

Amorphous silica nanoparticles aggregate human platelets: potential implications for vascular homeostasis

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Background: Amorphous silica nanoparticles (SiNP) can be used in medical technologies and other industries leading to human exposure. However, an increased number of studies indicate that this exposure may result in cardiovascular inflammation and damage. A high ratio of nitric oxide to peroxynitrite concentrations ($[NO]/[ONOO^-]$) is crucial for cardiovascular homeostasis and platelet hemostasis. Therefore, we studied the influence of SiNP on the platelet $[NO]/[ONOO^-]$ balance and platelet aggregation.

Methods: Nanoparticle–platelet interaction was examined using transmission electron microscopy. Electrochemical nanosensors were used to measure the levels of NO and $ONOO^-$ released by platelets upon nanoparticle stimulation. Platelet aggregation was studied using light aggregometry, flow cytometry, and phase contrast microscopy.

Results: Amorphous SiNP induced NO release from platelets followed by a massive stimulation of $ONOO^-$ leading to an unfavorably low $[NO]/[ONOO^-]$ ratio. In addition, SiNP induced an upregulation of selectin P expression and glycoprotein IIb/IIIa activation on the platelet surface membrane, and led to platelet aggregation via adenosine diphosphate and matrix metalloproteinase 2-dependent mechanisms. Importantly, all the effects on platelet aggregation were inversely proportional to nanoparticle size.

Conclusions: The exposure of platelets to amorphous SiNP induces a critically low $[NO]/[ONOO^-]$ ratio leading to platelet aggregation. These findings provide new insights into the pharmacological profile of SiNP in platelets.

Keywords: amorphous silica nanoparticles, nanotoxicology, nitric oxide, peroxynitrite, platelet aggregation

Introduction

It is increasingly recognized that engineered amorphous silica nanoparticles (SiNP) may have important implications for vascular homeostasis. We have recently demonstrated the ability of SiNP to induce endothelial cell dysfunction linked to increased peroxynitrite ($ONOO^-$) production.¹ As human blood platelets along with endothelium play a critical role in vascular homeostasis, we have now examined the biocompatibility of amorphous SiNP (10 nm, 10SiNP; 50 nm, 50SiNP; 150 nm, 150SiNP; 500 nm, 500SiNP) with human platelets.

The vascular NO, synergistically with prostacyclin, inhibits platelet activation and aggregation.^{2–5} This vasorelaxant and platelet regulator is essential for vascular homeostasis and an impairment of NO bioavailability is one of the earliest events in vascular diseases.^{6,7} Superoxide anion reacts rapidly with NO producing $ONOO^-$, a major component of oxidative stress, rapidly decreasing NO bioavailability.⁸

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A high ONOO^- production and depleted NO availability resulting in a low ratio of maximal NO to maximal ONOO^- concentrations ($[\text{NO}]/[\text{ONOO}^-]$) can be accurately used as an indicator of nitroxidative/oxidative stress and dysfunction of the cardiovascular system.^{1,9–11} The high $[\text{NO}]/[\text{ONOO}^-]$ balance is essential for maintaining platelet regulation, as high amounts of ONOO^- counteract the platelet-inhibitory effects of NO leading to platelet activation and aggregation.¹²

In the current studies, a direct release of NO and ONOO^- during nanoparticle-platelet interactions was measured electrochemically by nanosensors. The NO generation by platelets was followed by a greater ONOO^- production leading to a low $[\text{NO}]/[\text{ONOO}^-]$ ratio. Indeed, we demonstrated that amorphous SiNP induced activation of the glycoprotein IIb/IIIa (GPIIb/IIIa) and expression of the selectin P (SELP) on the platelet surface membrane leading to platelet activation and aggregation. The activation of GPIIb/IIIa is triggered off by the release of major platelet activators that mediate pathways of aggregation such as thromboxane A_2 (TXA_2),¹³ adenosine diphosphate (ADP)¹⁴ and matrix metalloproteinase 2 (MMP2)¹⁵ which amplify aggregation. Our studies showed that SiNP induce platelet aggregation via stimulation of ADP and MMP2 released from platelets.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich (Arklow, Ireland) unless otherwise indicated. Engineered amorphous SiNP with different sizes (10 nm, 50 nm, 150 nm, and 500 nm) were purchased from Polysciences (Eppelheim, Germany).

