Original Paper

Interaction of Amorphous Silica Nanoparticles with Erythrocytes *in Vitro*: Role of Oxidative Stress

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Key Words

Silica nanoparticles • Erythrocytes • Oxidative stress • Hemolysis

Abstract

Background/Aims: The use of engineered nanomaterials in the form of nanoparticles (NP) for various biomedical applications, as well as in consumer products, has raised concerns about their safety for human health. These NP are intended to be administered directly into the circulation following intravenous injection, or they may reach the circulation following other routes of administration such as oral or inhalation, and interact with circulating cells such as erythrocytes. However, little is known about the interaction of amorphous SiNP with erythrocytes. **Methods:** We studied the interaction of amorphous silica nanoparticles (SiNP) at various concentrations (1, 5, 25 and 125 µg/ml) with mouse erythrocytes *in vitro*. **Results:** Incubation of erythrocytes with SiNP caused a dose-dependent hemolytic effect. Likewise, the activity of lactate dehydrogenase was dose-dependently increased by SiNP. Transmission electron microscopy analysis revealed that SiNP are taken up by erythrocytes. Lipid erythrocyte susceptibility to *in vitro* peroxidation measured by malondialdehyde showed a significant and dose-dependent increase in erythrocytes. SiNP also enhanced the antioxidant activities of superoxide dismutase (SOD), catalase and reduced glutathione (GSH). Moreover, SiNP increased caspase 3, triggered annexin V-binding and caused a dose-dependent increase of cytosolic calcium concentration. **Conclusion:** It can be concluded that SiNP cause a dose-dependent hemolytic activity and are taken up by the erythrocytes. We also found that SiNP induce the occurrence of oxidative activity, apoptosis and increase cytosolic Ca²⁺, which may explain their haemolytic activity. Our *in vitro* data suggest that SiNP may, plausibly, lead to anemia and circulatory disorders *in vivo*.

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Introduction

Nanotechnology is an evolving science with the potential to manufacture and use engineered nanomaterials in the nanometer range (<100 nm) [1-3]. Nanoparticles (NP) have unique physicochemical properties, such as small size, large surface area to mass ratio, high reactivity, high carrier capacity and easy variation of surface properties [1-3]. They are being widely studied in various medical areas such as drug delivery, molecular diagnostics and gene therapy [1-3].

Amorphous silica nanoparticles (SiNP) are widely used in a variety of industries [3]. They are used as additives to cosmetics, drugs and food [3]. Moreover, they are currently tested for different medical applications, for instance drug delivery, cancer therapy and DNA transfection [3].

With medical applications, the intravenously injected SiNP can be distributed in the bloodstream, and cause the release of pharmacologically-active agents to targeted cells [3]. However, at the same time, NP can directly interact with circulatory cells such as erythrocytes, leukocytes or platelets and potentially induce undesirable toxicity [4-7]. Therefore, investigation of the direct impact of SiNP on erythrocytes is needed, and will help to understand the possible pathophysiological effects of those particles on erythrocytes.

It has been reported that exposure to particulate air pollution causes a decrease in erythrocyte antioxidant enzyme activity [8]. A decrease in erythrocytes in association with exposure to either particulate air pollution or nanoparticles in humans and experimental animals has been reported [9-11]. More recently, we showed that diesel exhaust particles (DEP) caused dose- and species-dependent erythrocyte hemolysis and oxidative stress [12].

Exposure of human platelets *in vitro* to amorphous SiNP induces oxidative effects leading to platelet aggregation [13]. We recently showed that intraperitoneal administration of SiNP causes proinflammatory and procoagulant responses *in vivo* and *in vitro* [14]. However, little is known about the interaction of amorphous SiNP with erythrocytes. It has only been demonstrated that SiNP cause hemolysis [15, 16].

The aim of this *in vitro* study was five-fold: (1) to evaluate the hemolytic activity of four graded doses of SiNP on mouse erythrocytes; (2) to assess the effect of SiNP on lactate dehydrogenase release by erythrocytes; (3) to evaluate whether SiNP are taken up by erythrocytes; (4) to appraise erythrocyte oxidant/antioxidant status following exposure to SiNP, including malondialdehyde (MDA), catalase, superoxide dismutase (SOD) and reduced glutathione (GSH) levels; and (5) to assess the effects of SiNP on erythrocytes cytosolic calcium concentration, annexin V and caspase 3.

