Comparative effects of macro-sized aluminum oxide and aluminum oxide nanoparticles on erythrocyte hemolysis: influence of cell source, temperature and size

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Effect of aluminum oxide nanoparticles on erythrocytes

Abstract

Al_2O_3 is the most abundantly produced nanomaterial and has been used in diverse fields, including the medical, military and industrial sectors. As there are concerns about the health effects of nanoparticles, it is important to understand how they interact with cells, and specifically with red blood cells. The hemolysis induced by three commercial nano-sized aluminum oxide particles (nanopowder 13 nm, nanopowder <50 nm and nanowire 2-6 nm × 200-400 nm) was compared to aluminum oxide and has been studied on erythrocytes from humans, rats and rabbits, in order to elucidate the mechanism of action and the influence of size and shape on hemolytic behavior. The concentrations inducing 50% hemolysis (HC_{50}) were calculated for each compound studied.

The most hemolytic aluminum oxide particles were of nanopowder 13, followed by nanowire and nanopowder 50. The addition of albumin to PBS induced a protective effect on hemolysis in all the nano-forms of Al_2O_3, but not on Al_2O_3. The drop in HC_{50} correlated to a decrease in nanomaterial size, which was induced by a reduction of aggregation

Aluminum oxide nanoparticles are less hemolytic than other oxide nanoparticles, and behave differently depending on the size and shape of the nanoparticles. The hemolytic behavior of aluminum oxide nanoparticles differs from that of aluminum oxide.

Key words: aluminum oxide nanoparticles, hemolysis, erythrocytes

Introduction

It is important to research the interactions of nanomaterials with membrane cells, because such interactions are critical in many applications such as biomedical imaging, drug delivery, disease diagnostics and DNA/protein structure probing (Verma and Stellaci, 2010). An increasing number of nanomaterials are being designed for biological applications, and this raises new concerns about the safety of nanotechnology (Nel et al. 2009). The small size, high surface curvature, large surface area, and abundant surface reactive sites may induce special reactions on the bio-surface.

Cell membranes are the most important biological surfaces that nanomaterials interact with when they come into contact with organisms. Therefore, the effects of nanomaterials on cell membranes should be assessed for nanomaterial applications and safety. As there are concerns about the health effects of NPs, it is important to understand how they interact with cells and specifically with red blood cells (RBC), which have a central role in blood functions and compare to the material not in the nano size.

Al_2O_3 is the most abundantly produced nanomaterial (NM). It is estimated to account for approximately 20% of the 2005 world market of NMs (Rittner, 2002). Al_2O_3 NMs have been used in diverse fields for medical, military and industrial purposes (Balasubramanyam et al. 2010). Recently, it has been demonstrated that Al_2O_3 NPs are effective bactericidal agents against ESBL-producing strains of E. coli, regardless of the drug-resistance mechanisms that confer importance to these bacteria as emerging pathogens (Ansari et al. 2014). Aluminum NMs act as drug delivery systems that could encapsulate drugs to increase solubility, evade clearance mechanisms, and allow site-specific targeting of drugs to cells (Tyner et al. 2004). One of the most important uses of nanometer-sized alumina in medicine may be in orthopedic/dental implants. Reducing the grain size of aluminum oxide not only mimics the physiological bone cell size, but also increases mechanical resistance to wear. Additionally, a higher specific surface area allows for better

integration of the implant surface with bone. It is believed that the use of nanometric aluminum oxide could solve the current problems associated with implantation by enhancing osseointegration and preventing graft rejection (Yamamoto et al. 2004). The study of human health impacts of NMs is essential, due to their widespread usage and the dearth of literature on the risks they pose at cellular and molecular level.

Some studies have reported the cytotoxic effect of aluminum oxide nanoparticles. It has been demonstrated that exposure to aluminum oxide nanoparticles caused dose-related cytotoxic effects through changes in lysosomal and mitochondrial dehydrogenase activity in CHO-K1 cells (Di Virgilio et al. 2010) . Other studies have demonstrated that they have a less cytotoxic effect than other nanoparticles (Zhang et al. 2011). Like other NPs, aluminum oxide nanoparticles pass through the cell membrane and accumulate in the cytoplasm of A549 cells after 6 h of exposure (Simon-Deckers et al. 2008) or after 48 hours of exposure in human fetal lung fibroblasts (Zhang et al. 2011). Aluminum oxide nanoparticles were demonstrated toxic to microalgae by interactions with the cell surface (Sadiq et al. 2011). NPs were observed in the cytoplasm of almost every cell. However, there are few studies in relation to its effects on membranes and this need to be explored

The hemolysis assay is recommended as a reliable test for material biocompatibility (Lu et al. 2009). In this study, we compared the effect on erythrocytes from human, rat and rabbit of three sizes of commercial aluminum oxide nano-particles and micro-sized aluminum oxide particles.

Experimental

Materials

Aluminum oxide nanopowder of <50 nm particle size (TEM) and >40 m2/g (BET), nanopowder of 13 nm particle size (TEM) and 85-115 m2/g (BET), nanowires 2-6 nm × 200-400 nm, and micro-sized aluminum oxide >98% purity, bovine albumin supplied by Sigma-Aldrich.

Characterization of the nanoparticles

The three commercial aluminum oxide nanoparticles of 13 nm diameter, 50 nm diameter and nanowires (NWs) were characterized by high resolution transmission electron microscopy (TEM), and atomic force microscopy (AFM).

The mean hydrodynamic diameter and the polydispersity index (PDI) of the NPs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). Before measurement, the NPs were appropriately diluted in phosphate buffered saline (PBS) and in PBS with albumin, and incubated for 24 hours at room temperature or at 37°C. Each measurement was performed using at least three sets of ten runs.