Zeta potential measurement

The zeta potentials of all the SiNP were determined by a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Measurements were performed six times for each particle size (100 $\mu\text{g/mL}$ in ultrapure water) at room temperature.

Transmission electron microscopy (TEM) of SiNP

Nanoparticle suspensions (10 $\mu\text{g/mL}$ in ultrapure water) were examined by a JEM 2100 transmission electron microscope (JEOL, Herts, UK). The diameter of 100 nanoparticles of each size on three TEM micrographs was measured by ImageJ an open-source, Java-based imaging software (<http://rsbweb.nih.gov/ij/>).

Isolation of human platelets

Blood was obtained from healthy volunteers who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Washed platelets were obtained as described previously.¹⁶ Briefly, blood was collected and anticoagulated using trisodium citrate (3.15% w/v; 9:1 v/v). The citrated blood was centrifuged at room temperature to prepare platelet-rich plasma (PRP). Washed platelets were prepared by centrifugation of PRP, containing prostacyclin at room temperature. Following isolation, platelets were resuspended in prostacyclin-free Tyrode's salt solution. Washed platelet concentrations were adjusted using Tyrode's salt solution to a final concentration of 2.5×10^8 platelets per milliliter.

Aggregation studies

Immediately before use, commercial stock suspensions of SiNP were sonicated for 2 minutes and nanoparticle dispersions in Tyrode's salt solution were prepared by serial dilution from these stock suspensions by vortexing. We tested the effect of different SiNP (10SiNP, 50SiNP, 150SiNP and 500SiNP) at different concentrations (1 to 200 $\mu\text{g/mL}$).

Platelets were incubated for 2 minutes at 37°C in a whole blood/optical lumi-aggregometer (model 700; Chrono-Log, Manchester, UK) prior to the addition of nanoparticles to test spontaneous aggregation. Platelet aggregation was studied for 15 minutes against blanks (Tyrode's salt solution) containing the same particle concentration as the test tubes. Collagen was used as a positive control for platelet aggregation studies. Aggregation data was analyzed by Chrono-Log AGGRO-LINK 8 software.^{17–19}

Selective platelet aggregation inhibitors (acetylsalicylic acid [ASA], phenanthroline, and apyrase) were added to test tubes. After 1 minute, SiNP were added to the appropriate test tubes.

TEM of human platelets

Platelet aggregation was terminated at 30% maximal response, as determined using an aggregometer, in platelets exposed to 10 $\mu\text{g/mL}$ 10SiNP. Unstimulated (resting) platelets were also prepared. Samples were fixed by mixing them with an equal volume of 3% glutaraldehyde in 0.1 M phosphate buffer for 1.5 hours at room temperature. After primary fixation, samples were centrifuged and pellets washed six times with phosphate buffer. Then, pellets were post-fixed with 2% osmium tetroxide in phosphate buffer for 30 minutes. Pellets were then dehydrated in a series of graded ethanol solution, infiltrated and embedded in agar 100 epoxy resin, using propylene oxide as a transitional fluid. Next, ultrathin sections

were cut from dried blocks with a diatom diamond knife on a LKB ULTRATOM III (LKB, Uppsala, Sweden), stained with 0.5% aqueous uranyl acetate followed by Reynold's lead citrate. Finally, sections were examined by a JEM 2100 transmission electron microscope.

Measurement of NO and ONOO⁻ concentrations using nanosensors

Concurrent NO and ONOO⁻ measurements were performed with electrochemical nanosensors (diameter: 300–500 nm). The designs are based on previously developed and well-characterized chemically modified carbon-fiber technology.^{20,21} Each of the sensors was made by depositing a sensing material on the tip of the carbon fiber: conductive film of polymeric nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrinic for the NO sensor²⁰ and a polymeric film of Mn (III)–paracyclophanyl-porphyrin for the ONOO⁻ sensor²². Amperometry was used to measure changes in NO and ONOO⁻ concentrations from its basal level with time (detection limit of 1 nM and resolution time <10 μs for each sensor). Linear calibration curves were constructed for each sensor from 5 nM to 3 μM before and after measurements with aliquots of NO and ONOO⁻ standard solutions, respectively. Measurements were performed into platelet suspensions. We tested the effect of different SiNP (10SiNP, 50SiNP, 150SiNP, and 500SiNP) at different concentrations (1 to 200 μg/mL).

