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Nanoparticles and the blood coagulation system. Part II: safety concerns

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Abstract

Nanoparticle interactions with the blood coagulation system can be beneficial or adverse depending on the intended use of a nanomaterial. Nanoparticles can be engineered to be procoagulant or to carry coagulation-initiating factors to treat certain disorders. Likewise, they can be designed to be anticoagulant or to carry anticoagulant drugs to intervene in other pathological conditions in which coagulation is a concern. An overview of the coagulation system was given and a discussion of a desirable interface between this system and engineered nanomaterials was assessed in part I, which was published in the May 2013 issue of *Nanomedicine*. Unwanted pro- and anti-coagulant properties of nanoparticles represent significant concerns in the field of nanomedicine, and often hamper the development and transition into the clinic of many promising engineered nanocarriers. This part will focus on the undesirable effects of engineered nanomaterials on the blood coagulation system. We will discuss the relationship between the physicochemical properties of nanoparticles (e.g., size, charge and hydrophobicity) that determine their negative effects on the blood coagulation system in order to understand how manipulation of these properties can help to overcome unwanted side effects.

Keywords

coagulopathy; disseminated intravascular coagulation; endothelial cell; leukocyte; nanoparticle; procoagulant activity platelet; thrombosis

When nanoparticles enter systemic circulation they immediately encounter blood cells, proteins and endothelial cells, as well as key components of the coagulation system such as platelets and plasma coagulation factors. If nanoparticles are engineered to specifically interact with cells and coagulation factors, their effect on the coagulation system will benefit treatment of certain coagulation disorders. Detailed coverage of this subject was provided in part I, which was published in the May 2013 issue of *Nanomedicine* [1]. If, however, nanoparticles induce undesirable alterations in the balanced function of these cells and proteins, they may cause severe and even life-threatening toxicities. Therefore, there are increasing concerns about nanoparticle-induced coagulopathies (i.e., coagulation disorders

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caused by perturbation of the blood coagulation system). For example, several studies have reported that environmental nanoparticles significantly increase the risk and worsen the prognosis of cardiovascular diseases due to the induction of thrombotic complications [2–5]. Additionally, there is an increasing number of studies reporting that engineered nanomaterials may cause severe toxicity through a shift of the hemostatic balance caused by perturbation of the coagulation system.

One such toxicity is known as disseminated intravascular coagulation (DIC) or consumptive coagulopathy. Acute DIC is characterized by depletion of coagulation factors through the formation of small intravascular clots, followed by abnormal hemorrhages. Chronic DIC is characterized by intravascular thrombosis [6]. When left untreated, DIC may lead to multiple organ failure and death. DIC is a common complication in sepsis and cancer, and was reported with intravenous (i.v.) administration of certain nanomaterials [7,8]. Another common coagulation disorder is deep vein thrombosis (DVT). DVT is a disorder characterized by clot formation in deep veins. Like DIC, DVT may be life threatening; a dislodged clot can move to the lungs and cause pulmonary embolism. Incidents of vascular thrombosis have also been reported for some nanomaterials [9].

In part II, we will focus on instances where coagulopathy is the dose-limiting toxicity of engineered nanomedicines. We will also discuss how manipulation of nanoparticle properties may help in overcoming coagulation-mediated toxicities to avoid common side effects caused by nanoparticle-induced alterations in the blood coagulation.

Undesirable interactions between nanoparticles & components of the coagulation system

Nanoparticle interactions with platelets, coagulation factors and endothelial cells may all contribute to undesirable coagulation-mediated toxicities (Figure 1).

The compatibility of administrated nanoparticles with components of the coagulation system depends on their physicochemical properties. Nanoparticle interactions with plasma proteins are important in evaluating undesirable interactions between the nanoparticles and the coagulation system, because protein binding can alter nanoparticle physicochemical properties [10,11], which, in turn, determines particle interaction with proteins [12–14]. Proteins involved in blood coagulation (e.g., fibrinogen) are common components of the ‘protein corona’ [14]. Corona profiles specific to nanoparticles have not been described so far, and protein binding to nanoparticles shares many features of that described for any other biomaterials. It is generally accepted that interactions with any surface may lead to changes in protein conformation, activation or inhibition of protein activity and exposure of epitopes, among others [11,15]. The impact of both bio- and nanomaterials on the coagulation system can be divided into two categories: contact with plasma coagulation factors and interaction with cells (e.g., platelets, epithelial cells and monocytes). Absorption/binding of coagulation factors onto nanoparticle surfaces may have two consequences: inactivation of the factors or reduced availability to other components of the coagulation cascade; or contact activation of the factors. The former may cause prolongation or deficiency in coagulation reactions, while the latter may cause undesirable coagulation. Nanoparticles’ physicochemical properties determine their effects on coagulation. For example, coagulation factor XII can undergo self-activation after interaction with an anionic surface [15]. Likewise, anionic polystyrene nanoparticles activate intrinsic coagulation [16]. Interestingly, this property is largely dependent on particle size, as only large (220 nm) nanoparticles, but not their small (24 nm) counterparts, activated intrinsic coagulation.