Preparation of red blood cell suspensions

Rat and rabbit blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. Human blood was obtained from healthy volunteers by venopunction. The procedure was approved by the institutional ethics committee. Red blood cells were isolated by centrifugation at 3000 rpm at 4°C for 10 min, and washed three times in isotonic PBS containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in distilled water (pH 7.4; 300 mOsmol/ l). The cell pellets were then suspended in PBS solution at a cell density of 8×10^9 cells/ ml.

Hemolysis assay

The membrane lytic activity of the aluminum oxide particles was examined by hemolysis assay. Twenty-five microliter aliquots of erythrocyte suspension were exposed to various concentrations of the compounds dissolved in PBS solution in a total volume of 1 ml. Two controls were prepared by suspending erythrocytes either in buffer alone (negative control) or in distilled water (positive control). The samples were incubated at 37°C and at room temperature under constant shaking for 1, 3 and 24 hours, and then centrifuged at 10,000 rpm for 5 min. Supernatants were taken, the absorbance of the hemoglobin release was measured at 540 nm using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan), and the percentages of hemolysis were determined by comparison with positive control samples that were totally hemolyzed with distilled water. Dose-response curves were obtained from the hemolysis results, and concentrations inducing 50% hemolysis (HC_{50}) were calculated (Nogueira et al. 2011). To discard possible interferences of aluminum oxide nanoparticles with hemoglobin, we determined the spectrum of hemoglobin and of the nanoparticles in a UV-spectrophotometer. The UV–vis absorption spectra were recorded on a UV–vis spectrophotometer (Shimazu, Tokyo, Japan) with a 1.0 cm quartz cell.

Albumin effect

The effect of albumin on the hemolytic activity of the nanomaterials was assessed under the same conditions, except that albumin at a concentration of 0.5 mg/ml was added to PBS. The hemolysis was determined for each product in the presence and absence of albumin in the medium, and the HC_{50} was calculated.

Statistical data evaluation

All measurements are reported as mean ± standard deviation Un-paired two-tailed Student's t-test with unequal variance was used for statistical analysis of results. $p < 0.05$ was considered significant.

Results and discussion

Nanoparticle characterization

We characterized the nanomaterials by TEM. Figure 1 shows the three nanomaterials. The nanopowder of 50 nm had smaller nanoparticles of about 8-10 nm, forming agglomerates of about 50 nm. The nanowires were shorter than the length declared by the supplier, at about 100 nm. The difference in primary size as given by the suppliers' data and experimentally obtained data may be due to aggregation of the nanoparticles in the medium (PBS).

The surface of the samples was characterized in detail using x quantitative roughness parameters, determined with special image analysis software for AFM (Nanoscope Analysis 1.5, Bruker), as shown in Figure 2.

The determination of size by dynamic light scattering (DLS) corroborated the results observed by TEM, which were similar to the sizes declared by the suppliers (Table 1). In the case of nanopowder 50 nm, the values determined by DLS corresponded to the aggregates observed in the TEM images. There was a high degree of polydispersity, expressed by PDI values above 0.3 for all conditions and all NPs. The determination of size under different conditions shows that an increase in temperature from room temperature to 37°C induced a decrease in the size of the three nanoparticles studied.

Hemolysis

The hemolytic assay has been used as a measure of particle reactivity because this method measures the ability of the particles to destroy the integrity of red blood cells as a predictor for the in vivo inflammatory potential of nanoparticles (Hedberg et al. 2010). Firstly, we determined the hemolytic effect of aluminum oxide nanoparticles in rabbit after 1, 3 and 24 hours of incubation at room temperature. The hemolytic concentration inducing 50% of hemolysis (HC_{50}) was determined for each substance. There was a clear time effect on the rabbit erythrocyte hemolysis, with decreases in HC_{50} values after a longer time exposure (Figure 3). These nanoparticles were less hemolytic than others studied in the literature, such as silver (Choi et al. 2011) or cuprous oxide nanoparticles (Chen et al. 2013). The HC_{50} of aluminum oxide nanoparticles in rabbit erythrocyte was in the range of 10 to 24 mg/ml after 24 hours of incubation at room temperature. Due to the low hemolytic effect observed after 1 and 3 hours incubation, the following studies were performed at 24 hours incubation.

The hemolytic effect varied in rabbit, rat and human erythrocytes (Figure 4). When we compared the three species, rat had more resistant erythrocytes, as demonstrated by the higher HC_{50} values, and was more similar to human erythrocytes (Figure 5). Rat erythrocytes had HC_{50} values that were around 4 to 6 times higher than rabbits for the Al_2O_3 of 50 nm and the Al_2O_3 nanowire, respectively. In the case of macro-sized aluminum oxide there are no statistical differences between species, by contrary in the case of nanoparticles there are significant differences between hemolytic behavior of rabbit and human erythrocytes ($p<0.01$).

In human erythrocytes, macro-sized aluminum oxide is more hemolytic than the nanoparticles. Actually it can be said that macroscopic Al_2O_3 has more hemolytic effect than nanopowder 50. When particle size decreased to 13 nm hemolytic effects increased.

The limitations of hemolysis methods for studying nanoparticles have been discussed (Dobrovolskaia et al. 2008) . Nanoparticles should be well-dispersed and stabilized in a physiological solution before a hemolysis study. The crucial point is to use well-mixed samples. There are two general methods for mixing samples: using a tube rotator or rotating the tubes every 30 minutes (Choi et al. 2011). We consider that a tube rotator is the best of these methods, as it ensures that the samples are well-mixed throughout the process, especially when incubation times are long, and to avoid sedimentation with high concentrations. The tube rotator is better than other proposed methods based on constant shaking (Sharma et al. 2014) where sedimentation could be present. Another important point is the type of tubes that are used to mix the samples with the erythrocyte suspension. The best tubes are those with round bottoms for the same reasons as have been exposed before.