Selectin P and GPIIb/IIIa analyses by flow cytometry

Flow cytometry was performed using a FACSAArray™ bioanalyzer (Becton, Dickinson and company [BD], Oxford, UK) on single stained platelet samples as described previously.^{17,23} Briefly, to analyze SELP expression, platelet aggregation was terminated at 30% maximal response, as determined using an aggregometer, in platelet exposed to different SiNP (10SiNP, 50SiNP, 150SiNP, and 500SiNP) at different concentrations (1 to 200 μg/mL). Platelet suspensions and anti-selectin-P antibody (BD) were then mixed and incubated in the dark at room temperature for 5 minutes. Platelets were identified by forward and side scatter signals, and 10,000 platelet-specific events were analyzed by the flow cytometer for fluorescence.

To analyze GPIIb/IIIa activation, platelet aggregation was terminated at 30% maximal response, as determined using an aggregometer, in platelets exposed to different SiNP as above. Platelet suspensions and PAC-1 antibody (BD) were mixed and incubated in the dark at room temperature

for 15 minutes. Afterwards, an APC Rat Anti-Mouse IgM (secondary antibody) was added and samples incubated in the dark at room temperature for another 15 minutes. Platelets were identified by forward and side scatter signals, and 10,000 platelet-specific events were analyzed by the flow cytometer for fluorescence.

Nonactivated and activated platelets were gated so as not to analyze platelet aggregates. Silica nanoparticles were not detected by the bioanalyzer.

Statistical analyses

All data are presented as group of means ± standard error of the mean of $n > 3$. Statistical analysis of the mean difference between multiple groups was determined by one-way ANOVA followed by Tukey-Kramer multiple comparison tests; and between two groups by two-tailed Student's *t*-tests. The alpha level for all tests was 0.05. A *P* value < 0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego, CA) and Origin (version 6.1 for Windows; OriginLab, Northampton, MA).

Results

Nanoparticle characterization

In order to confirm the commercially-provided nanoparticle size, we used TEM to measure the size of SiNP. The TEM analysis of 10SiNP, 50SiNP, 150SiNP, and 500SiNP resulted in sizes of 10.50 ± 0.19 nm, 52.67 ± 0.66 nm, 148.20 ± 2.19 nm, and 495.90 ± 5.87 nm, respectively.¹ Furthermore, Zetasizer reported high negative zeta potentials (more negative than −30 mV) for all SiNP tested in ultrapure water.¹

Silica nanoparticle–platelet interaction

We studied nanoparticle–platelet interactions and uptake using TEM (Figure 1). Electron micrographs showed amorphous SiNP forming agglomerates. These agglomerates were located within platelet aggregates, dispersed among platelets and interacting with the plasma membrane (Figure 1B–D). Nanoparticles also internalized and distributed into the platelet cytoplasm (Figure 1C and D). We also observed degranulated platelets indicating platelet activation (Figure 1C and D).

Silica nanoparticles induce a [NO]/[ONOO⁻] imbalance in platelets

Using electrochemical nanosensors, we examined in situ, real-time dynamic interaction of amorphous SiNP with human platelets by measuring directly NO and ONOO⁻