Materials and Methods

Amorphous silica nanoparticles

Amorphous silica nanoparticles (50 nm) were purchased from Polysciences (Warrington, PA, USA). Their characteristics were recently studied [13, 17]. As indicated by the supplier (Polysciences, Warrington, PA, USA), the commercially available negative surface-charged silica nanoparticles used in our studies were amorphous solid (nonporous) pure silicon dioxide synthesized by a precipitation process. Using the same type of SiNP from the same source, Corbalan et al. [13, 17] confirmed the size stated by the supplier and found that the zeta potential of the silica nanoparticles was more negative than -30 mV.

Blood Collection

This project was reviewed and approved by our Institutional Review Board, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

TO mice (30–35 g, HsdOla:TO, Harlan, UK) were housed in light (12-h light:12-h dark cycle) and temperature-controlled (22 ± 1 °C) rooms. They had free access to commercial laboratory chow and were provided tap water *ad libitum*. For blood collection, the animals were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4 %).

Hemolysis experiments

The hemolysis experiment has been performed according to a previously described method [12, 18]. Mouse blood was mixed separately by gentle inversion of the tube and centrifuged at $1,200 \times g$ for 10 min. The plasma supernatant was discarded and the erythrocytes were washed 4 times by suspending them in saline (0.9%) before centrifugation at $1,200 \times g$ for 10 min. The final suspension consisted of 5% by volume erythrocyte in saline. Flat-bottomed 12-well plates were used. To each well, 900 μ l of erythrocyte suspension was added in duplicate. One hundred μ l SiNP suspensions (1, 5, 25 and 125 μ g/ml) were added, and mixed gently by pipetting. Negative control wells consisted of normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %), and positive control wells consisted of 0.1% Triton-X 100. The plates were then positioned in an MS 3 digital microtiter shaker (IKA WERKE GmbH & CO, Stauffer, Germany) and rotated at 300 rpm for 30 min at room temperature. After that, the samples were transferred into 1.5 ml eppendorf tubes, and were centrifuged for 5 min. Finally, 90 μ l were carefully removed from each tube and transferred to a clean plate. The amount of hemoglobin released into the supernatant was determined spectrophotometrically at a wavelength of 540 nm. The percent hemolysis was calculated using the equation of a straight line, $y = mx + c$, where % hemolysis (x) = [optical density (y) – negative control optical density (c)] / [(positive control optical density – negative control optical density) / 100] (m) [12, 18].

Erythrocytes analysis by transmission electron microscopy (TEM)

In separate animals, to assess whether SiNP are internalized by erythrocytes, mouse erythrocytes were incubated for 30 min with either saline containing Tween 80 (0.01%) or SiNP, as described above. The samples were then collected and fixed in Karnovsky's fixative. Fixed cells were later rinsed in 0.1 M phosphate buffer, dehydrated in a graded series of ethanol concentrations, post-fixed in 1% OsO₄ in 0.1 M cacodylate buffer for 1 h, and embedded in Araldite. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy [19, 20].

Measurement of LDH and markers of oxidative stress

Nine hundred μ l of the erythrocyte suspension was incubated with SiNP (100 μ l) at final concentrations of 1, 5, 25 and 125 μ g/ml, in a 12-well plate. The erythrocytes treated with normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %) were taken as control. The plate was positioned in an MS 3 digital microtiter shaker (IKA WERKE GmbH & CO, Stauffer, Germany) and rotated at 300 rpm for 30 min at room temperature. At the end of the incubation period, the cell suspensions were centrifuged, and aliquots of the resulting supernatants were subjected to LDH assay and markers of oxidative stress. LDH activities were determined using standard clinical laboratory methods. Erythrocytes malondialdehyde (MDA) was quantified colorimetrically following its controlled reaction with thiobarbituric acid [21] with a commercially available kit (Cayman Chemicals, Ann Arbor, MI, USA). SOD activity (Cayman Chemicals, Ann Arbor, MI, USA), reduced GSH concentration (Sigma Chemicals, St. Louis, MO), and catalase activity (Cayman Chemicals, Ann Arbor, MI, USA) were analyzed spectrophotometrically according to methods described by the manufacturers.