Other mechanisms of particle interference with the assay have been identified (Dobrovolskaia et al. 2008), including the adsorption of hemoglobin by particles. This phenomenon could be observed with some nanoparticles, in presence of hemoglobin obtained from erythrocytes and incubated with the nanoparticles. In these cases there is a reduction in the absorbance of the hemoglobin in the supernatant after centrifugation, because hemoglobin is adsorbed by the pellet including the nanoparticles. In our study, we did not observe these interferences, which made the test suitable for studying this kind of nanoparticles.

The most common mechanism of interference is nanoparticle absorbance at or close to the assay wavelength (540 nm). This has been observed for gold nanoparticles, some nanoemulsions, fullerene derivatives, and doxorubicin-loaded particles (Dobrovolskaia et al. 2008). To discard this interference, we measured the absorption of aluminum oxide and nanoparticles alone, without red blood cells, at the higher concentration tested. For these products, absorption was not significant, as it corresponded to a maximum of 2% of the absorption found for hemoglobin (data not shown).

Influence of size and shape

Hemolytic behavior differs depending on the size of the nanoparticles and the species studied. Macro-sized aluminum oxide has similar HC_{50} values, irrespective of the species, but this phenomenon was not observed for the nanoparticles. The most hemolytic aluminum oxide particles expressed by the lowest HC50 were of nanopowder 13, followed by nanowire and nanopowder 50. Nanopowder 50 showed at TEM that it consisted of particles of 8-10 nm, but organized in clusters of about 50 nm, with less of the surface in contact with the erythrocyte membrane than nanopowder 13, which determined the lower hemolytic effect.

The shape and size of nanoparticles has been found to greatly influence their cellular uptake. In a study by Chithrani et al., uptake of 14-, 50-, and 74-nm gold nanoparticles was investigated in Hela cells (Chithrani et al. 2007). It was found that the kinetics of uptake and the saturation concentration varied with nanoparticle size. The uptake of the 50-nm particles was the most efficient, which indicates that there might be an optimal size for efficient nanomaterial uptake into cells. The effect of nanoparticle shape on its internalization was also examined: spherical particles of similar size were taken up 50% more than rod-shaped particles, which is explained by the greater membrane wrapping time required for the elongated particles. In other studies, nanoparticle size was shown to strongly affect the binding and activation of membrane receptors and subsequent protein expression (Jiang et al. 2008). It is crucial to examine the influence of the shape and size of nanomaterials on cell interactions, as this could have implications for toxicity (Nel et al. 2009).

Particle size and surface area are key factors that affect hemolysis. In the case of silver nanoparticles, it was observed that the hemolytic properties of nano-sized particles were considerably greater than those of micron-sized particles at equivalent mass concentration (Choi et al. 2011). It was found that hemolysis caused by large agglomerates of protein-stabilized silver particles was significantly less than for smaller agglomerates (Zook et al. 2011). Porosity and the surface characteristics of the nanoparticles are the major factors that influenced the cellular

association of the nanoparticles. The impact of nanoparticle geometry became pronounced as the concentration further increased (Yu et al. 2011).

One study supported surface-dependent hemolysis of silica, and suggested that mesoporous silica particles reduce hemolytic activity on RBC because of their lower external surface area (Slowing et al. 2009). Membrane fluidity and lateral organization may be influenced by nanoparticle attachment. Changes in membrane fluidity and lateral organization could be one of the reasons for NP toxicity.

Influence of albumin

The addition of albumin to PBS induced a protective effect on hemolysis in all the nano-forms of Al_2O_3, but not on micron-sized Al_2O_3 (Figure 6). The protective effect was demonstrated by increases in the HC_{50} values in the presence of albumin. These increases were of about 40 to 60%. In the case of the nanowire, albumin induced the highest protection against hemolysis ($p< 0.01$).

Some studies have demonstrated that treatment of RBC with polystyrene nanoparticles induces (dose- and particle size-dependent) hemolysis in a protein-free medium, but supplementation of the suspension medium by albumin inhibits PS-NP hemolytic activity even at very low protein concentrations (0.05% wt). This shows that even when the protein corona is not fully formed and the surface of the NP is only partly covered by protein, the presence of protein molecules on the NP surface strongly modulated their interaction with biological cells (Barshtein et al. 2011). In a similar way, it has been demonstrated that the addition of serum albumin, which comprises about half of the blood serum protein, markedly attenuated hemolysis induced by fullerene (C60) nanoparticles. Albumin could prevent the interaction of nanoparticles with erythrocytes and subsequent damage to their membrane (Trpkovic et al. 2010).

The surfaces of nanomaterials in a biological environment are modified by the adsorption of biomolecules (such as proteins and lipids), resulting in what is known as a particle-corona

complex (Lundqvist et al. 2008; Kapralov et al. 2012). The protective effect of serum or plasma was first observed many years ago for silica-induced hemolysis in human RBC (Harley and Margolis 1961). The lower toxicity of silica nanoparticles in the presence of serum has also been attributed to the higher agglomeration state and lower cellular uptake of silica nanoparticles (Dutta et al. 2007). There is considerable synergy between the NP surface chemical functionalities and the protein corona, leading to dramatically attenuated hemolytic behavior of NPs (Saha et al. 2014).

Nanomaterial surface properties, size, material, morphologies etc. strongly influence the protein corona composition (Monopoli et al. 2011; Walczyk et al. 2010).

The presence of this protein corona should clearly reduce the hemolytic activity of the aluminum oxide nanoparticles studied in this work, compared to the absence of effect observed in the case of micro-sized aluminum oxide, in which the protein corona is not formed.