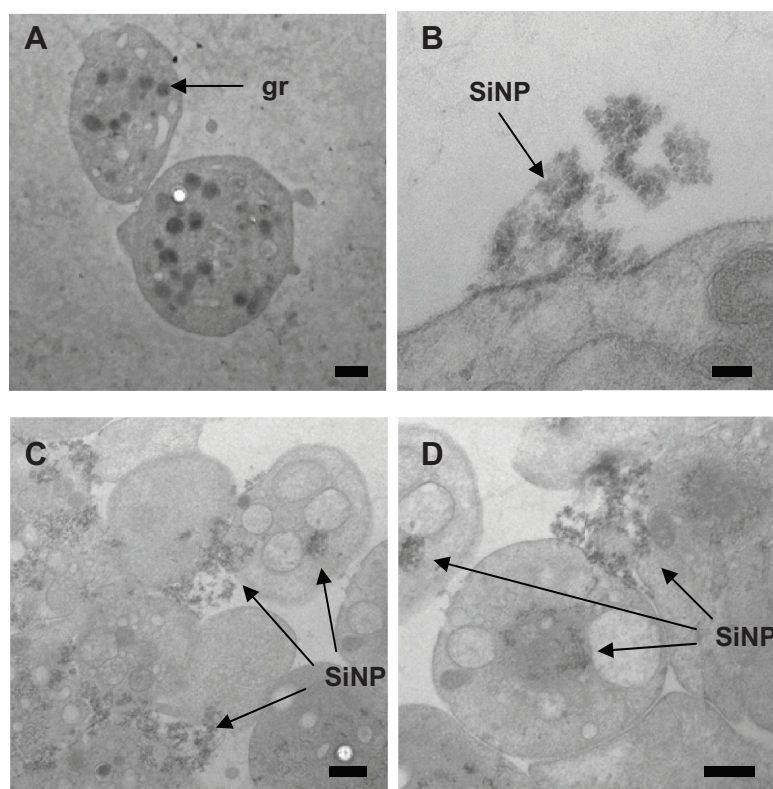


Figure 1 Amorphous SiNP interact with human platelets. As shown by TEM, after exposure of human platelets to 10SiNP (10 $\mu\text{g/mL}$), nanoparticles rapidly interacted with the platelet surface membrane (**B**) and induced aggregation (**C** and **D**). Unstimulated (resting) platelets were also presented (**A**).

Notes: Scale bars represent 500 nm in **A**, **C**, and **D**; and 100 nm in **B**.

Abbreviations: SiNP, silica nanoparticles; gr, platelet granule; TEM, transmission electron microscopy.

concentrations in a platelet suspension. We demonstrated with time resolution better than 10 μs that after collision with platelets, these nanoparticles rapidly stimulated NO release (Figure 2B) followed by a greater production of ONOO⁻. An increased ONOO⁻ production was observed when nanoparticle concentration was increased and size decreased (Figure 2C). The exposure of human platelets to SiNP induced an unfavorable shift of the ratio of [NO]/[ONOO⁻], as nanoparticle size decreased. Indeed, 10 $\mu\text{g/mL}$ 10SiNP induced a greater decrease in [NO]/[ONOO⁻] than an equal concentration of 50SiNP (Figure 2D). Moreover, 50SiNP (100 $\mu\text{g/mL}$) induced a lower ratio than an equal concentration of larger nanoparticles (Figure 2E).

Silica nanoparticles induce platelet aggregation

We studied the ability of SiNP to induce platelet aggregation. Our results demonstrate that amorphous SiNP induce activation of GPIIb/IIIa as well as expression of SELP in the platelet surface membrane (Figure 3A and B). Importantly, platelet receptor activation and expression was inversely proportional to nanoparticle size and directly proportional

to particle concentration. Indeed, the smallest nanoparticles (10SiNP) induced GPIIb/IIIa activation and SELP expression when used at a low concentration (10 $\mu\text{g/mL}$). In contrast, an equal concentration of larger nanoparticles (50SiNP, 150SiNP, and 500SiNP) did not influence platelet receptors. However, when used at a high concentration (100 $\mu\text{g/mL}$), 50SiNP induced both GPIIb/IIIa activation and SELP expression. Moreover, 200 $\mu\text{g/mL}$ 150SiNP and 500SiNP induced SELP expression.

Furthermore, we demonstrated that amorphous SiNP directly induce platelet aggregation and that this effect is greater when nanoparticle size decreases and particle concentration increases (Figure 3C). Indeed, 10SiNP induced platelet aggregation when used at a concentration of 10 $\mu\text{g/mL}$ or higher. Also, 50SiNP and 150SiNP induced platelet aggregation when used at a concentration of 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$, respectively. However, 500SiNP did not induce platelet aggregation even when used at a very high concentration of 200 $\mu\text{g/mL}$.

Knowing that 10SiNP at 10 $\mu\text{g/mL}$ were able to induce platelet aggregation, further experiments with selective inhibitors of the pathways leading to platelet aggregation mediated