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ was measured after incubation with either vehicle or various concentrations of SiNP, as described above, according to a previously described technique [22]. Briefly, erythrocytes were washed in Ringer solution and then centrifuged for 5 min at $1,200 \times g$. The erythrocyte pellets were resuspended with 2 ml of 5 mM Ringer and then added with 2 μ M of fura 2AM (Calbiochem; La Jolla, CA). The cells were then incubated at 37°C for 15 min. After that, the cell suspension was centrifuged for 5 min at $1,200 \times g$. The fura 2AM-loaded erythrocytes were resuspended in 2 ml Ringer solution containing 1 mM CaCl₂, and incubated 30 min in dark. Finally, Ca²⁺-dependent fluorescence intensity was then monitored with a fluorometer (model SFM 25, Kontron; Zurich, Switzerland) set at 340-nm excitation and 510-nm emission [23].

Caspase-3 activity assay

The activity of caspase-3 was determined using a Caspase-3 activity colorimetric assay kit according to the manufacturer's instruction (Invitrogen, MD, USA).

Fig. 1. Hemolytic action of silica nanoparticle (SiNP) in the incubation medium of erythrocytes isolated from the blood of mice. The results are expressed as % of positive control (0.1 % Triton-X 100). Data are mean $\pm$ SEM (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

Fluorescence-activated cell sorter (FACS) analysis of annexin V-binding

Cell membrane phospholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface was estimated from binding of fluorescent annexin V- fluorescein isothiocyanate (FITC). The preparation of samples for FACS analysis was performed according to a recently described method [24]. Annexin V-FITC was determined according to the manufacturer's instruction (BD Pharmingen, San Jose, CA, USA) using FITC annexin V apoptosis detection kit I.

Statistics

All statistical analyses were performed with GraphPad Prism Software version 5 (San Diego, CA, USA). To determine whether parameters were normally distributed, the Kolmogorov-Smirnov statistic normality test was applied. Comparisons between groups were performed by one way analysis of variance (ANOVA), followed by Dunnett's multiple range tests. LDH data, which were not normally distributed, were analyzed with the Kruskal-Wallis test followed by Dunn's multiple comparison test. *P*-values <0.05 were considered as significant. All the data in the figures were reported as mean $\pm$ SEM.

Results

Effect of SiNP on erythrocytes hemolysis

Incubation of erythrocytes with SiNP caused significant and dose dependent hemolytic effects (Fig. 1). The degree of hemolysis observed was 8% (1 $\mu\text{g/ml}$ SiNP), 13% (5 $\mu\text{g/ml}$ SiNP), 39% (25 $\mu\text{g/ml}$ SiNP) and 52% (125 $\mu\text{g/ml}$ SiNP).

Erythrocytes analysis by electron microscopy

Figure 2 illustrates the TEM analysis in control (A) and SiNP (B-H) exposed erythrocytes. The incubation of erythrocytes with SiNP (1-125 $\mu\text{g/ml}$) for 30 min revealed the presence of particles either around or within the erythrocytes (Fig. 1B-H).

Effect of SiNP on LDH activity

Figure 3 illustrates the effect of SiNP on the activity of LDH in mouse erythrocyte suspensions. LDH was used as a marker of erythrocytes damage. The incubation of erythrocytes with SiNP showed a dose-dependent increase in LDH activity. The effect was significant at 25 ($P<0.01$) and 125 $\mu\text{g/ml}$ SiNP ($P<0.0001$).

Effect of SiNP on concentrations of MDA and GSH and activities of catalase and SOD

Figure 4A represents the effect of DEP on the concentrations of MDA in mouse erythrocyte suspensions. MDA was used to assess the proneness of erythrocytes lipid to *in vitro* peroxidation. The incubation of erythrocytes with SiNP showed a significant and dose-dependent increase in MDA concentrations (Fig. 4A). The effect was statistically significant at 25 $\mu\text{g/ml}$ ($P<0.0001$) and 125 $\mu\text{g/ml}$ ($P<0.0001$).

Fig. 2. Transmission electron micrographs (TEM) of erythrocytes after *in vitro* incubation with saline containing either 0.01% of Tween 80 (A) or silica nanoparticles (SiNP) 1 $\mu\text{g}/\text{ml}$ (B), 5 $\mu\text{g}/\text{ml}$ (C and D), 25 $\mu\text{g}/\text{ml}$ (E and F) and 125 $\mu\text{g}/\text{ml}$ (G and H). The presence of SiNP either outside or within the erythrocytes is indicated by arrows. Scale bars in A, B, C, E and G: 2 μm . Scale bars in D, F and H: 1 μm .

