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IN VITRO TOXI - AND ECOTOXICOLOGICAL ASSESSMENT OF PORPHYRINE NANOMATERIALS BY FLOW CYTOMETRY USING NUCLEATED ERYTHROCYTES

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Abstract: The use of nanoparticles for biological and medical applications has rapidly increased and the potential for human and ecological toxicity is a growing area of investigation. For assessing cytotoxicity of nanoparticles we developed a new experimental cell system based on the use of nucleated erythrocytes (RBCs) from fish and batrachians, which are directly exposed to pollutants or nanoparticles absorbed by different ways and we have evaluated the toxic effects by flow cytometric analysis. The two modes of cell death (apoptosis and necrosis) differ fundamentally in their morphology, biochemistry and biological relevance. We and others have recently shown that programmed cell death of nucleated erythrocytes is related to an apoptotic mechanism. The toxicological analysis were performed comparatively on porphyrin base or metalloporphyrin and for each porphyrin bare derivative on two correspondent porphyrin-silica-hybrid nanomaterials obtained by one step acid catalysis and by two steps acid-base catalysis. To evaluate cell-nanoparticles interactions, nucleated RBCs were exposed to different concentrations of nanocomposites and analyzed by flow cytometry, after 24h incubation endpoints for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and viability (using calcein-AM method). The investigation showed that the type of cellular death of nucleated erythrocytes is related to an apoptotic mechanism and that flow cytometric analysis of nucleated RBCs viability and cell death discrimination could provide a rapid and accurate analytical tool for evaluating *in vitro* the biological responses towards of nanoparticles for environmental protection. Nucleated erythrocytes can be a new experimental cellular model easy to use, with no costs for culture and for maintaining in the culture. The results reported in the present study indicate that our new flow cytometric protocols can be used to create dose-response curves which allow us to determine EC50 for toxicity or eco-toxicity tests. It is also generally applicable for identifying harmful effects associated with general antropic impact for the aquatic environment and for its biomonitoring.

Key words: nanoparticles; nucleated erythrocytes; apoptosis; cell viability; flow cytometry; toxicity; ecotoxicology; nanomaterials.

1. INTRODUCTION

Improvements in nanoscale materials synthesis and characterization have given scientists great control over the fabrication of materials measuring between 1 and 100 nm, unlocking many unique size-dependent properties and, thus, promising many new and/or improved technologies

(Oberdörster et al., 2005; ASTM E 2456-06 2006). Recent years have found the integration of such materials into commercial goods and a current estimate suggests there are over 800 nanoparticle-containing consumer products. The production of nanoparticles will increase from 2 300 tons produced today to 58 000 tons by 2020 (Maynard, 2006). Manufactured nanomaterials (nanoparticles, nanotubes, nanosheetsand nanowires) have recent applications in drug delivery, medical devices, cosmetics, chemical catalysts, optoelectronics, electronics and magnetics. Some nanomaterials have been found to be toxic to humans and other organisms either upon contact or after persistent environmental exposure (Oberdörster 2004; Zhu et al., 2006; Griffitt et al., 2007; Usenko et al., 2007).

Despite this increase in the prevalence of engineered nanomaterials, little is known about their potential impact on environmental health and safety (Moore 2006; Crosera et al., 2009). The field of nanotoxicology has formed in response to this lack of informations and resulted in a flurry of research studies. Nanotoxicology is an emerging discipline (Oberdörster et al., 2005), a gap between the nanomaterials safety evaluation and the nanotechnology development that produces new nanomaterials, new applications and new products. Nanotoxicology relies on many analytical methods for the characterization of nanomaterials as well as on their impact on *in vitro* and *in vivo* functions (Lewinski et al., 2008; Hassellöv et al., 2008).

Flow cytometry is an analytical tool widely used to obtain detailed information on bioprocesses, using light-scattering, fluorescence and absorbance measurements.