Influence of temperature

It is essential to understand temperature behavior at physiological conditions of particles that are in contact with the human body. The hemolysis assay is usually performed at body temperature (37°C) (Koziara et al. 2005) or room temperature (20-25°C) (Hedberg et al. 2010; Slowing et al. 2009).

The effect of temperature on hemolysis has been demonstrated in human red blood cells, which show an increase in hemolysis with temperature. At lower concentrations, hemolysis was similar at 37°C and at room temperature, but an increase in hemolysis was observed at increasing concentrations. The hemolytic activity of the nanoparticles in this study increased with temperature, which was in line with other studies performed with silica particles (Shi et al. 2012). Interestingly, the temperature dependence of hemolysis varied among the aluminum oxide nanoparticles, as can be observed by comparing the HC_{50} for each individual nanoparticle (Figure

7). The greatest effect was observed in the case of nanopowder 50 nm ($p< 0.05$). At a higher temperature, the aggregates of about 50 nm observed at room temperature were disaggregated. The small particles of 8-10 nm which are the constituents of the compound have a greater surface area and the hemolysis value was closer to the hemolysis observed for nanopowder 13 nm.

Some studies have estimated the free energy profile for a structured assembly. The results clearly show that the successful assembly of NPs is energetically favorable at a lower temperature (Patra et al. 2014). By contrast, the aggregation of ZnO nanoparticles was found to be induced by temperature (Majedi et al. 2014).

Studies performed to investigate the influence of temperature on the formation and composition of the protein corona on magnetic NPs have shown that changes in the incubation temperature can have significant effects, although this is not necessarily always the case. The effects are specially pronounced at physiological temperature, which suggests that studies on the formation of a protein corona should be carried out at well-controlled temperatures (Mahmoudi et al. 2013).

The hemolytic behavior was higher after incubation at 37°C than at room temperature, as shown by the lower HC_{50} observed at higher temperature. The drop in HC_{50} correlated to a decrease in nanomaterial diameter, which was induced by a reduction of aggregation (Figure 8). There is a correlation between the percentage of decrease in hemolysis and the percentage of decrease in the size represented by a linear fit with an r of 0.98. By contrast, this phenomenon is not observed in macro-sized Al_2O_3.

Conclusions

Aluminum oxide nanoparticles are less hemolytic than other oxide nanoparticles, and behave differently depending on the size and shape of the nanoparticles. The hemolytic behavior of aluminum oxide nanoparticles differs from that of micro-sized aluminum oxide. Temperature is an important factor that influences the hemolytic behavior of aluminum oxide nanoparticles, with

increase hemolysis at 37ºC compared to room temperature. The conditions of the experiments should be clearly established when comparing different nanoparticles.

Competing interests

The authors declare that they have no competing interests

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Table 1. Characterization of nanoparticles by DLS after 24 hours incubation at room temeperature and 37ºC and in presence of 0.5 mg/ml of albumin.

Nanoparticle	***Supplier especification***	***PBS 24h Room Tº (nm) DLS***	***PBS 24h 37ºC (nm) DLS***	***PBS+Alb 0.5 mg/ml 24h Room Tº (nm) DLS***
Nanopower 50 nm	<50 nm (TEM)	17.40 ± 4.66	10.39 ± 2.43	17.17 ± 3.08
Nanopower 13 nm	13 nm (TEM)	43.00 ± 0.88	36.10 ± 2.56	28.76 ± 0.72
Nanowire	2-6 nm × 200-400 nm (TEM)	12.51 ± 0.98	10.04 ± 2.50	9.94 ± 1.40

Figure captions

Figure 1 TEM of aluminum oxide nanoparticles: nanopowder 13 nm (a), nanopowder 50 nm (b) and nanowire (c)

Figure 2 Representative AFM topography images (2D and 3D) and particle size histograms in (a) Al_2O_3 13 nm commercial NPs; (b) Al_2O_3 50 nm commercial NPs; and (c) Al_2O_3 commercial NWs show the presence of individual nanoparticles and aggregates

Figure 3 Hemolytic effect of aluminum oxide nanoparticles compared to macroscopic aluminum oxide as a function of incubation time in rabbit erythrocytes (mean ± SD of at least three independent experiments)

Figure 4 Behavior of human (▲), rabbit (●) and rat (■) erythrocytes exposed to different aluminum oxide nanoparticles and macroscopic aluminum oxide

Figure 5 Hemolytic effect of aluminum oxide nanoparticles and micro-sized aluminum oxide expressed as HC_{50} in human, rat and rabbit erythrocytes after 24 hours of incubation

Figure 6 Effect of albumin addition on the hemolysis induced by Al_2O_3 nano-forms and micro-sized Al_2O_3 on rabbit red blood cells

Figure 7 Effect of temperature of incubation on the HC_{50} induced by Al_2O_3 nano-forms and micro-sized Al_2O_3 on human red blood cells

Figure 8 Correlation between the decrease in HC_{50} and the decrease in Al_2O_3 nanoparticle size