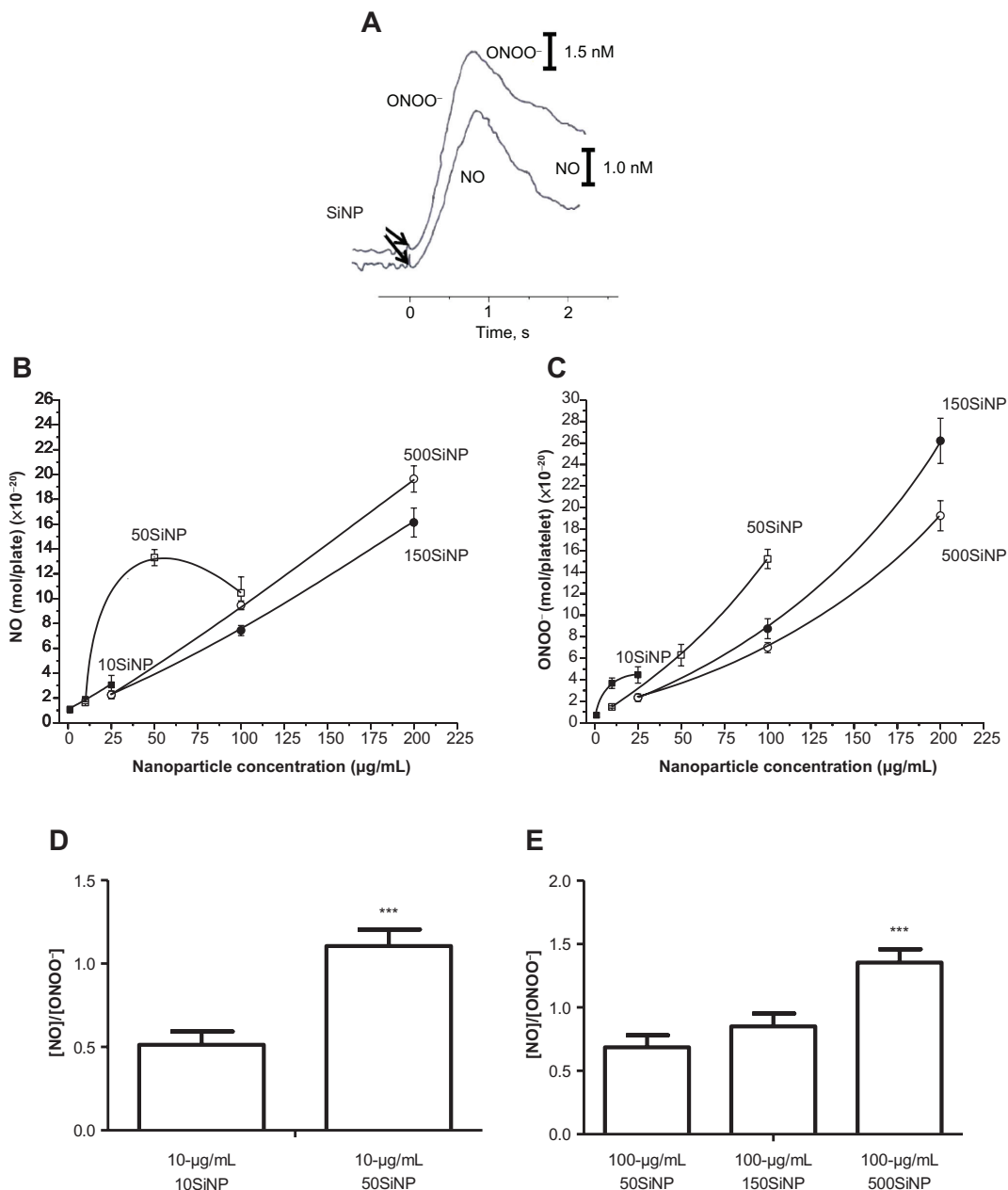


Figure 2 Amorphous SiNP induce NO and ONOO⁻ production and unfavorably shift the [NO]/[ONOO⁻] balance in human platelets. **(A)** Representative recordings of NO and ONOO⁻ measured in situ by specific nanosensors after the addition of 10 µg/mL 10SiNP to the platelet suspension (2.5×10^8 platelets per milliliter). **(B and C)** Maximal NO **(B)** and ONOO⁻ **(C)** release from a single platelet following stimulation by SiNP: 10SiNP (■), 50SiNP (□), 150SiNP (●), 500SiNP (○). **(D and E)** The [NO]/[ONOO⁻] ratio of the maximal NO and ONOO⁻ concentrations measured upon stimulation of platelets by SiNP at 10 µg/mL **(D)** and 100 µg/mL **(E)**.

Notes: All values are mean \pm standard error of the mean (SEM) of $n = 4$. One-way ANOVA and Tukey-Kramer multiple comparison tests were used for analysis **(B and C)**. All values are mean \pm SEM of $n = 4$. Two-tailed Student's t -test: *** $p < 0.001$ compared with 10 µg/mL 10SiNP **(D)**. One-way ANOVA and Tukey-Kramer multiple comparison test: *** $p < 0.001$ compared with 100 µg/mL 50SiNP **(E)**.