For assessing cytotoxicity of nanoparticles 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. The two modes of cell death (apoptosis and necrosis) differ fundamentally in their morphology, biochemistry and biological relevance. We and others have recently shown that programmed cell death (PCD) of nucleated erythrocytes is related to an apoptotic mechanism (Bratosin et al. 2004). In the present study, to evaluate cell-nanomaterials interactions, nucleated RBCs were exposed to different concentrations of nanocomposites and analyzed by flow cytometry, after 24h incubation endpoints for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and viability (calcein-AM method). The toxicological analysis were performed comparatively on porphyrin base or metalloporphyrin and for each porphyrin bare derivative on the correspondent porphyrin-silica-hybrid nanomaterials obtained by sol-gel synthesis in one step acid catalysis or by two steps acid-base catalysis, using tetraethylorthosilicate (TEOS) as silica precursor.

2. MATERIALS AND METHODS

2.1. Chemicals

Fluorescein-conjugated Annexin-V and propidium iodide were from PharMingen (San Diego, CA, USA) and the fluorogenic dye calcein acetoxymethyl ester (calcein-AM), was purchased from Sigma Aldrich (Saint Louis, Mo, USA).

Nanoparticles based on porphyrins and their hybrids: *meso*-tetra-tolylporphyrin (P1); hybrid silica- meso-tetratolylporphyrin by sol-gel reaction in two steps acid-base catalysis HCl:TEOS = 0.02:1; NH_3:TEOS=0.0142:1 (P2); Zn(II)-meso-tetrakis(4-pyridyl)porphyrin (P3); hybrid silica-Zn(II)-meso-tetrakis(4-pyridyl)porphyrin, acid catalysis HCl:TEOS=0.01:1 (P4); hybrid silica- Zn (II)-meso-tetrakis (4-pyridyl) porphyrin, acid/base catalysis HCl:TEOS=0.01:1; NH_3:TEOS=0.015:1 (P5); *meso*-tetra(3,4 dimethoxy-phenyl)porphyrin (P6); hybrid silica *meso*-tetra(3,4-dimethoxy-phenyl)porphyrin acid catalysis (P7); hybrid silica-*meso*-tetra(3,4-dimethoxy-phenyl) porphyrin acid/base catalysis (P8) were synthesized according to the previous reported literature (Fagadar-Cosma et al., 2007; Fagadar-Cosma et al., 2009; Enache et al., 2010; Fagadar-Cosma et al., 2009).

2. 2. Erythrocytes collection and cell treatments

Erythrocytes from Batracian species *Rana esculenta* blood collected under ether anesthesia on heparin were sedimented by centrifugation (1000g, 4°C, 5min) and washed three times with Dulbecco's phosphate buffered saline solution pH 7.4 (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4).

In order to evaluate porphyrins nanoparticles effects, nanomaterials where incubated in sterile saline physiological solution (9 g Sodium Chloride/L) for 24 hours, after UV activated for 10 minutes and supernatants where used for obtaining serial dilutions (between 0. 008 and 0.0005 g/ml) in culture plates. Nucleated RBCs were incubated for 24 h at 20°C in these supernatants dilutions.

2. 3. Flow cytometric analysis

Flow cytometric analysis was performed on

a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest Pro software for acquisition and analysis. The light-scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale, a minimum of 5000 cells being analysed in each condition. Erythrocyte size and density were assessed using forward and side-angle scatters (FSC *versus* SSC).

2.4. 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).

2. 5. 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. Erythrocytes (4 x10^5 in 200 µl PBS buffer) were incubated with 10 µl calcein-AM working solution (final concentration in calcein-AM 5 µM) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

2. 6. Flow cytometric cell death assays

Cell death was determined using an Annexin-V-FITC/propidium iodide apoptosis kit. Annexin-V is a Ca^{2+}- dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both Annexin-V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin-V-FITC and PI are alive and not undergoing measurable apoptosis.

For analysis of Annexin-V- FITC and propidium iodide labeling, erythrocytes were washed twice with cold PBS buffer pH 7.4 and resuspended (1x10^6 cells) in HEPES buffer pH 7.4 containing 2.5 mM calcium chloride for 30 min at room temperature in the dark with 10 µl (0.1 µg) of FITC–annexin V and 10 µl (50 µg/ml) of propidium iodide solution. After incubation, cells were directly gated for biparametric histograms FL1 *versus* FL2. All studies were performed at least three times, with three replicates each time.

