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Polystyrene nanoparticles affecting blood coagulation

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Abstract

The association of nanoparticles (NPs) with blood coagulation proteins may influence the natural balance between pro- and anticoagulant pathways. We investigated whether polystyrene NPs, when added to human plasma, affected the generation of thrombin in plasma. Amine-modified NPs were found to decrease the thrombin formation due to binding of factors VII and IX to the NPs, which resulted in depletion of the respective protein in solution. In contrast, carboxyl-modified NPs were able to act as a surface for activation of the intrinsic pathway of blood coagulation in plasma. These results highlight the influence of NPs on a biologically important pathway.

From the Clinical Editor: With the expected introduction of several nanoparticle-based therapeutic and diagnostic methods in the near future, their impact on basic homeostasis and obligatory biological processes needs to be carefully and accurately studied. In this study, the effects of polystyrene NP-s was investigated on blood coagulation pathways, and critically important biomodulatory effects were found.

Key words: Polystyrene; Nanoparticle; Blood coagulation; Nanosafety; Nanotoxicology

The production and use of nanoparticles (NPs) are increasing rapidly. Still, relative little is known about the interaction of NPs with biological systems and about the potential hazards of their use.[1,2] When NPs enter a biological fluid, e.g., plasma, they become coated with different biomolecules forming what is known as "the protein corona." This protein corona may be the biologically active entity rather than the NP itself,[3,4] and it is important to identify the corona composition. Biophysical methods for investigation of the interaction between NPs and proteins have been established.[4,5] Latex NPs are polystyrene-based particles generated at different sizes and with surface modifications such as amine- and carboxyl-modifications. The protein coronas around different polystyrene NPs in plasma have been identified, implicating binding of proteins involved in the lipid metabolism, complement systems, and the blood coagulation system, among others.[6-9] The effects of blood coagulation by biomaterials consisting of self-assembled monolayers with different ratios of hydrophobicity and negative charge have been of interest in different studies.[10,11] The study reported in this article investigated the effect of polystyrene NPs of different size and surface modifications on the blood coagulation system.

Blood coagulation is activated in response to tissue damage and leads to the generation of thrombin and a fibrin clot that will stop the bleeding. Upon tissue damage, tissue factor (TF) forms a complex with activated factor VII (FVIIa) and initiates the formation of thrombin.[12,13] This pathway is known as the extrinsic pathway and dominates in vivo. Blood coagulation can also be activated through the intrinsic pathway through contact activation by negatively charged surfaces.[14] Anticoagulant pathways regulate blood coagulation at different levels, including tissue factor pathway inhibitor, antithrombin, and activated protein C.[15-17]

We studied the influence on blood coagulation by the presence of different polystyrene NPs in plasma. We demonstrate distinct effects on blood coagulation depending on particle size and surface modification. This highlights the importance of identifying the protein corona around NPs to formulate hypotheses on which biological systems might be perturbed.

Methods

Reagents

Sulfonated polystyrene NPs were purchased from Bangs Laboratories Inc. (Fishers, Indiana). NPs used included:

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sulfonated 200 nm and 23 nm particles, amine-modified 57 nm and 330 nm particles, and carboxyl-modified 220 nm and 24 nm particles. Human plasma was withdrawn from healthy volunteers into citrate vacutainer tubes. Platelet-poor plasma was prepared by centrifugation at 2000*g* at 25°C for 15 minutes twice. Natural phospholipids, phosphatidylserine (PS, brain), phosphatidylethanolamine (PE, egg extract), and phosphatidylcholine (PC, egg extract) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Recombinant tissue factor (TF, thromboplastin) was from Dade Innovin (Marburg, Germany) with a concentration of 8.5 nM TF.[18] The thrombin fluorogenic substrate I-1140 Z-Gly-Gly-Arg-AMC·HCl was purchased from Bachem (Bubendorf, Switzerland). BSA and kaolin were provided by Sigma-Aldrich (St. Louis, Missouri). Antibody for detection of factor VII was from R&D Systems (Minneapolis, Minnesota). Monoclonal antibody to factor IX was a kind gift from Professor Johan Stenflo. Corn trypsin inhibitor (CTI) was purchased from Haematologics Technologies Inc. (Essex Junction, Vermont).