Abbreviations: SEM, standard error of the mean; SiNP, silica nanoparticles.

by TXA₂ (ASA), ADP (apyrase), and MMP2 (phenanthroline) were carried out to characterize the mechanism of SiNP-induced platelet aggregation (Figure 3D). Our results show that incubation of platelets with both phenanthroline and apyrase, but not ASA, prior to their exposure to nanoparticles, inhibited nanoparticle-induced platelet aggregation (Figures 3D and 4).

Discussion

Platelets and endothelial cells are crucial for the correct maintenance of vascular homeostasis. To exert their function, platelets survey the vascular system and adhere where alterations of the endothelial cell lining, accompanied or not by exposure of subendothelial matrix components, are detected.²⁴

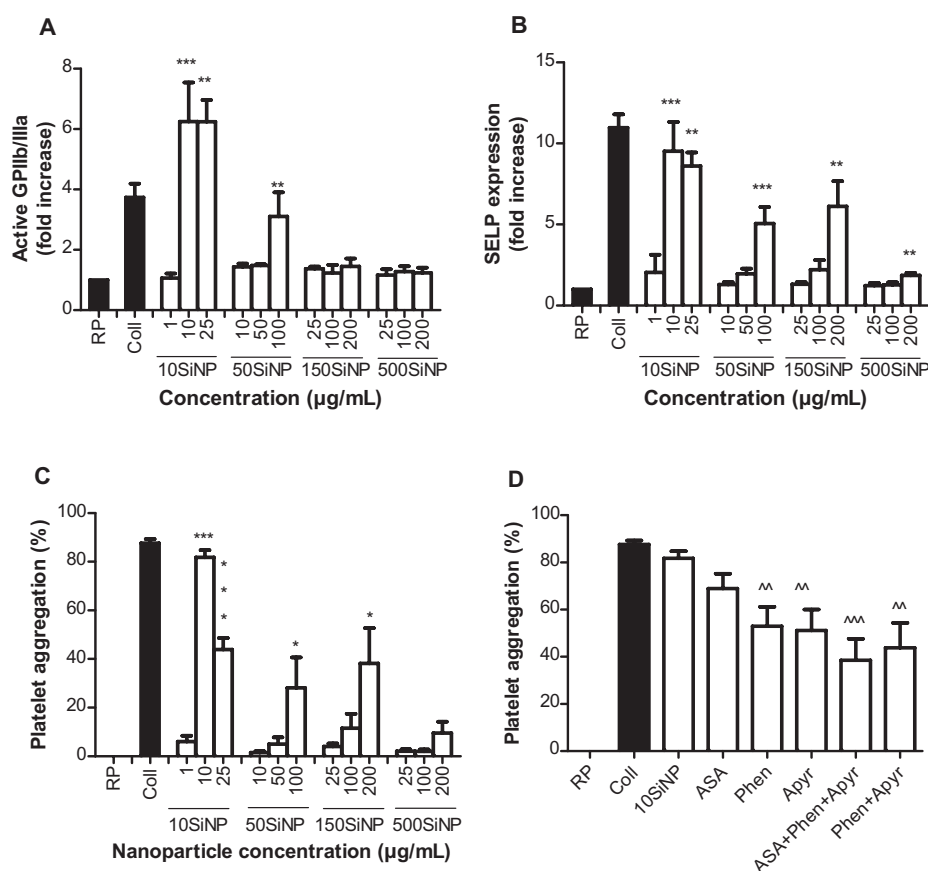


Figure 3 Amorphous SiNP upregulate surface receptors and induce platelet aggregation. Nanoparticles induce GPIIb/IIIa activation (A), SELP expression (B), and platelet aggregation (C). The use of phenanthroline (Phen) and apyrase (Apyr), but not acetylsalicylic acid (ASA), inhibits 10SiNP-induced platelet aggregation (D). A mixture of these three inhibitors also inhibited nanoparticle-induced platelet aggregation (D). Collagen (Coll) was used as a positive control for platelet aggregation. The GPIIb/IIIa activation (A) and SELP expression (B) induced by each nanoparticle treatment is expressed relative to unstimulated (resting) platelets.

Notes: All values are mean \pm SEM of $n = 4$. One-way ANOVA, Tukey-Kramer post test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with resting platelets (RP) (A–C). One-way ANOVA, Tukey-Kramer post test: [^] $P < 0.01$; ^{^^} $P < 0.001$ compared with 10 μ g/mL 10SiNP (D).