3. RESULTS AND DISCUSSIONS

3. 1. Detection of altered morphology by light scattering flow cytometry

Multiparametric flow cytometric analysis which discriminates and quantifies viable, apoptotic and necrotic cells via measurement of forward and side light scatter (proportional to cell diameter and internal granularity, respectively) is a very rapid and sensible method. As shown in Figure 1, flow cytometric analysis announce significant morphological changes of nucleated RBCs incubated for 24 h in saline supernatants of different nanomaterials (P1-P8) compared to nucleated RBCs incubated only in saline isotonic solution (T24h).

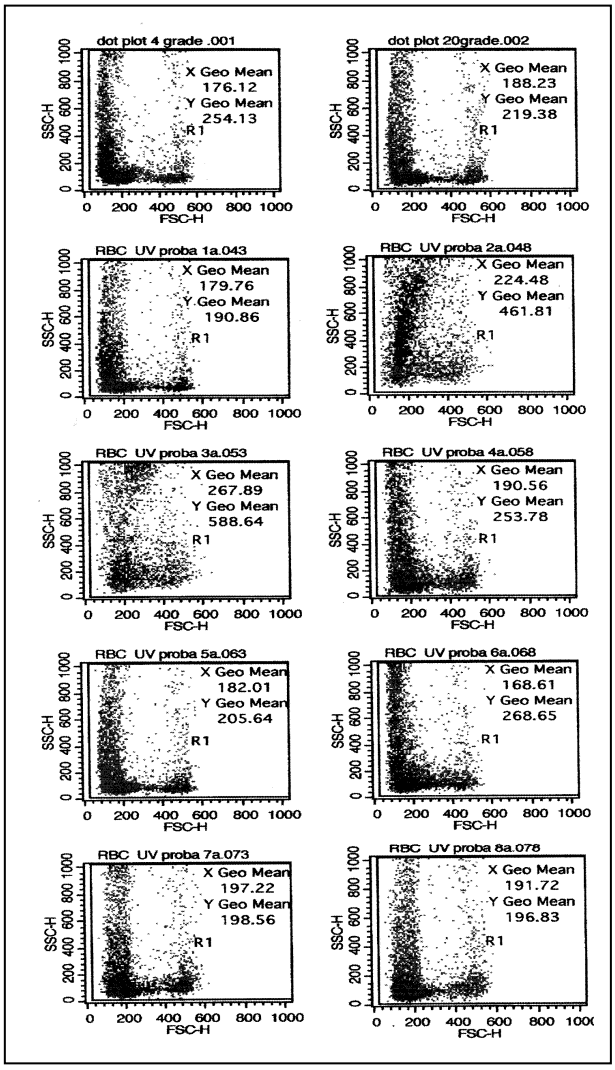

Figure 1. Comparative flow cytometric analysis of morphological cytograms of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P8) at 0,008 g/ml. Dot-plot analysis FSC/SSC of cells shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, 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.

In fact, as demonstrated in figure 2, the XGeo Mean values (cell side scatter) vary from 168 (P6) to 268 for P3 as compared to the statistical value of normal RBCs, i.e. 182±6. In the same way, the YGeo Mean values (cell density scatter) vary from 190 for (P1) to 461 (P2) or 588 for P3 as compared to the statistical value of normal RBCs, i.e. 237±17.

Optical microscopy presented in Figure 3 entirely confirmed these data and showed that morphological changes of nucleated erythrocytes were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of characteristic features of apoptosis. Images of microscopic analyses of nucleated erythrocytes incubated in supernatants obtained by preincubation of nanomaterials in saline solutions show that highlights the morphological changes are not uniform for all samples, neither the intensity nor that the manner of expression, showing that they accurately reflect the toxicity of different samples. Change of discoid morphology to rounded forms, brings to mind an apoptosis phenomenon. They are very numerous in samples P2 and P3, and when they are accompanied by a transparent appearance, providing that these cells are dead.