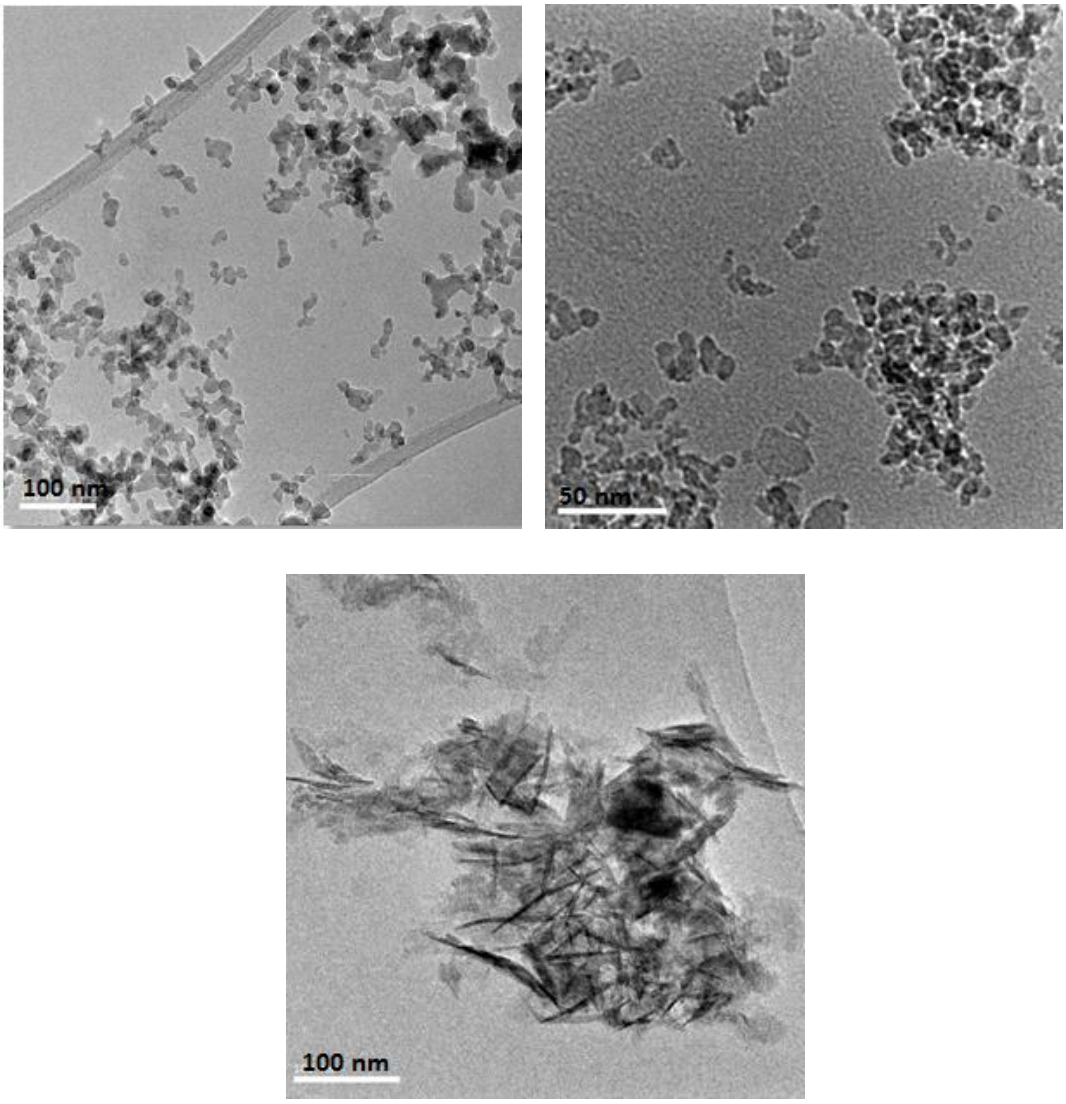


Figure 1

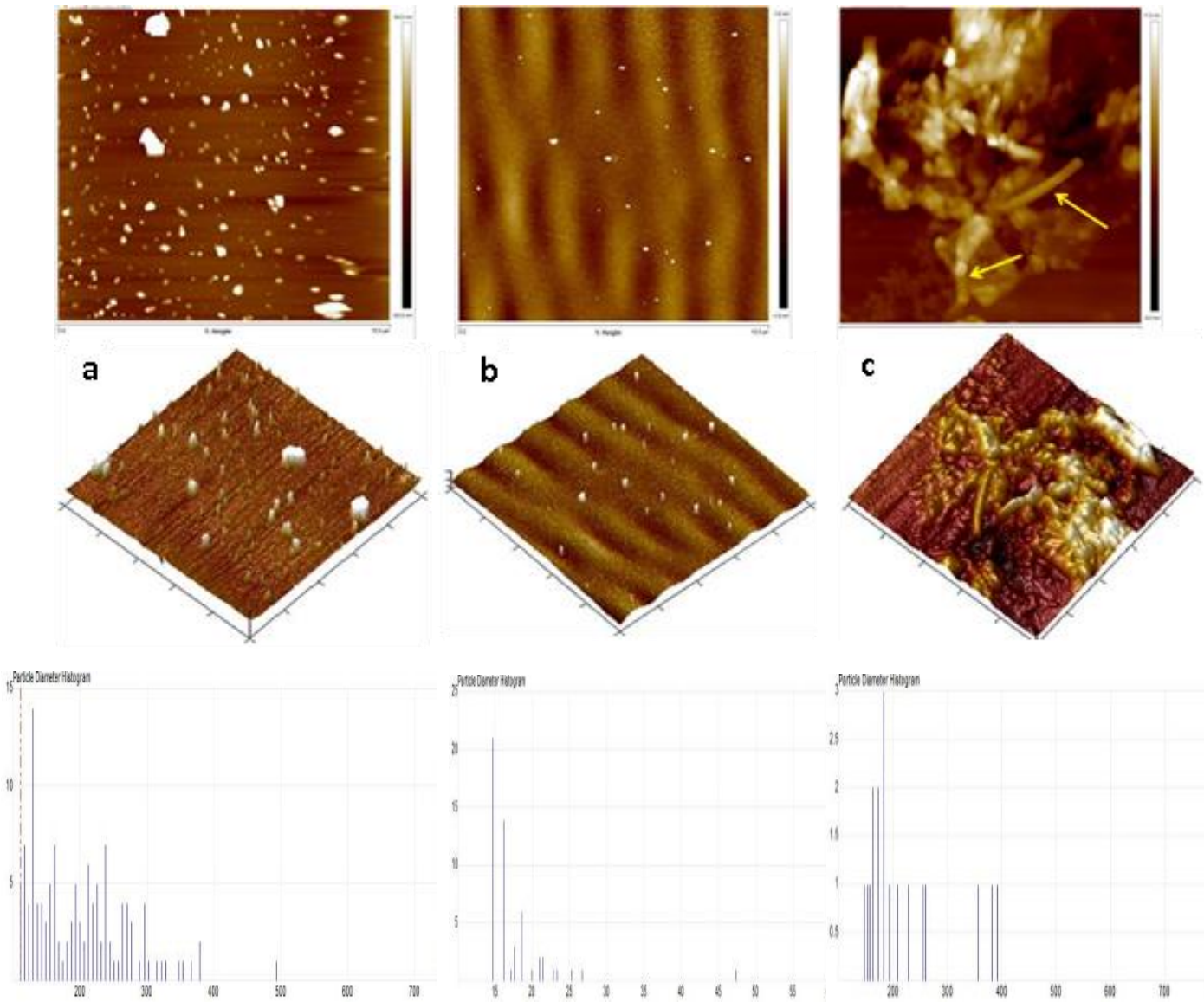
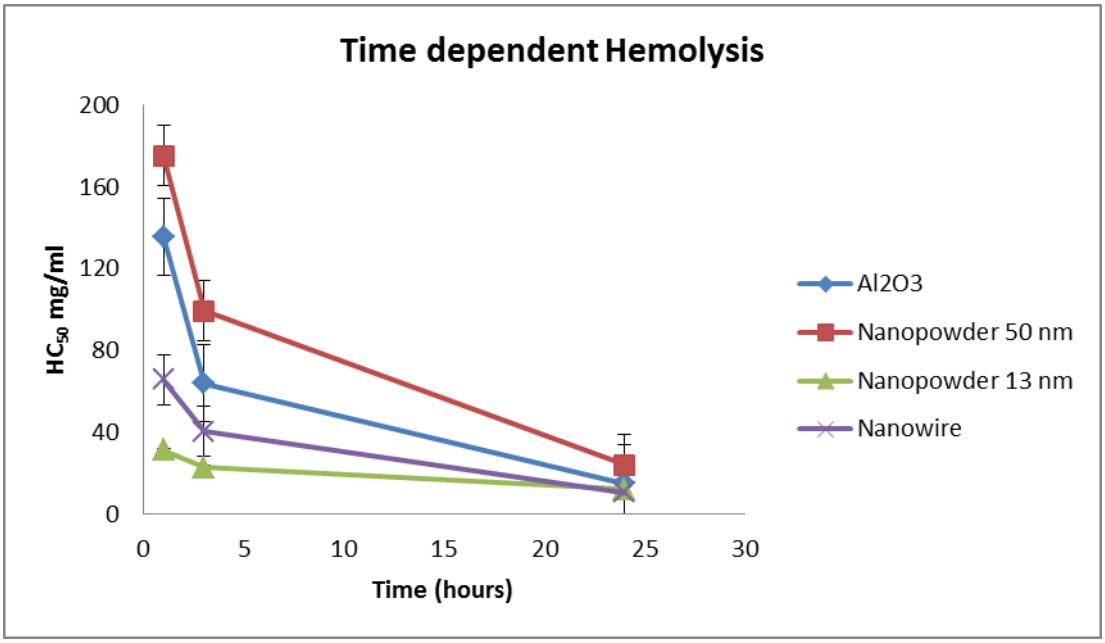

a
b
c
Particle Diameter Histogram
Particle Diameter Histogram
Particle Diameter Histogram

Figure 2


Time dependent Hemolysis
200
160
120
80
40
0
HC50 mg/ml
0
5
10
15
20
25
30
Time (hours)
Al2O3
Nanopowder 50 nm
Nanopowder 13 nm
Nanowire