Abbreviations: SEM, standard error of the mean; SiNP, silica nanoparticles.

On the other hand, by the secretion or surface expression of a series of specific molecules (NO, prostacyclin), endothelial cells ensure that under normal conditions blood flow is appropriately regulated and that intravascular platelet activation and blood coagulation are avoided.

The deleterious effect of SiNP on endothelial cells found in our previous work¹ prompted us therefore to study the effect of SiNP on human platelets. In this current study, we found that amorphous SiNP when interacting with human blood platelets have the ability to stimulate the release of NO and to a greater extent ONOO⁻ leading to a low [NO]/[ONOO⁻] ratio that correlated well with the SiNP-induced platelet aggregation. Indeed, we observed that SiNP stimulated GPIIb/IIIa activation and SELP expression and led to platelet aggregation via ADP- and MMP2-dependent mechanisms. All of these effects augmented with increased nanoparticle concentration, but were inversely proportional to the size of SiNP. In fact, the smallest SiNP (10 nm) induced

the most severe effects on platelets. Recently, our studies on the effects of amorphous SiNP on human endothelial cells also reported the role of the [NO]/[ONOO⁻] ratio in the nanoparticle-induced endothelial inflammatory and cytotoxic responses.¹

Amorphous SiNP have a noncrystalline structure, a high surface-area-to-volume ratio and a large negative surface charge. A high surface-area-to-volume ratio, which decreases in the opposite direction to size, favors the formation of the “protein corona” with fundamental significance for bionanointeractions. The “protein corona” along with fluid characteristics and particle surface area and zeta potentials can affect the formation of nanoparticle agglomerates in the biological environment and may influence nanoparticle toxicity.^{25,26}

Our TEM study shows that SiNP interacted with the platelet surface membrane, internalized and distributed within the platelet cytoplasm. The interaction of SiNP with platelet surface membranes raised the possibility that nanoparticles

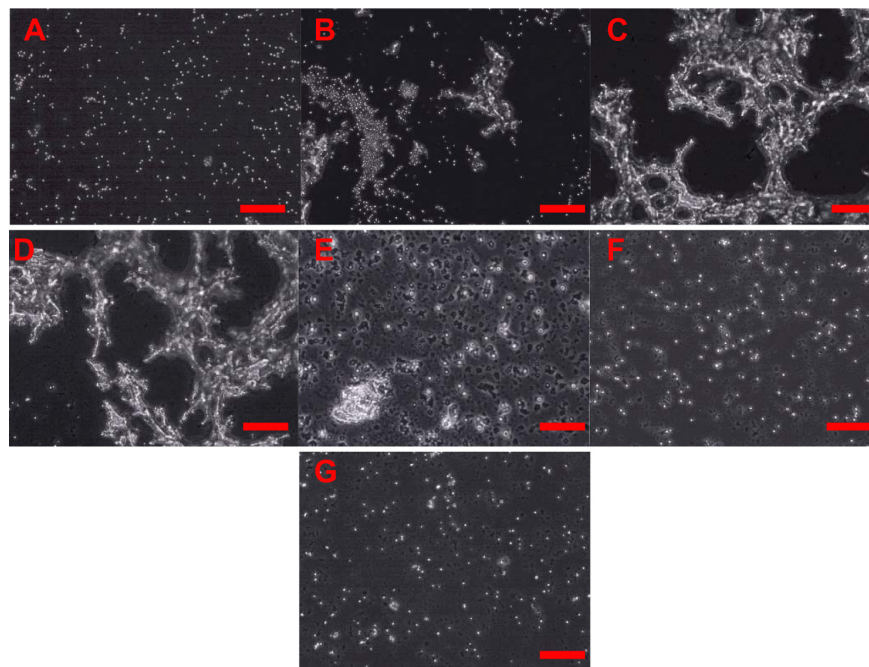


Figure 4 Amorphous SiNP induce platelet aggregation. Platelets were exposed to 10 µg/mL 10-nm SiNP in the absence (C) or present of inhibitors of platelet aggregation (D–G). Unstimulated (resting) platelets (A) and platelet aggregation induced by collagen (B) are also shown. Previous to the exposure to 10SiNP, platelets were incubated with acetylsalicylic acid (D), phenanthroline (E), apyrase (F) and a mixture of these three inhibitors (G).

Note: Scale bars represent 100 µm.