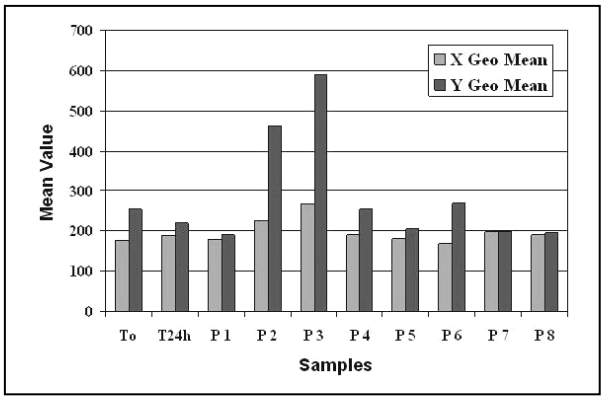

Figure 2. Histogram of X-and YGeoMean values of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P8) at 0,008 g/ml, refer to dot-plot analyses of Figure 1. Results presented are from one representative experiment of three performed.

Samples P4 and P7 induce an unexpected morphological aspect, comparable to a "bicycle wheel" which can be later clarified by TEM analysis. Very interesting, in the sample P5, the nanomaterials produce even more bizarre forms, a sort of "mega pores" or "holes". The same phenomenon is also observed in P8 sample, but less obvious.

3. 2. Influence of porphyrins on cell viability measured with calcein-AM assay

We recently devised 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 derivative 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).