Fig. 3. Effect of silica nanoparticles (SiNP) on lactate dehydrogenase (LDH) activity measured in the incubation medium of erythrocytes. Data are mean $\pm$ SEM (n=6 in each group). Statistical analysis by Kruskal-Wallis test followed by Dunn's multiple comparison test.

Figure 4B shows the effect of SiNP on the concentration of GSH. Compared with the control group, the concentration of GSH in erythrocytes was significantly and dose-dependently increased. The level of significance was achieved at all the studied SiNP doses, i.e. 1 $\mu\text{g}/\text{ml}$ ($P<0.05$), 5 $\mu\text{g}/\text{ml}$ ($P<0.0001$), 25 $\mu\text{g}/\text{ml}$ ($P<0.0001$) and 125 $\mu\text{g}/\text{ml}$ ($P<0.0001$). Likewise, compared with the control group, a significant increase of catalase activity was seen following the incubation of erythrocytes with 5 $\mu\text{g}/\text{ml}$ ($P<0.05$), 25 $\mu\text{g}/\text{ml}$ ($P<0.0001$) and 125 $\mu\text{g}/\text{ml}$ ($P<0.0001$) SiNP (Fig. 4C). Figure 4D illustrates the effect of SiNP on erythrocyte SOD activity. Incubation of erythrocytes with SiNP induced a dose-dependent increase of SOD, and the plateau/maximum effect was achieved at a 25 $\mu\text{g}/\text{ml}$ dose. The effects were significant at all the studied doses, i.e. 1 $\mu\text{g}/\text{ml}$ ($P<0.05$), 5 $\mu\text{g}/\text{ml}$ ($P<0.0001$), 25 $\mu\text{g}/\text{ml}$ ($P<0.0001$) and 125 $\mu\text{g}/\text{ml}$ ($P<0.0001$).

Fig. 4. Effect of silica nanoparticles (SiNP) on the concentrations of malondialdehyde (MDA, A) and reduced glutathione (GSH, B), and activities of catalase (C) and superoxide dismutase (SOD, D) measured in the incubation medium of erythrocytes. Data are mean $\pm$ SEM (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

Fig. 5. Effect of silica nanoparticles (SiNP) on caspase 3 activity measured in the incubation medium of erythrocytes. Data are mean $\pm$ SEM (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

Effect of SiNP on caspase 3 activity

The effect of SiNP on erythrocyte caspase 3 activity is represented in Figure 5. Compared with the control group, the incubation of erythrocytes with SiNP caused a significant increase of caspase 3 activity at all the studied doses ($P < 0.0001$). The maximum effect was already observed at 1 $\mu\text{g/ml}$ SiNP, and the effects were comparable between the various doses studied (1 $\mu\text{g/ml}$ –125 $\mu\text{g/ml}$).

Effect of SiNP on annexin V-binding

Exposure of phosphatidylserine at the cell surface was estimated from binding of fluorescent annexin V-FITC. Incubation of erythrocytes with SiNP triggered annexin V-binding (Fig. 6). The effect was significant at all the studied doses, i.e. 1 $\mu\text{g/ml}$ ($P < 0.005$), 5 $\mu\text{g/ml}$ ($P < 0.05$), 25 $\mu\text{g/ml}$ ($P < 0.0001$) and 125 $\mu\text{g/ml}$ ($P < 0.005$).

Fig. 6. The quantification of the FACS analysis of the apoptotic annexin V-fluorescein isothiocyanate (FITC) positive erythrocytes following the incubation with SiNP. FITC-annexin V binding to erythrocytes is given as a percentage of annexin V-positive cells. Data are mean $\pm$ SEM (n=3-5 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

Fig. 7. Effect of silica nanoparticles (SiNP) on cytosolic calcium concentration from Fluo3 fluorescence measured in the incubation medium of erythrocytes. Data are mean $\pm$ SEM (n=6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

Effect of SiNP on intracellular calcium

Figure 7 illustrates the effect of SiNP on cytosolic calcium concentration from Fluo3 fluorescence. Compared with the control group, the incubation of erythrocytes with SiNP caused a significant increase in cytosolic calcium concentration. The level of significance was achieved at 25 $\mu\text{g/ml}$ ($P < 0.0001$) and 125 $\mu\text{g/ml}$ ($P < 0.0001$).

