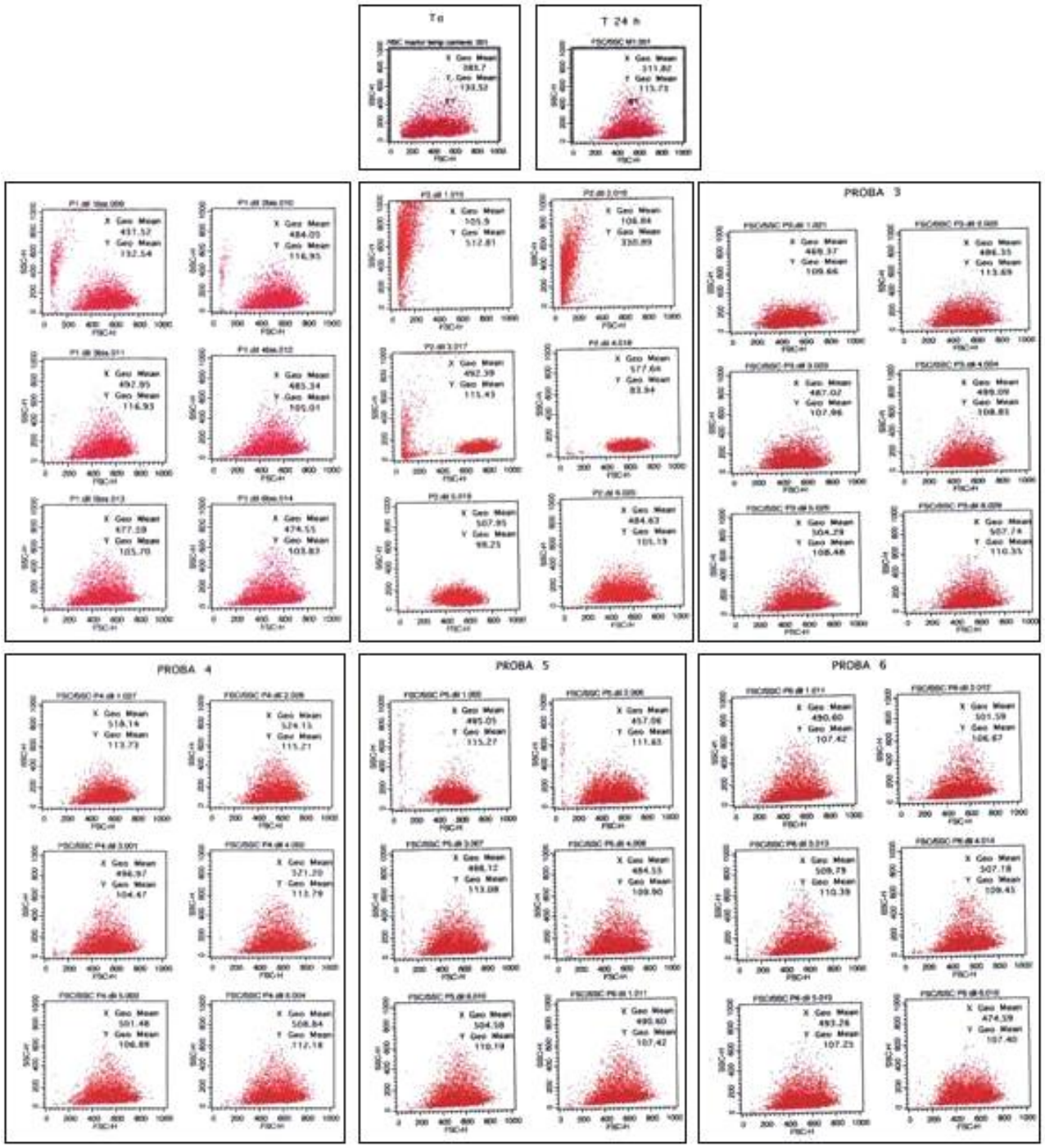

Fig 1. Comparative flow cytometric analysis of morphological cytograms of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P6) at 6 dilutions. Dot-plot analysis FSC/SSC of cells shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). Data are representative of three analysis giving similar results. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