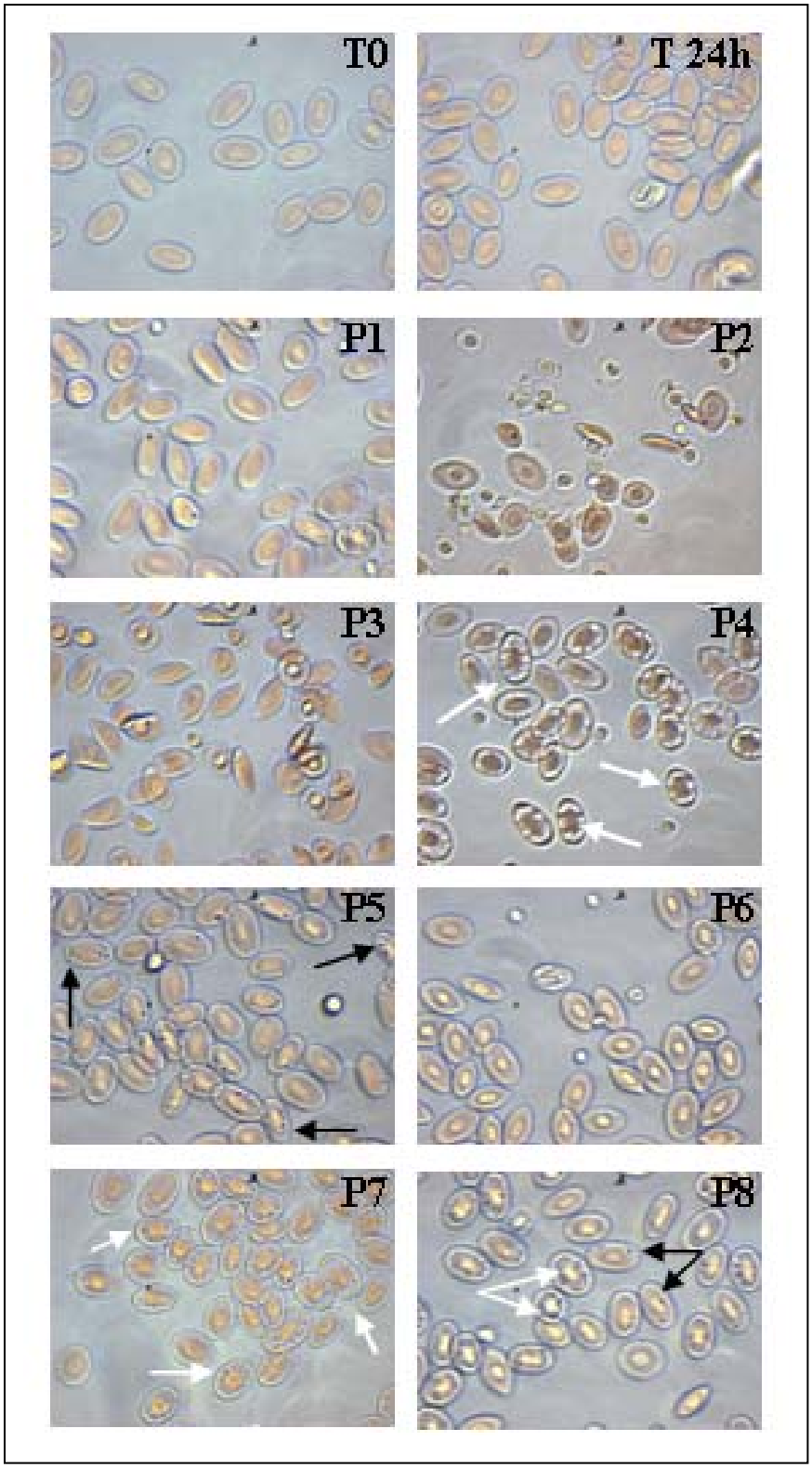

Figure 3. Morphological shape changes analyses by optical microscopy of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P8) at 0,008 g/ml, refer to dot-plot analyses of figure 1. Black arrows: erythrocytes with "mega pores" or “holes” White arrows: "bicycle wheel" erythrocyte shape. Results presented are from one representative experiment of three performed. Cells were visualized using an inverted microscope MCX 1600 for bright field (Micros Autrich).

Application of this assay for analysing the effect of nanomaterials practised on nucleated erythrocytes 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).

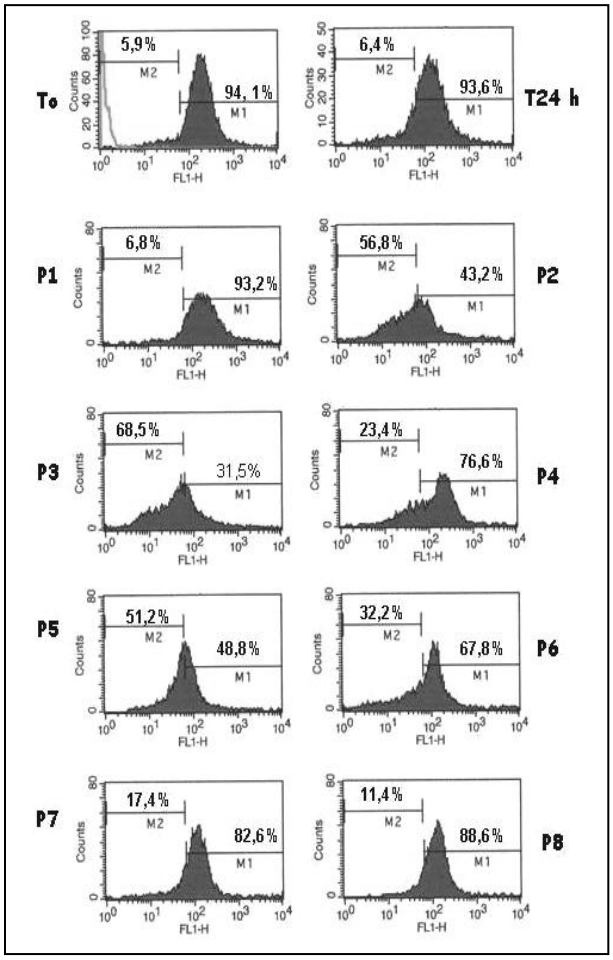

Figure 4. Comparative flow cytometric histogram analysis of calcein-AM cell viability of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials (P1 to P8). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged 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 figure 4, the number of viable cells (region M1) in population decreased drastically as an expression of toxicity of nanomaterials especially for P3 (around 31.5%) or P2 (around 43.2%) as compared to normal erythrocytes population (around 94%).