Dynamic light scattering (DLS)

The size of the particles (the z-average molecular size in terms of hydrodynamic diameter) and the zeta potential were determined using a Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, United Kingdom). Samples were diluted 100 times in MilliQ dH_2O before measurements, and the measurements were made at 25°C using 1 cm cuvettes. The results are the mean value from 5 measurements.

Incubation of plasma with NPs

NPs were diluted in HEPES buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.4) and incubated with equal volume of plasma, at 37°C for 15 minutes. Final concentration of NPs during plasma incubation is stated in figures, ranging from 0.0 to 0.5 mg/mL. When indicated, plasma/NP solution was pelleted by centrifugation at 20,000*g* for 10 minutes to separate the NPs from the remaining supernatant.

Routine coagulation tests

Plasma was incubated with NPs (or HBS as a control) as described above, yielding a final NP concentration of 0.5 mg/mL. Pelleted NPs were discarded, and the remaining plasma supernatant was analyzed by measuring activated partial thromboplastin time (APTT), prothrombin time (PT) and coagulation factor levels, using standard methods at the local hospital coagulation laboratory. The concentrations of the blood clotting factors were determined by measuring the ability of the sample plasma to correct for the coagulation time of plasma being deficient for the blood-clotting factor of interest. The clotting time was then converted to a concentration expressed as an international unit per mL, IU/mL, using a standard curve constructed using dilutions of normal plasma as sample.

Immunoblotting analysis of NP-associated proteins

Plasma (100 μL) was incubated with NPs (100 μL, 1 mg/mL) as described above followed by centrifugation. The NP pellet was washed three times using 500 μL HBS and the remaining washed pellet was dissolved in 100 μL loading buffer. The dissolved pellet was analyzed for associated proteins by SDS/PAGE (7% nonreduced gel) with immunoblotting using appropriate primary antibodies followed by HRP-conjugated secondary antibodies.

Table 1
Characteristics of polystyrene and COOH- and NH_2-modified polystyrene particles

Bangs Polystyrene	Size according to Bangs (nm)	Z-ave* (nm)	PdI†	PdI Width (nm)	Zeta-potential
23	23	23.6	0.058	2.8	−45.6
200	200	224.2	0.046	23.4	−52
24-COOH	24	27.8	0.201	6.2	−49.2
220-COOH	220	223.9	0.025	16.1	−43.5
57-NH_2	57	57.1	0.049	6.3	36.5
330-NH_2	330	284	0.164	57.4	40.1

* Z-average size, hydrodynamic diameter.
† Polydispersity index.

Thrombin generation assay

The amount of thrombin formed in plasma/NP samples were monitored using the thrombin generation assay as previously described[18] with the following modifications. Natural phospholipids, 20/20/60 PS:PE:PC, were mixed and prepared. Plasma (40 μL) was incubated with 40 μL NPs as described above. Fluorogenic substrate (Z-Gly-Gly-Arg- AMC·HCl), 20 μL, was added to samples and coagulation was initiated with 20 μL of phospholipids/TF/$CaCl_2$ mixture in a final volume of 120 μL. All reagents were diluted in HBSBSA (HBS buffer supplemented with 5 mg/mL BSA) and final concentrations were approximately 1.17 pM TF, 4.2 μM phospholipids, 300 μM fluorogenic substrate, and 16 mM $CaCl_2$. When indicated, a modified thrombin generation assay was performed in the absence of tissue factor, replaced with HBSBSA buffer, to study the effects of the activation of the intrinsic pathway by NPs.

Results

Plasma was incubated with different polystyrene NPs to determine if they could affect the reactions of blood coagulation. Blood coagulation was monitored by the generation of thrombin using the thrombin generation assay. Polystyrene NPs that were tested included sulfonated particles of 200 nm or 23 nm, amine-modified particles of 57 nm or 330 nm, and carboxyl-modified particles of 220 nm or 24 nm. The polystyrene NPs were characterized using DLS (see Table 1).