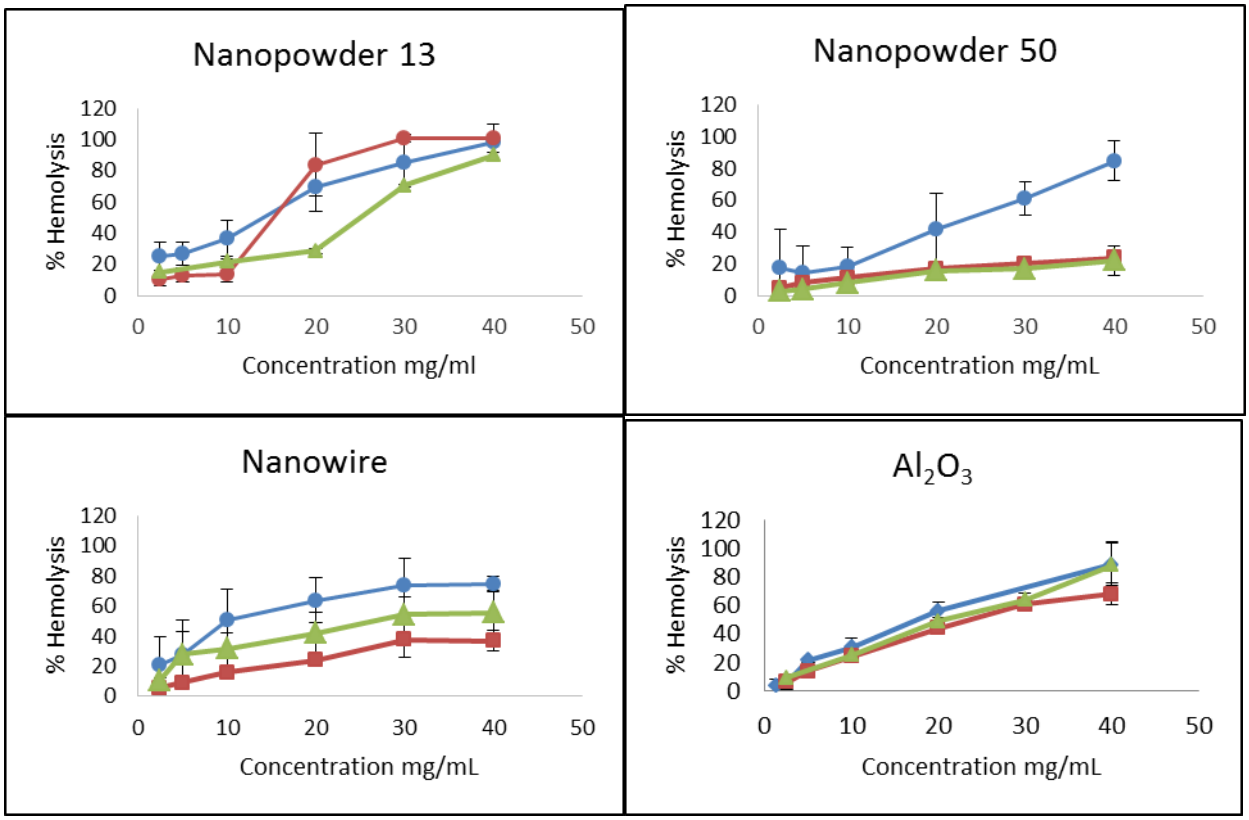

Nanopowder 13
% Hemolysis
120
100
80
60
40
20
0
0
10
20
30
40
50
Concentration mg/ml
Nanopowder 50
% Hemolysis
120
100
80
60
40
20
0
0
10
20
30
40
50
Concentration mg/mL
Nanowire
% Hemolysis
120
100
80
60
40
20
0
0
10
20
30
40
50
Concentration mg/mL
Al_2O_3
% Hemolysis
120
100
80
60
40
20
0
0
10
20
30
40
50
Concentration mg/mL

Figure 4

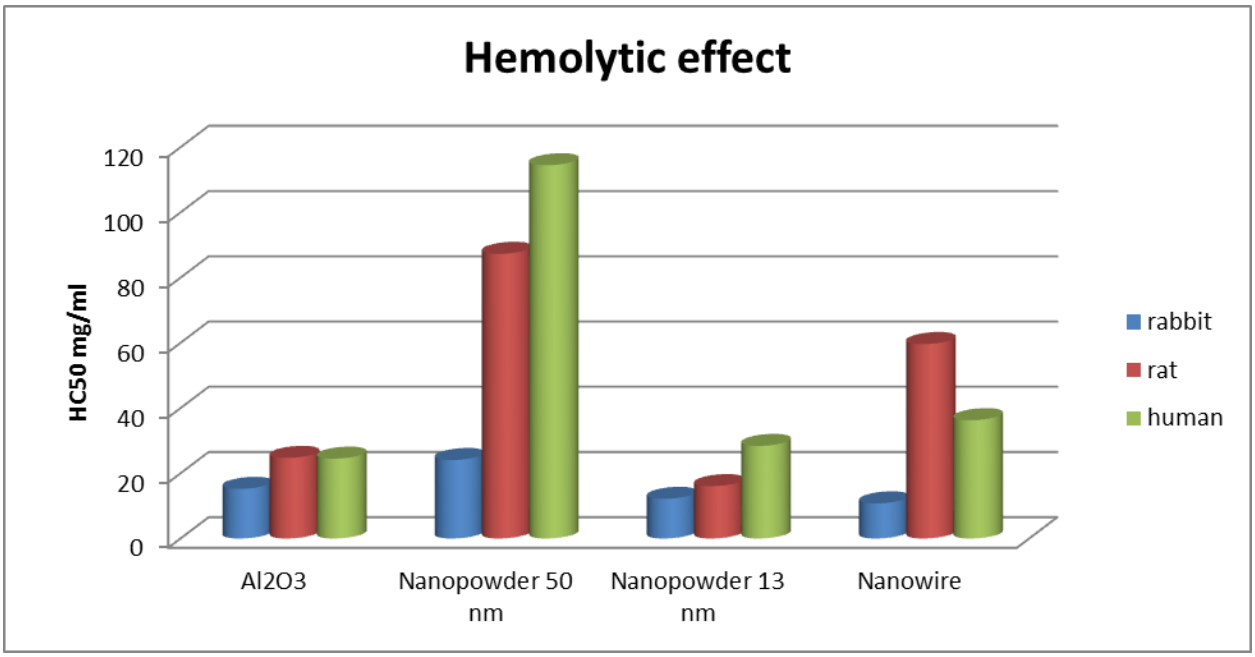

Hemolytic effect
HC50 mg/ml
120
100
80
60
40
20
0
Al2O3
Nanopowder 50 nm
Nanopowder 13 nm
Nanowire
rabbit
rat
human