To get an evident grasp of nanomaterials 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 (Fig. 5).

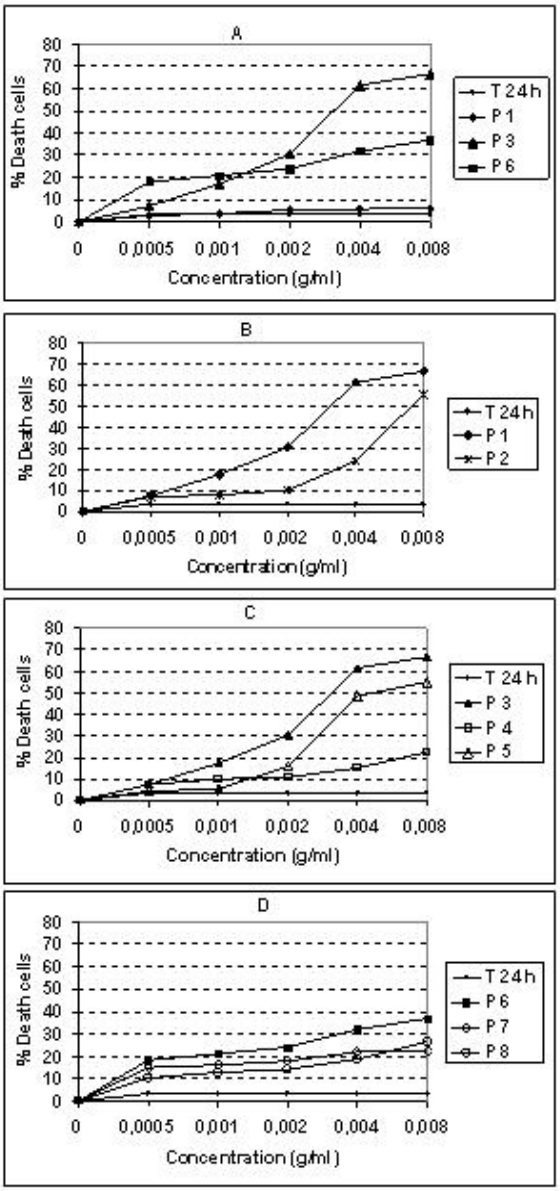

Figure 5. Curves dose-response for the calcule of EC_{50}. Abscissae: concentration of nanomaterials. Ordinates: % of death cells coresponding of M2 region from flow histograms presented in Figure 4. Results presented are from one representative experiment of three performed.

3. 3. Study of erythrocytes death by Annexin V-FITC and propidium iodide double-labelling

To investigate the mode of cell death induced by porphyrins, we applied simultaneous staining of erythrocytes with annexin-V and propidium iodide. Normal and incubated erythrocytes were analyzed by flow cytometry for phosphatidylserine (PS) exposure (Annexin-V labelling) and membrane permeabilization (PI-labelling). Phosphatidylserine residues are exposed

in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally observed during late apoptosis and cell necrosis.

Figure 6 shows comparative flow cytometric analyses of normal (N) and incubated erythrocytes with porphyrins. The number of living cells (Annexin$^-$/PI$^-$) decreased drastically from 96% (normal erytrocytes) to 23% for P3.