Decreased thrombin generation in plasma containing amine-modified NPs

Thrombin generation was decreased in plasma incubated with amine-modified NPs (Figure 1) in comparison with plasma incubated with buffer or all the other NPs. Furthermore, concentration-dependent studies with amine-modified NPs showed that the 57-nm NPs were more effective in decreasing thrombin generation in comparison with the larger 330-nm particles when comparing similar mass concentrations (Figure 1). The activation of coagulation in plasma incubated with amine-

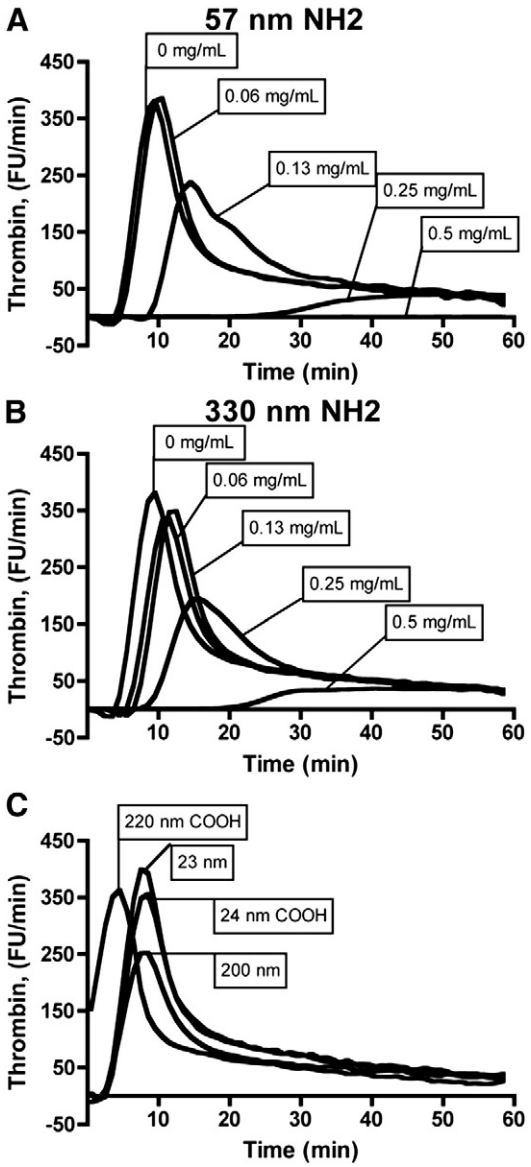


Figure 1. Plasma containing amine-modified NPs has decreased thrombin generation. Plasma was incubated in the presence or absence of NPs and tested for thrombin generation using the thrombin generation assay. The first derivative, fluorescence units/min, is shown (means of $n = 3$) for plasma with 57-nm and 330-nm amine-modified NPs (**A** & **B**, respectively) at different concentrations as indicated in the figure. The 0.5 mg/mL line is hidden under the x-axis in **(A)**. Plasma incubated with 23-nm, 200-nm particles, either the sulfonated (just denoted their size) or the carboxyl-modified particles at 0.5 mg/mL **(C)**.

modified NPs was also measured using the traditional coagulation assays activated partial thromboplastin time (APTT) and prothrombin time, see Table 2. The 57-NH_2 particles prolonged the clotting times of both assays, whereas the 330-NH_2 particles only prolonged the APTT time.

Coagulation factors VII and IX bind selectively to amine-modified NPs

The decreased thrombin formation in plasma incubated with amine-modified NPs indicated that the NPs might have bound one or more proteins involved in the blood coagulation cascade. The concentrations of different coagulation proteins were analyzed in plasma after incubation with and removal of 57-nm amine-modified NPs. The analysis indicated that the NPs bind FVII and FIX and deplete the plasma from these proteins (Figure 2). The levels of factor VIII, factor XI, protein S, and protein C were also decreased in plasma incubated with NPs in comparison with control plasma, but the difference was not as large as for FVII and FIX. The binding of FVII and FIX to amine-modified NPs was confirmed by western blot analysis of the washed NP pellets from plasma incubations (Figure 3, *A*). The binding to FVII and FIX was selective for amine-modified particles as seen by the lack of binding to 200 nm sulfonated particles and carboxyl-modified NPs (Figure 3, *B*).

Table 2
Coagulation assays of plasma incubated with buffer (HBS) or NH_2-modified polystyrene particles. Values are means ± SD from triplicate measurements

Plasma incubated with:	APTT* (sec)	PT† (sec)
HBS	47 ± 1.0	28 ± 0.6
57-NH_2	66 ± 2.1	41 ± 0.6
330-NH_2	57 ± 3.1	22 ± 1.2

* Activated partial thromboplastin time (APTT).
† Prothrombin time (PT).