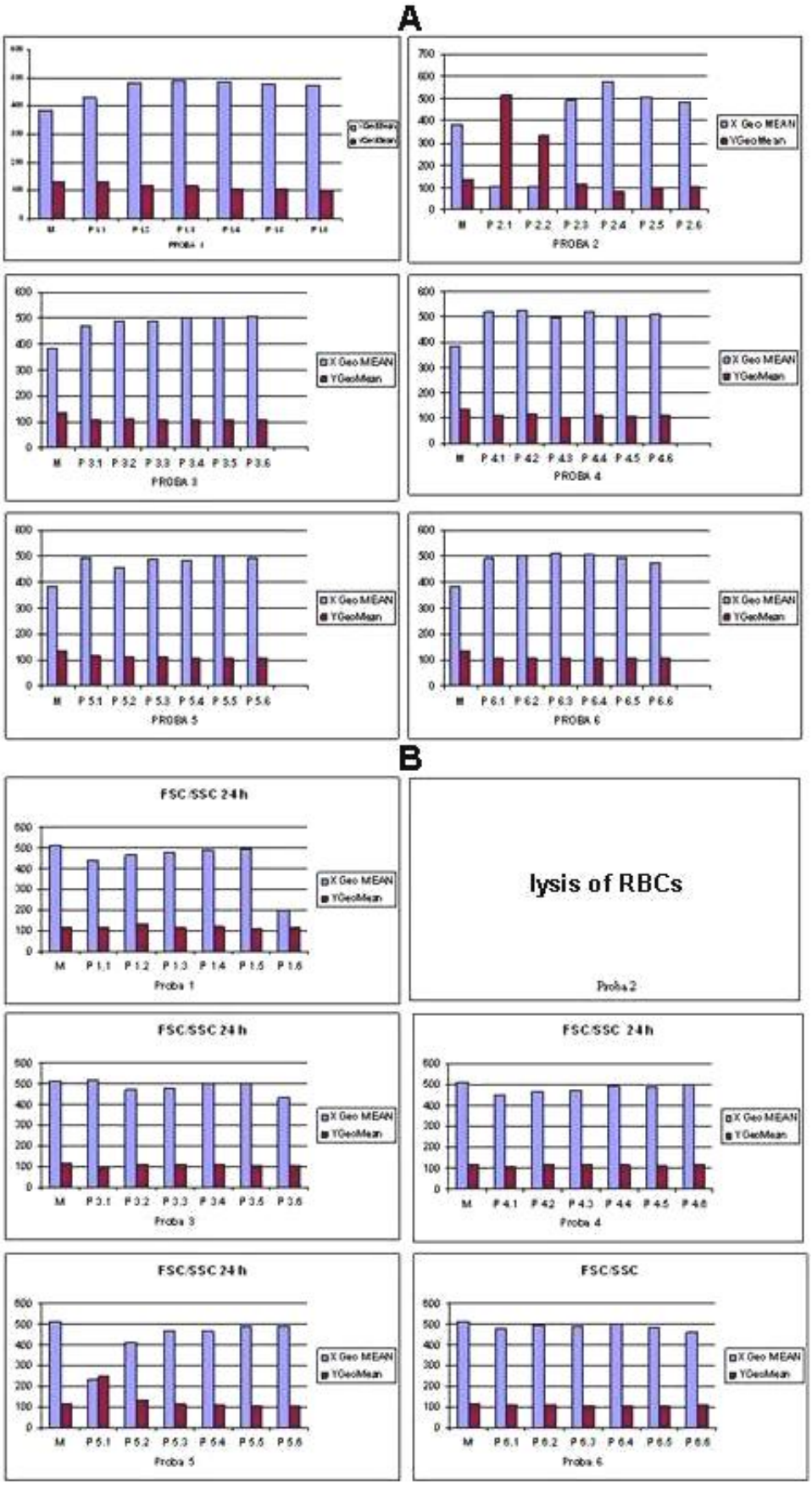

Fig 2. Histogram of XGeoMean and YGeoMean values of normal human erythrocytes (M) and exposed to nanoparticles (P1 to P6) for 3h (A) and 24h (B) at serial dilutions refer to dot-plot analyses similar to those from Fig 1. Results presented are from one representative experiment of three performed.