Discussion

In this work, we show that SiNP are able to penetrate erythrocytes, and induce oxidative stress, apoptosis and increase cytosolic Ca^{2+} leading to hemolysis.

Several studies have reported that NP may have unpredicted biological effects compared with larger particles [1, 3]. It has been reported in human endothelial cells that monodispersed SiO_2 -NP caused cytotoxic cell damage and decreased cell survival in a concentration-dependent manner [25]. SiO_2 -NP with sizes of 14, 15, and 16 nm exhibited higher toxicity as compared with larger SiO_2 -NP of 104 and 335 nm [25]. We have recently demonstrated that amorphous SiNP cause systemic inflammation, coagulation events and alter vascular reactivity. The effects observed with 50 nm SiNP were more pronounced than those with 500 nm SiNP [14].

The use of nanoparticles can happen via different routes such as skin penetration, ingestion and inhalation. It has been previously reported that SiNP could penetrate the skin and enter various tissues [26]. Even after inhalation, nanoparticles have been reported to rapidly translocate to the systemic circulation and reach different organs [4, 5, 27, 28]. Moreover, with medical applications, following injection, nanoparticles can be distributed by the bloodstream and affect vascular homeostasis [3]. Therefore, studies on the interaction of nanoparticles with erythrocytes are essential.

In vivo studies in mice using intravenously injected SiNP reported doses of 29.5-177.5 mg/kg [29] and 10-30 mg/kg [30]. For a 25 g mouse, this would correspond to 737-4437 μg and 250-750 μg , respectively. The approximate blood volume of a mouse is 77-80 $\mu\text{l/g}$, and for a 25 g mouse this is equivalent to 1.9-2.0 ml. Therefore, we assume that the concentration of intravenously injected SiNP in blood would be 368-2,218 $\mu\text{g/ml}$ [29] and 125-375 $\mu\text{g/ml}$

[30], respectively. The SiNP concentrations that we used here were 1, 5, 25 and 125 $\mu\text{g/ml}$ in a final suspension consisting of 5% erythrocytes. The hematocrit measured after blood collection in mice was on average 35 %. Thus, by taking into account the dilution factor (i.e. 7), we assume that SiNP concentrations used would be 7, 35, 175 and 875 $\mu\text{g/ml}$ in a final suspension consisting of 35% of erythrocytes. Regarding human exposure scenario (e.g. nanomedicine), pharmacokinetic data related to the liposome-encapsulated doxorubicin nanoparticles, used in cancer therapy, established plasma concentrations of more than 10 $\mu\text{g/ml}$ [31]. Therefore, at least one concentration used in our in vitro study is probably relevant to mice and human exposures.

Our data show that SiNP caused a significant and dose-dependent hemolytic activity. Our findings corroborate recent studies which reported that SiNP induce hemolysis [15, 16]. We have recently reported that DEP caused dose- and species-dependent erythrocyte hemolysis [12]. Moreover, a direct hemolytic effect of TiO_2 nanoparticles has been previously reported in rabbit erythrocytes [32]. In the present study, LDH activity was used as a marker of erythrocytes damage and hemolysis. In line with the hemolysis findings, LDH activity was significantly and dose-dependently increased following the incubation with SiNP.

In the present study, because of the observed hemolytic effect of SiNP, we also wanted to verify whether and to what extent SiNP can penetrate the erythrocytes. To this end, we performed TEM analysis of erythrocytes following the incubation with SiNP. Our data show the presence of SiNP within the erythrocytes. We have recently reported that DEP are taken up by erythrocytes [12]. Following inhalation, TiO_2 nanoparticles were localized within blood capillaries and erythrocytes [33]. In addition, 100 nm MCM-41-type mesoporous silica nanoparticles were found to adsorb to the surface of erythrocytes [34].