Carboxyl-modified NPs are activators of the intrinsic pathway of coagulation

The intrinsic pathway is activated by negatively charged surfaces, e.g., provided by glass, polyphosphates,[19] collagen, or kaolin. Polystyrene NPs were studied in their ability to activate the intrinsic/contact pathway of coagulation using a modified thrombin generation assay, without the addition of tissue factor. Carboxyl-modified NPs of larger size, 220 nm, were able to act as a surface for initiating the intrinsic pathway, in comparison with plasma incubated with buffer (Figure 4, *A*) or all the other NPs (Figure 4, *D*). Carboxyl-modified NPs of smaller size, 24 nm, were not able to act as an activating surface. Initiation requires activation of factor XII (FXIIa) by the presence of not only negatively charged surfaces but also prekallikrein and high molecular weight kininogen.[14] CTI is a specific inhibitor of FXIIa that forms a tight 1:1 complex with FXIIa. The ability of carboxyl-modified NPs to activate the intrinsic pathway was blocked by the presence of CTI, indicating that the NPs activate coagulation at the level of FXIIa through the intrinsic pathway (Figure 4, *B*). Finally, NPs were compared with kaolin in their ability to activate the intrinsic pathway of coagulation. Carboxyl-modified NPs at 50 μg/mL in plasma were as efficient as 1 μg/mL kaolin in plasma (Figure 4, *C*).

Discussion

Given the rapid increase in the use of nanomaterials, relatively little is known about the interaction of NPs with biological system and the potential hazards. Consequently, the field of nanotoxicology and nanosafety is now emerging. Regulation of blood coagulation can have unwanted consequences, and it is important

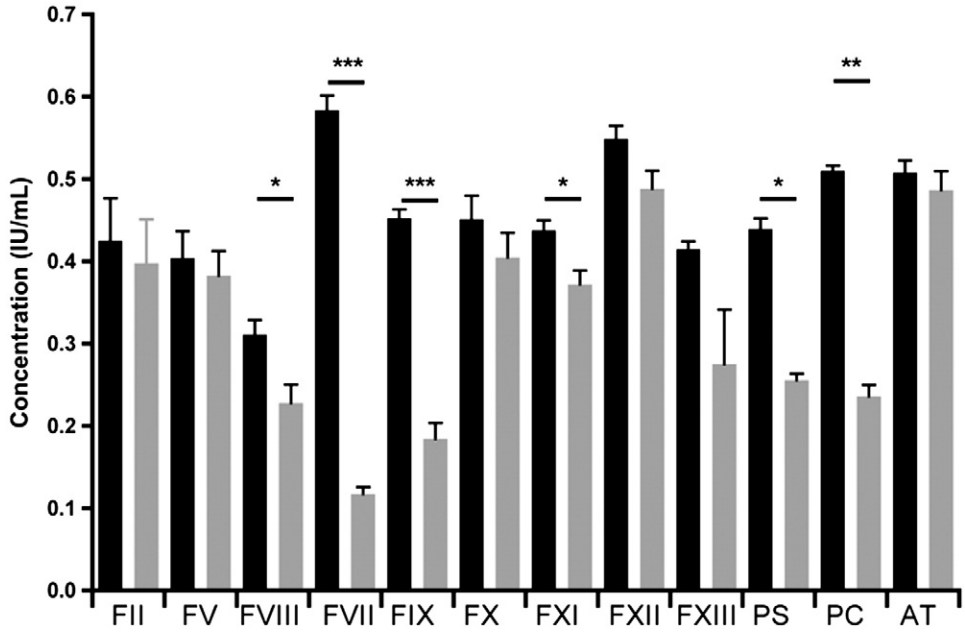


Figure 2. Analysis of blood coagulation proteins in plasma incubated with 57-nm amine-modified NPs. Plasma was incubated in the presence (*gray bars*) or absence (*black bars*) of 57-nm amine-modified NPs (0.5 mg/mL) for 15 min at 37°C. NPs were pelleted by centrifugation at 20,000*g* for 10 min, and the remaining plasma was analyzed for coagulation factor concentrations. Concentrations are expressed as International Units/mL, means ± SEM of $n = 3$ or 4. Significance was determined by unpaired *t*-test (*** < 0.001, ** < 0.01, * < 0.05). PS = protein S, free; PC = protein C; AT = antithrombin.