Optical microscopy presented in Fig. 3 as an illustration, entirely confirm data obtained by flow cytometry and proves that morphological changes of erythrocytes were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of characteristic features of apoptosis. Images of microscopic analyses of erythrocytes highlights that the morphological changes are not uniform for all samples, neither the intensity nor the manner of expression, showing that they accurately reflect the toxicity of different samples. Change of disc-shaped, biconcave of morphology to rounded forms, brings to mind an apoptosis phenomenon. They are very numerous in samples P2 and P5, and when they are accompanied by a transparent appearance, means that these cells are dead by lyses.

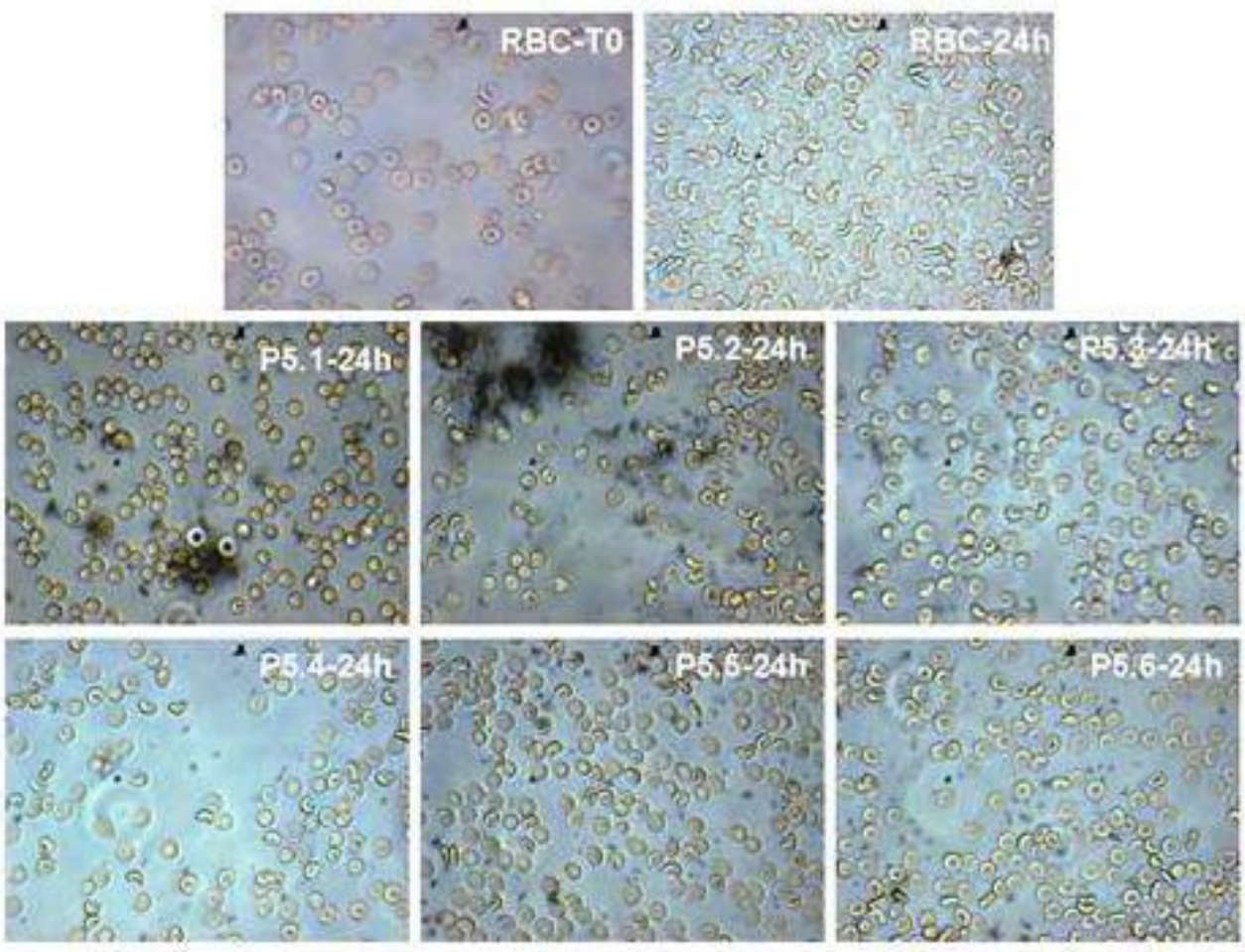

Fig 3. Morphological shape changes analyses by optical microscopy of normal erythrocytes (To and T24h) and exposed to nanoparticles of P3 at diferents dilution (1-6) refer to dot-plot analyses of Figure 1. Results presented are from one representative experiment of three performed. Cells were visualized using an inverted microscope MCX 1600 for bright field (Micros Autrich).