Abbreviation: SiNP, silica nanoparticles.

can affect calcium channels as well as the endothelial nitric oxide synthase (eNOS) that is associated with caveolae.²⁷ Indeed, there is convincing evidence that modulation of mechanosensitive ion channels involves NO and ONOO[−].²⁸ Therefore, we hypothesized that amorphous SiNP collide with the platelet surface membrane leading to opening of calcium channels, and eventually, uncoupling of eNOS. The kinetics of the penetration of the platelet membrane by SiNP most likely involved a flux of calcium ions into the cytoplasm.²⁹ Therefore, SiNP are likely to stimulate a calcium-dependent NO release in human platelets. The process of increased and prolonged NO production leads to depletion of enzymatic substrates and cofactors, and eventually, uncoupling of eNOS.³⁰ The uncoupled eNOS can generate superoxide¹⁰ which can rapidly react with NO to produce ONOO[−] and change critically the balance of [NO]/[ONOO[−]]. At high level of cytoprotective NO and/or low level of cytotoxic ONOO[−], this ratio is high (>2.0) indicating coupled eNOS and normal endothelial and platelet function. A low ratio (<1.0) is associated with uncoupled eNOS, dysfunctional cardiovascular system and high nitroxidative/oxidative stress.^{1,9,10} Our current experimental results support this hypothesis and show that amorphous SiNP unfavorably shift the [NO]/[ONOO[−]] balance to low levels. There is convincing evidence that platelet aggregation is favored by nitroxidative/oxidative stress induced by ONOO[−]

.^{11,12} Indeed, platelet-derived and exogenous ONOO[−] induce TXA₂ formation,³¹ nitration of tyrosine-containing proteins,³² SELP expression and GPIIb/IIIa activation.¹² The activation of GPIIb/IIIa (a change from the low- to high-affinity conformation) and translocation of SELP to the platelet surface membrane is crucial for platelet aggregation to occur.^{33,34} Indeed, GPIIb/IIIa is the major platelet surface transmembrane receptor and it plays an important role in platelet aggregation.³⁵ In addition, platelets contain a pool of this glycoprotein stored in α-granules.³⁶ Furthermore, SELP, that is also stored in platelet α-granules, mediates platelet-leukocyte aggregation since it is rapidly translocated to the surface upon platelet activation.³⁷ Our studies demonstrate that SiNP have the ability to induce GPIIb/IIIa activation and SELP expression on the platelet surface membrane leading to platelet aggregation. Interestingly, carbon nanoparticles, including nanotubes, have a similar ability.^{38,39} The activation of GPIIb/IIIa is triggered off by the release of major platelet activators that mediate pathways of aggregation such as TXA₂,¹³ ADP,¹⁴ and MMP2¹⁵ which amplify aggregation. In fact, we have previously shown that MMP2 translocates to the platelet surface during platelet aggregation.⁴⁰ Indeed, it has been also demonstrated the interaction of MMP2 with the integrin αIIbβ3 on the platelet membrane.⁴¹ We demonstrate here that SiNP can stimulate platelet aggregation via ADP- and MMP2-dependent mechanisms.

However, in contrast to agonist-induced aggregation which can be abolished by combination of ASA, apyrase, and phenanthroline,¹⁵ SiNP-induced aggregation is not completely dependent on the release of TXA₂, ADP, and MMP2. Interestingly, nanoparticles can directly interact with proteins and ion channels and these effects can also contribute to the profile of SiNP actions on platelets.^{42,43}

In conclusion, engineered amorphous SiNP penetrated the platelet plasma membrane and stimulated a rapid and prolonged NO release leading to eNOS uncoupling, ONOO⁻ over-production and eventually, a low [NO]/[ONOO⁻] ratio and high nitroxidative/oxidative stress. The unfavorable situation, thus produced, favored receptors activation (GPIIb/IIIa) and expression (SELP) on the platelet surface membrane leading to platelet activation and aggregation. Furthermore, SiNP-induced platelet aggregation is mediated via the MMP2 and ADP pathways. Importantly, these effects were all inversely proportional to the amorphous SiNP size and directly proportional to nanoparticle concentration. These findings, along with our study on endothelial cells,¹ conclusively highlight the importance of NO/peroxynitrite imbalance in the pathomechanism of nanotoxicological effects of amorphous SiNP with profound implications in the disruption of vascular homeostasis.

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Disclosure

The authors report no conflicts of interest in this work.

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