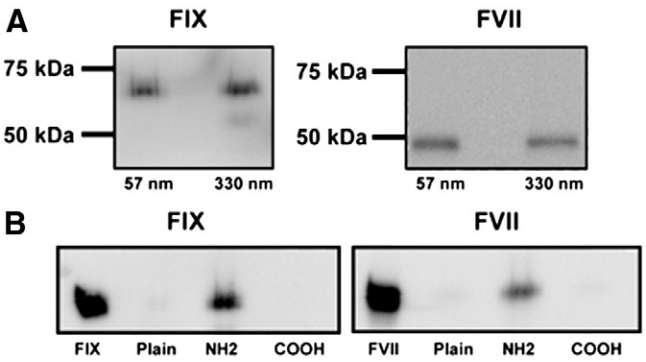
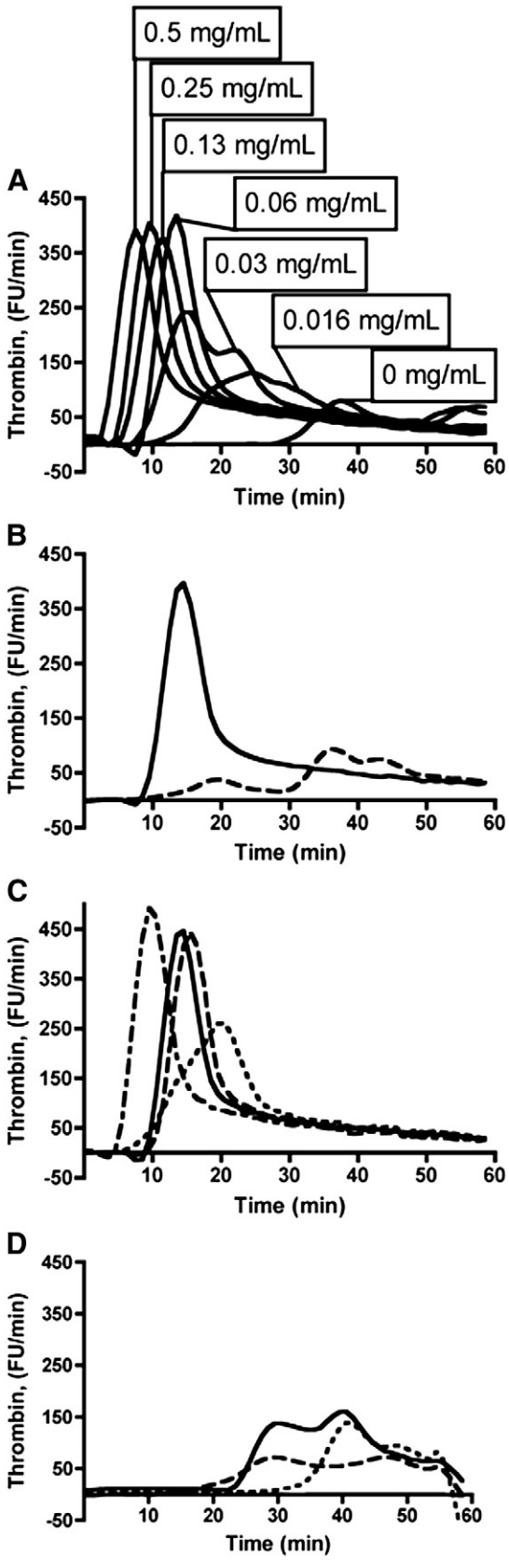


Figure 3. Amine-modified NPs selectively bind FVII and FIX. Plasma was incubated in the presence of NPs (0.5 mg/mL) for 15 min at 37°C. NPs were pelleted by centrifugation at 20,000*g* for 10 min, and the pellet was washed three times with 500 μL HBS buffer and then dissolved in loading buffer and analyzed for FIX and FVII content by SDS/PAGE (7% nonreducing gel) followed by immunoblotting. **(A)** 57-nm and 330-nm amine-modified NPs. **(B)** 200-nm sulfonated particles, 330-nm amine- and 220-nm carboxyl-modified NPs. As a control, 10 ng of either FIX or FVII was loaded on gel.

to characterize the effect of NPs on these reactions. Association of NPs to proteins involved in blood coagulation might influence the balance between pro- and anticoagulant state and needs to be further analyzed. In this report, we demonstrate that polystyrene NPs having different surface charges strongly affect reactions of blood coagulation in human plasma.