Influence of nanoparticles on cell viability measured with calcein-AM assay

We have recently developed a new flow cytometric assay for the measurement of cells viability using calcein-AM (Bratosin *et al.*, 2005). The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivate and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes. In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure (Bratosin *et al.*, 2005).

Application of this assay for analysing the effect of nanoparticles practised on erythrocytes at T0 and T24h (Fig. 4.) showed that two regions could be clearly and unambiguously defined: the region of fluorescent erythrocytes with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) and the region of nonfluorescent dead cells with damaged cell membranes (region M2).

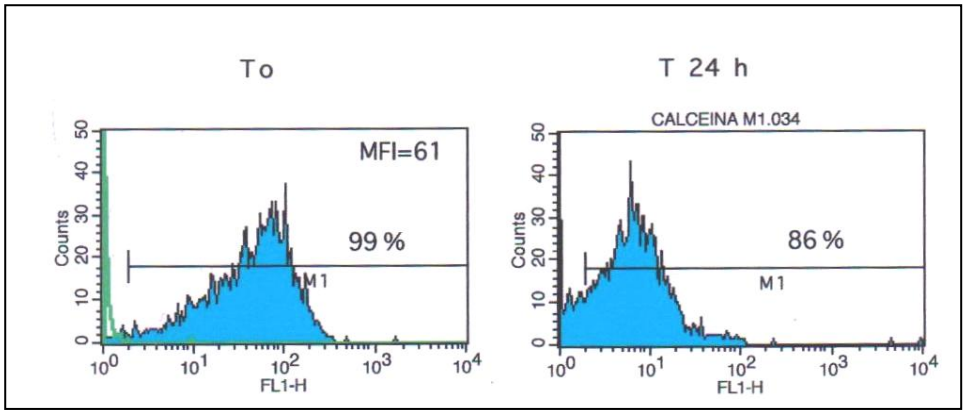

Fig 4.
Comparative flow cytometric histogram analysis of calcein-AM cell viability of normal erythrocytes (To and T24h). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Abscissae: log scale green fluorescence intensity of calceine (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

As shown in Fig. 4, the number of viable cells (region M1) in population decreased by 24h incubation, from 99% viable cells at moment T0 at 86% viable cells after 24h incubation.

The number of viable cells (region M1) in population decreases drastically as an expression of toxicity of nanoparticles especially for P1, P2 or P3 as compared to normal erythrocytes population.

To get an evident grasp of nanoparticles toxicity, a quantitative dose–response curve was adopted for comparison. For this reason, this test can be a test of toxicity or eco-toxicity, allowing us to determine EC50 (Figure 5).