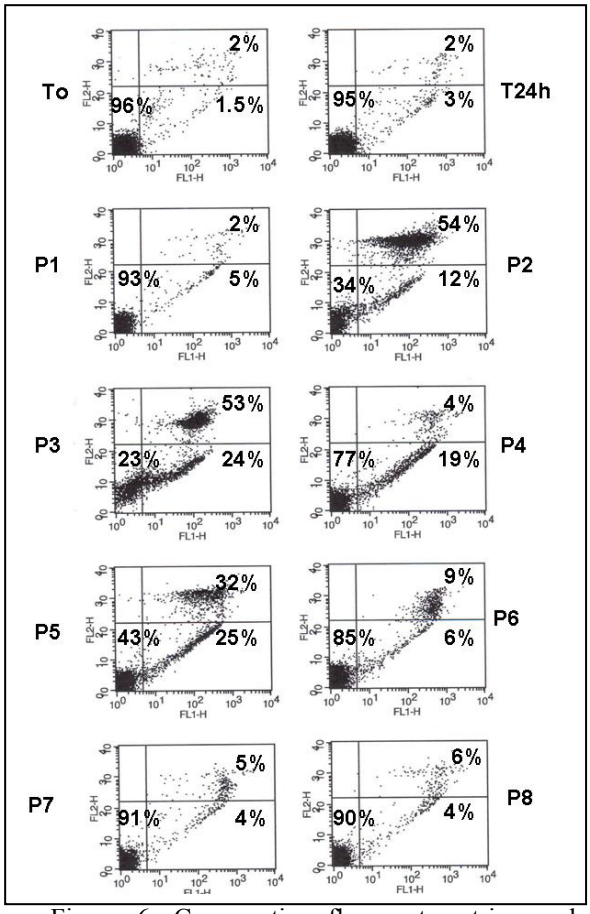

Figure 6. Comparative flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials (P1 to P8). Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

As shown in figure 7, we can see that porphyrin base or porphyrin-nanomaterials has *in vitro* serious deleterious effect on nucleated erythrocytes in a dose-dependent, allowing calculating EC50.

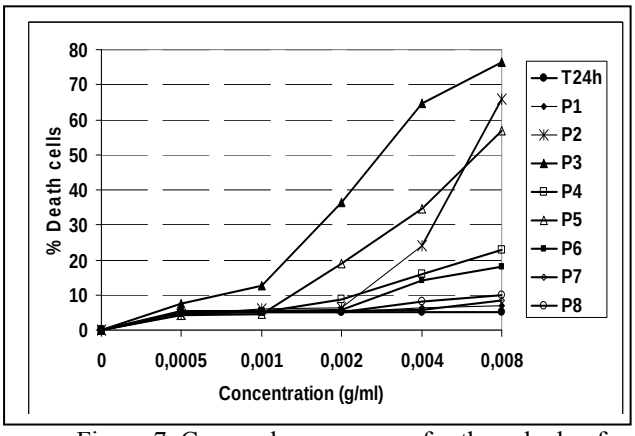

Figure 7. Curves dose-response for the calcule of EC_{50} conforming to % of death erythrocytes determined by Annexin V-FITC and propidium iodide double-labelling. Abscissae: concentration of nanomaterials. Ordinates: % of death cells refers to the % of total cells (100%) less % of viable cells (low left quadrant: viable cells (annexin-V and propidium iodide negative cells) from flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained presented in Figure 6. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

Our results demonstrate that nucleated RBCs can be a new experimental cellular model easy to use, with no costs for culture and for maintaining in the culture. Our results indicate that the sensitivity of nucleated RBCs to nanomaterials was further increased and the information could be potentially useful for the development of low cost and rapid ecotoxicity assays and erythrocyte apoptosis can be an efficient ecotoxicological biomarker providing significant information of environmental stress.

With the rapid development of nanotechnology, there is an increasing risk of human and environmental exposure to nanotechnology- based materials and products.

First step for understanding how the nanoparticles will react in the body is cellular testing, easier to control and reproduce. In the case of cytotoxicity it is very important to note if the cell cultures are sensitive, to note that the concentration of the potentially toxic agent being tested and conducting multiple tests to ensure valid conclusion.

The simplest test applied for evaluating the cytotoxicity of single-walled carbon nanotubes and gold nanoparticles involved inspection of the cells with bright-field microscopy for assessment of cellular and nuclear morphology (Fiorito et al., 2006; Altman et al., 1993; Bottini et al., 2006; Goodman et al., 2004). However, most cytotoxicity assays measure cell death via

colorimetric methods by measuring plasma membrane integrity and mitochondrial activity [Borenfreund E (1985), Flahaut et al., (2006); Monteiro-Riviere & Inman (2006)] and the most widely used test is the MTT viability assay (Flahaut et al., (2006), Monteiro-Riviere N & Inman A (2006), Jia et al., (2005), Tian FR et al., (2006), Sayes CM et al., (2009)].