Amine-modified polystyrene NPs were shown to bind to coagulation factors FVII and FIX, and to negatively affect blood coagulation with a decreased thrombin formation as a consequence. Amine-modified NPs of two different sizes were tested using the same mass concentration, and the observed larger effect on blood coagulation by the 57 nm in comparison with 330-nm particles is most likely related to the larger surface area-to-volume ratio of the smaller particles. The surface area-to-volume ratio is inversely dependent on the radius of NPs, and thus if the results in Figure 1 should be compared at the same surface area-to-volume-ratio, the larger effect of the 57-nm amine-modified NPs is lost (compare 0.25 mg/mL of 330-nm amine-modified particles with 0.06 mg/mL 57-nm amine-modified particles in Figure 1). This indicates that smaller amine-modified NPs will have a larger effect on blood coagulation if distributed at the same mass concentration. The larger effect on the prothrombin time of the 57-nm particles in comparison with the 330-nm particles is also likely related to the larger surface area-to-volume ratio of the smaller particles (compare prothrombin time in Table 2). Binding of FVII to NPs will deplete FVII in plasma, thus decreasing the amount of thrombin formed, which potentially could lead to a state of bleeding. Binding of FIX has little effect on the thrombin formation measured by the TF-induced thrombin generation assay, because FIX is mainly important in the intrinsic/contact pathway. Therefore, the ability of plasma to clot through the intrinsic/contact pathway was analyzed by the activated partial thromboplastin time assay. Plasma incubated with amine-modified NPs had a prolonged clotting time in comparison with control plasma (Table 2), indicating that the binding of FIX to amine-modified NPs also decreased the ability to generate thrombin through the intrinsic/contact pathway (see Figure 5).

In contrast to the amine-modified NPs, the 220-nm carboxyl-modified NPs were able to initiate the intrinsic pathway of coagulation, whereas the smaller particles of 24 nm were unable to



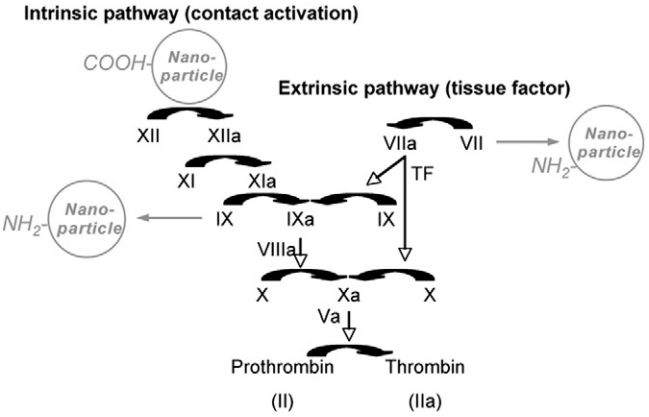


Figure 5. Schematic picture of the different effects on blood coagulation by polystyrene NPs. Schematic picture of the different effects on blood coagulation by polystyrene NPs. Blood coagulation can be activated either through the intrinsic/contact pathway or through the extrinsic pathway. Amine-modified NPs bind FVII and FIX, which leads to a decrease in thrombin formation. Carboxyl-modified NPs are able to activate the intrinsic pathway of coagulation by activation of FXII.