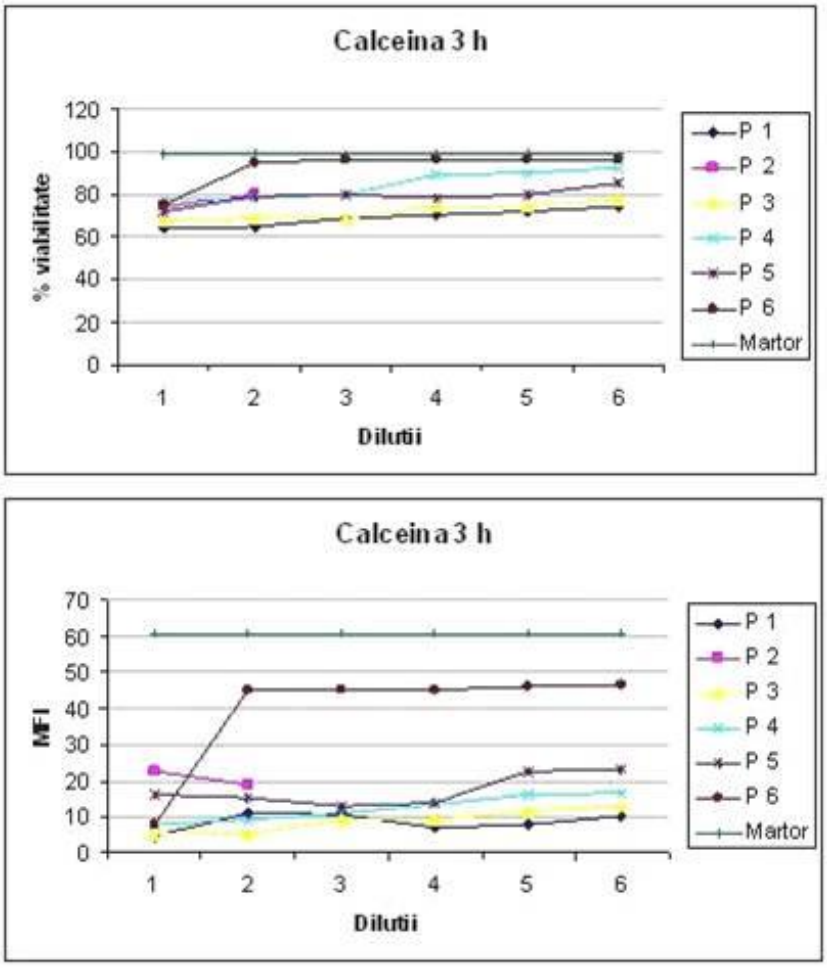

Fig. 5. Curves dose-response for the calculation of EC_{50}. Abscissae: concentration of nanoparticles. Ordinates: % of viable cells coresponding of M1 region from flow histograms resulting of its viability test with calcein-AM acording to methods of Bratosin *et al.,* 2005 or MFI (Mean Fluorescence Intensity) as expression of viability. Results presented are from one representative experiment of three performed.

DISCUSSION AND CONCLUSION

Is evident that in present and especially in the future, with the development of nanotechnologies, human exposure to nanoparticles from natural and anthropogenic sources will be increasingly higher. Exposure monitoring and control strategies are necessary. Indeed, there is a need for a new discipline - nanotoxicology - that would evaluate the health threats posed by nanoparticles, and would enable safe development of the emerging nanotechnology industry (Donaldson et *al.*, 2004). The development of nanotechnology and the study of nanotoxicology, requires a systematic scientific approach to the study of nanoparticle toxicity correlation of the physical and chemical characteristics of nanoparticles with their toxicity. Understanding the potentially toxic effects of nanoparticles requires a multidisciplinary approach, necessitating a dialogue between nanomaterial fabrication scientists, chemists, toxicologists, epidemiologists and environmental scientists.

First step for understanding how the nanoparticles will react in the body is cellular testing, easier to control and to reproduce. In the case of cytotoxicity, it is very important to note if the cell cultures used for bioassay are susceptible to determine the toxic potential of nanoparticles, to determine the potentially toxic concentration of the tested agent and carrying out multiple tests in order to ensure correct validation of the toxicity The ability of nanoparticles to enter cells and affect their biochemical function makes them important tools at the molecular level.

As we know, blood plays a vital role in carrying oxygen from lungs to tissues or organs in order to satisfy the metabolic needs, erythrocyte, dominant (99%) cell in the blood, can be vulnerable to toxicity (Rothen-Rutishauser *et al.*, 2006) and the erythrocytes treated with nano-TiO_2 presented morphological change from biconcave shape and underwent abnormal sedimentation, hema-gglutination and dose dependent hemolysis (Li SQ *et al.*, 2008).

In our paper, from this series of experiments, we can conclude that studied nanoparticles have seriously deleterious effect on erythrocytes in a dose-dependent *in vitro*, and consequently the erythrocyte is extremely vulnerable. To emphasize the toxic effect of nanoparticles we examined cell viability measurement using calcein-AM by flow cytometry.

The results reported in the present study indicate that the exposure of human RBCs to nanoparticles induces cell death depending of the doses. The changes of all the erythrocyte parameters investigated appear to be strongly correlated with increasing concentration of nanoparticles and flow cytometric analysis of RBCs viability could provide a rapid and accurate analytical tool for evaluating *in vitro* the biological responses towards of nanoparticles, for assessment of their toxicity and biosafety.

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