A third cytotoxicity assay used in several carbon-nanoparticle studies is lactate dehydrogenase (LDH) release monitoring (Sayes et al. 2004; Muller et al., 2005). Other cytotoxicity assays determine the genotoxic potential of nanoparticles measuring the extent of DNA damage by flow cytometry using a membrane-impermeable dye (Kastarelos et al., 2007; Cui et al., 2005) or by comet assay in individual cells using gel electrophoresis (Fairbairn et al., 1995). The major biological effects involve interactions with cellular components such as the plasma membrane, organelles or macromolecules, but, because the different nanoparticles can trigger distinctive biological responses, it is very important that cytotoxicity studies are conducted for each nanoparticle type (Lewinski et al., 2008) with more different cell types. The nanoparticles and nanomaterials could be absorbed by respiratory tract (inhalation), digestive tract (ingestion) and dermal (penetration) and were subsequently distributed as noted in such key organs as lung (Warheit et al., 2007), lymph nodes (Bermudez et al., 2004), liver (Wang et al., 2007), brain (Thomas et al., 2006).

As we know, blood plays a vital role in carrying oxygen from lungs to tissues or organs to meet 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). Unfortunately, up to now, nothing was known about the interaction of nanoparticles or nanomaterials with nucleated erythrocytes from fishes and batrachians which are directly exposed to pollutants or to nanoparticles absorbed by different ways.

3.4. Impact of our results on aquatic environment under the influence of nanoparticles as potential aquatic pollutant

The nanotechnology industries start to come on line with larger scale production and it is inevitable that nanoscale products and by-products will enter the aquatic environment (Moore et al., 2004). Consequently, environmental release of nanoparticles into aquatic systems rise many questions, mainly what will be the implications of nanoparticle exposure for organism health and ecosystem integrity? Other question is if the particle size and surface properties may be significant factors in determining toxicity and pathogenesis of nanoparticles in aquatic organisms?

Uptake of nanoparticles in aquatic animals includes direct ingestion or entry across epithelial boundaries such as gills, olfactory organs or body wall. Recent studies with fish have indicated that C60-fullerene may be internalized by these routes, although this was a very limited investigation (Oberdörster, 2004).

Predicting the behaviors of nanoparticles is likely to be much more difficult than predicting those of conventional chemical pollutants, which is still often a major challenge. Consequently, until we can effectively discount specific or generalized hazards associated with various types of nanoparticle we should invoke a precautionary approach (Colvin, 2003 and Howard 2004). This will require testing of existing and new nanomaterials to determine individual level impacts on animal health status.

In our paper, from this series of experiments, we can conclude that studied porphyrin base or porphyrin-nanomaterials has seriously deleterious effect on nucleated 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 and consecutively apoptotic processes. Particular attention has been accorded to evaluation of porphyrine or porphirine-nanomaterials action in relation to initial and late apoptotic phases and with cell viability measurement using calcein-AM by flow cytometry.

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

All the modifications observed *in vitro* with our tests can be induced *in vivo* in aquatic organisms under the action of nanoparticles

released in the aquatic environment

Consequently, the higher level of damage produced to animal health, ecological risk and possible food chain risks for humans require generalized application of toxicity and ecotoxicity testing protocols to identify harmful effects associated with nanoparticles.

4. CONCLUSION

Now, a major challenge for ecotoxicologists will be the derivation of toxicity thresholds for nanomaterials and determining whether or not currently available biomarkers of harmful effect will also be effective for environmental nanotoxicity and new methods are required to assess the toxicity and ecotoxicity of nanomaterials.

The results reported in the present study indicate that our new flow cytometric protocols can be used to create dose-response curves which allow us to determine EC50 for toxicity or eco-toxicity tests. Also, this new tests are generally applicable for identifying harmful effects associated with general antropic impact for the aquatic environment and for its biomonitoring with consequences for environmental protection.

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