do so. This was probably due to their smaller size and more curved surfaces. Thus, to activate the intrinsic pathway, the particles need to be large enough to bind the initiating molecule FXII and assemble the multimolecular complexes of the intrinsic/contact pathway, which apart from FXII include the proteins FXI, prekallikrein/kallikrein, and high molecular weight kininogen (HMWK). The effective (hydrodynamic) radius of a protein can be calculated from its molecular weight and was used to theoretically calculate if the smaller carboxyl-modified particles could potentially assemble intrinsic complexes.[20] The activation of FXII requires at least the assembly of both kallikrein (80 kDa, 7.5 nm in diameter) and FXII (80 kDa, 7.5 nm in diamter). In addition, the activation of FXI (160 kDa, 9.5 nm in diameter), which requires HMWK (120 kDa, 8.6 nm in diameter) and FXIIa (80 kDa, 7.5 nm in diameter) also takes place on negatively charged surfaces. Taken together, it is evident that a larger negatively charged surface of 200 nm supports the activation much better than a 24-nm particle. Even though both the activating complexes do not exceed 25 nm in total, it might be difficult for the 24-nm particles to bind all the different proteins in close proximity due to the high curvature of small particles.

Figure 4. Carboxyl-modified NPs activate the contact pathway of coagulation in plasma. Plasma was incubated in the presence or absence of NPs and tested for the ability to activate contact pathway of coagulation using a modified thrombin generation assay without the addition of tissue factor. The first derivative, fluorescent units/min, is shown (means of $n = 3$). **(A)** 220-nm carboxyl-modified NPs at different concentrations, as indicated in the figure. **(B)** Plasma was pre-incubated in the absence (*solid line*) or presence (*dashed line*) of corn trypsin inhibitor (100 μg/mL plasma for 15 min on ice), followed by addition of 220-nm carboxyl-modified NPs (50 μg/mL). **(C)** Plasma was incubated with 220-nm carboxyl-modified NPs (50 μg/mL, *solid line*) or kaolin (0.5 μg/mL, 1 μg/mL, and 5 μg/mL; *dotted, dashed*, *dotted/dashed lines* respectively). **(D)** NPs incubated with plasma at 0.5 mg/mL. 23-nm sulfonated particles (*solid line*), 200-nm sulfonated particles (*dashed line*), 24-nm carboxyl-modified particles (*dotted line*). The lines for amine-modified particles of 57 nm and 330 nm are hidden under the x-axis.

Kaolin, which is used in the APTT test, is a silicate mineral that has a high negative surface charge that activates the intrinsic/-contact pathway. The carboxyl-modified NPs also have a negative charge and thus activate coagulation in a manner similar to that of kaolin. Besides promoting blood coagulation, FXIIa also activates the complement cascade through activation of C1 and, in addition, activates the fibrinolytic system and kinin generation through the generation of α-kallikrein.[14] The fact that the carboxyl-modified NPs are able to activate blood coagulation through the contact pathway might indicate that other reactions involved in the contact activation are activated as well, e.g., the generation of the vasoactive peptide bradykinin from HMWK. Recent studies have indicated that polystyrene NPs can have an effect on the primary hemostasis with an increase in platelet aggregation.[21,22] Carbon NPs have also been reported to induce platelet aggregation and vascular thrombosis.[23]

NP size and surface properties have been shown to influence the protein corona[6-9,24] and, in addition, we now show that these properties of NPs also have an impact on an important biological system such as blood coagulation. From the characterization data, Table 1, it can be concluded that both the carboxyl-modified particles and the 23- and 200-nm sulfonated particles have a total negative charge. Despite their similar charge, it is only the carboxyl-modified particles that can activate the intrinsic pathway of coagulation. This suggests that the observed effects on blood coagulation are related to the surface modifications of polystyrene NPs. The effects of NPs on blood coagulation might have a clinical relevance if the particles come in contact with the blood, e.g., if particles come in contact with an open wound. The overall aim of the study was to study the toxicology effects of polystyrene NPs, and determine whether the particles could have any extreme effects on a biological system such as blood coagulation. Polystyrene NPs were used as a model system to study the effects on blood coagulation in terms of surface modifications and size. The observed effect of the carboxyl-modified NPs, i.e., that larger particles support the activation of the intrinsic pathway more efficiently is interesting because the general idea of NPs is that smaller particles are more prone to have larger effects due to their high surface area-to-volume ratios.

In conclusion, polystyrene NPs activate or inhibit blood coagulation in different ways depending on their size and surface chemistry (see Figure 5). The ability of amine-modified NPs to bind FVII and FIX could potentially lead to an increased risk of bleeding, whereas the ability of carboxyl-modified NPs to activate the intrinsic pathway of coagulation could instead lead to coagulation activation and a thrombotic state.

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