It is well-established that SiNP cause hemolysis [15, 16]. Nevertheless, the mechanism related to this effect is poorly understood. In the current study, we assessed the effect of SiNP on the biomarkers of oxidative stress, including MDA, GSH, SOD and catalase. Following the occurrence of oxidative stress, erythrocyte membranes are susceptible to lipid peroxidation that involves cleavage of polyunsaturated fatty acids at their double bonds leading to the formation of aldehydes, MDA, and 4-hydroxynonenal [21]. These derivative substances may modify the structural and functional integrity of cells [21]. The measurement of MDA is customarily used as a method for the quantification of lipid peroxidation [21]. Our data corroborate the hemolysis findings; erythrocytes incubated with SiNP exhibited a significant and dose-dependent increase in MDA. This suggests that SiNP induced oxidative stress to the erythrocyte; the plasma membrane is often the initial site of damage, and the resulting peroxidation of membrane lipids causes hemolysis and cross-links between protein and lipid molecules to different extents [35]. We have recently reported that incubation of erythrocytes with DEP (1-100 $\mu\text{g/ml}$) caused a significant and dose-dependent increase in MDA in erythrocytes of rats, but not humans or mice [12]. The last finding was explained by the fact that human and mouse erythrocytes are resistant to peroxidation of membrane lipids. The results of the present study shows a significant effect at 25 and 125 $\mu\text{g/ml}$ SiNP; such an effect was absent in our previous study using mouse erythrocytes incubated with up to 100 $\mu\text{g/ml}$ DEP [12], suggesting that the pro-oxidant potency of engineered SiNP is higher than that of pollutant DEP.

Erythrocytes contain intracellular antioxidant enzymes such as SOD, catalase and GSH, that work together to prevent oxidative damage to the cell [35]. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, while catalase converts hydrogen peroxide into water [35]. GSH is a sensitive biomarker of cell functionality and viability, and is implicated in the cellular defence against hydroperoxides and free radicals [35]. Our data show a significant and dose dependent increase in SOD and catalase activities, and GSH concentrations. This indicates that the development of oxidative stress induced by SiNP is accompanied by an adaptive response that counterbalances the potentially damaging activity of oxygen free radicals by antioxidant defence mechanisms. We have reported an increase of antioxidant in various organs, including the lungs, heart liver and kidney following short-term exposure to cigarette smoke [36, 37].

Caspases are aspartate-specific cysteine proteinases that exist as latent zymogens, but once activated by apoptotic signals, they promote apoptosis. Caspase 3 is synthesized as a 32 kDa zymogen that is processed to mature 20/17 kDa and 12 kDa subunits by cleavage at Asp9, Asp28 and Asp175 [38]. It is well established that erythrocytes express caspases [38, 39]. In the present study, we found that incubation of erythrocytes with SiNP stimulates caspase 3. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing phosphatidylserine to the external cellular environment [40]. Annexin V is a Ca_2^+ dependent phospholipid-binding protein that has a high affinity for phosphatidylserine, and binds to cells with exposed PS [40]. Our data show that exposure of erythrocytes to SiNP triggered annexin V-binding. This finding corroborates the increase of caspase 3 and confirms the occurrence of apoptosis following the exposure to SiNP. The last could be the consequence of the oxidative stress induced by SiNP. Indeed, it has been previously reported that caspases are activated by oxidative stress [41].

Erythrocytes do not have nuclei and mitochondria, which are crucial components in the apoptotic process. Despite that, it has been shown that exposure of erythrocytes to the Ca^{2+} ionophore ionomycin causes cell shrinkage, membrane blebbing and phosphatidylserine exposure, all typical features of apoptotic nucleated cells [40]. Our data show a significant increase in erythrocyte cytosolic calcium activity following incubation with SiNP. It has been reported that eryptosis (suicidal erythrocyte death) could be triggered by increased calcium activity, and that calcium may enter through non-selective cation channels which can be activated by oxidative stress [40, 42, 43]. Thus, we can speculate that the increase in oxidative stress caused by SiNP may have induced an increase in cytosolic calcium and subsequent erythrocyte death. Additional studies are required to clarify this issue.

It can be concluded that SiNP cause a dose-dependent hemolytic activity and are taken up by the erythrocytes. We also found that SiNP induce oxidative activity and apoptosis, and increase cytosolic Ca^{2+} , which may explain the hemolysis induced by these nanoparticles. Surface modification/functionalization, and protein corona need in-depth evaluations. Recently, It has been shown that protein corona can significantly modify the responses to biomedical nanoparticles. It would be of interest, in our future experiments to investigate further the role of oxidative stress by using antioxidants and that of calcium using calcium chelators.

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Disclosure Statement

No conflicts of interest to disclose.

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