Figure 5

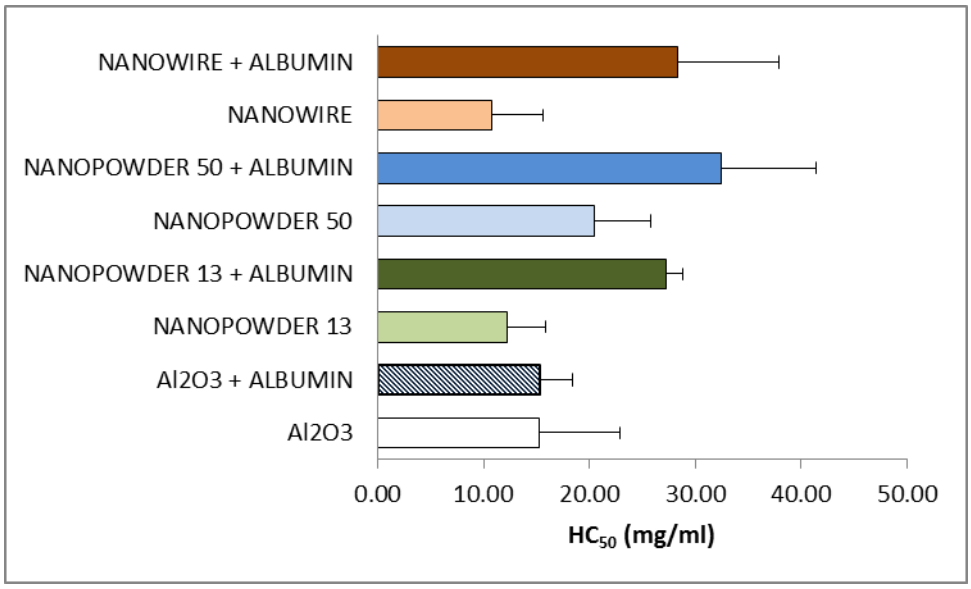

NANOWIRE + ALBUMIN
NANOWIRE
NANOPOWDER 50 + ALBUMIN
NANOPOWDER 50
NANOPOWDER 13 + ALBUMIN
NANOPOWDER 13
Al2O3 + ALBUMIN
Al2O3
0.00
10.00
20.00
30.00
40.00
50.00
HC50 (mg/ml)

Figure 6

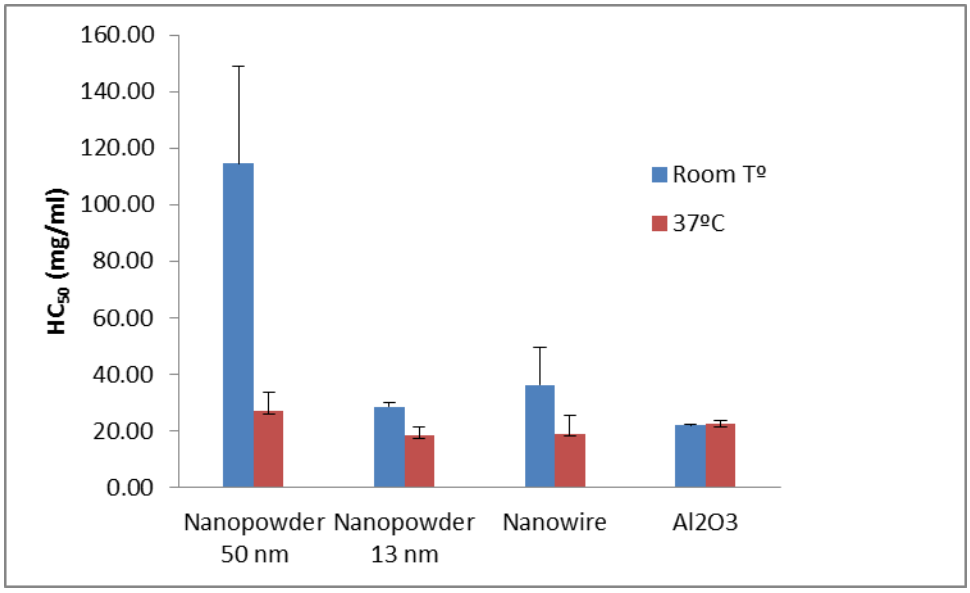

160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
HC50 (mg/ml)
Room Tº
37ºC
Nanopowder 50 nm
Nanopowder 13 nm
Nanowire
Al2O3

Figure 7

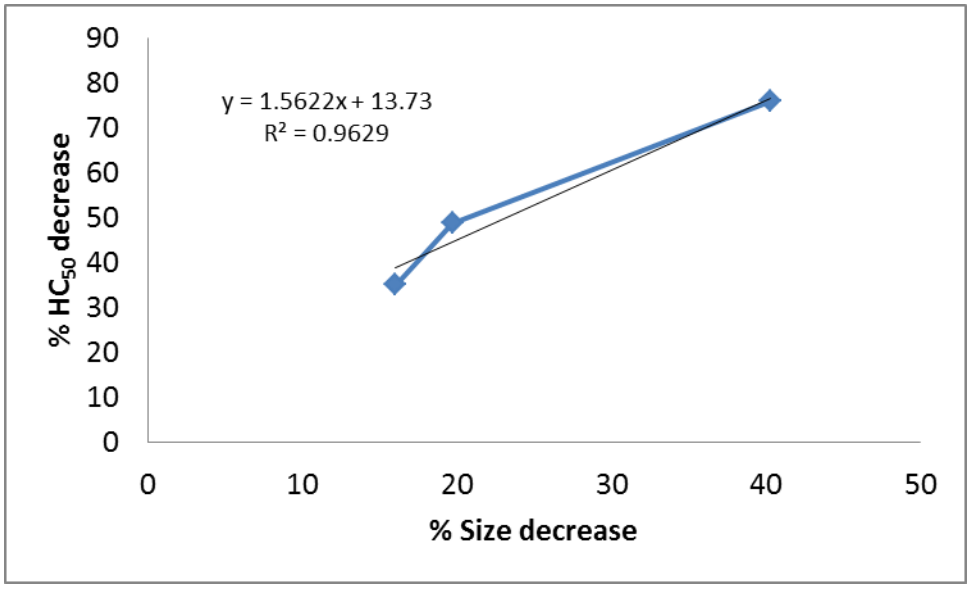

y = 1.5622x + 13.73
R² = 0.9629
% HC50 decrease
% Size decrease
0
10
20
30
40
50
60
70
80
90
0
10
20
30
40
50

Figure 8