Since each nanoparticle is unique and mechanisms of interactions with various components of the coagulation system may vary even for nanomaterials of the same category, we grouped literature findings based on the type of nanoparticles: polymers (chitosan, dendrimers and latex); liposomes; nanoemulsions; carbon-based nanomaterials; quantum dots (QDs); metallic (iron oxide, gold and silver nanoparticles); and ceramic (silica) nanoparticles. Since the pro-coagulant activity (PCA) of nanomaterials is elucidated from their impact on the main components of the coagulation system – endothelial cells, coagulation factors and platelets – we will review studies describing nanoparticle interactions with these components. Wherever data are available, we will highlight trends and mechanisms common for different categories of nanomaterials.

Polymeric nanomaterials

Polymeric nanomaterials are widely used for drug delivery due to their easily controlled synthesis.

Dendrimers—As with other nanomaterials, dendrimer interaction with components of the coagulation system is determined by their physicochemical properties. It has been shown that cationic dendrimers are cytotoxic *in vitro* [17], whereas anionic and neutral dendrimers are not. The cytotoxic effect of cationic dendrimers on endothelial cells was attributed to cell membrane damage, which is common for other cationic nanomaterials. The interaction of cationic G5 polypropylenimine dendrimers with the membrane of endothelial cells (human umbilical vein endothelial cells [HUVEC]) caused time-dependent changes in membrane permeability. Perforation of the cell membrane by these nanoparticles depended on particle surface properties and the incubation time [18]. The biocompatibility of poly(amido amine) (PAMAM) dendrimers *in vivo* is also size and charge dependent. For example, i.v. and oral administration of G4 and G7 amine-terminated PAMAM dendrimers resulted in DIC in mice [7]. This toxicity was dose dependent and observed when dendrimers were injected at dose levels exceeding their maximum tolerated dose [7]. The mechanism of DIC is very complex and involves coagulation factors, leukocytes, endothelial cells and platelets. The ability of cationic dendrimers to induce platelet aggregation and leukocyte PCA was studied *in vitro* [19–21]. In agreement with their DIC-like toxicity *in vivo*, only cationic PAMAM dendrimers, but not their anionic and neutral counterparts, induce platelet aggregation [20,21] and PCA in HL-60 cells [19] *in vitro*. The toxicity of cationic dendrimers observed in these *in vitro* studies was also dependent on their size, in that larger particles with higher densities of surface amines were more reactive with platelets and leukocytes [19–21]. The mechanism of platelet aggregation by cationic PAMAM dendrimers is common for other cationic nanomaterials; in other words, it occurs through disturbance of membrane integrity [20]. *In vitro* studies demonstrated that the mechanism of PCA induction in leukemia cells depends on *de novo* protein and RNA synthesis, and the presence of phospholipid and tissue factor on the cell surface [19]. Of interest is the finding that cationic PAMAM dendrimers do not induce PCA in normal leukocytes, but exaggerate endotoxin-induced PCA [19]. The mechanism behind the exaggeration of endotoxin-induced PCA by dendrimers remains unknown. Understanding this phenomenon is important, because exaggeration of other endotoxin-mediated inflammatory reactions has been reported for several types of nanomaterials [22–24]. It is also important to the nanomedicine field, because endotoxin contamination is very common in engineered nanomaterials, and amino groups are often used for drug, ligand and imaging agent conjugation to nanoparticle surfaces. Available data warrant special care to ensure that nanoparticle-based products are essentially endotoxin-free and do not have free, nonreacted amines on their surfaces in order to avoid DIC-like toxicities.

Other polymeric nanomaterials—Several other polymeric nanomaterials have been studied in blood coagulation tests with some challenges. For example, cationic polystyrene nanoparticles aggregated in the presence of serum, which resulted in drastic changes in their size versus that measured under pristine conditions [10]. In contrast to theoretical expectations, activation of platelets was independent of nanoparticle surface charge [10]. Another study reported that polystyrene latex nanoparticles of the same size induce activation of platelets through different pathways that depend on the nanoparticles' surface charge. Cationic polystyrene latex nanoparticles induced platelet activation and aggregation through perturbation of the cellular membrane, while their anionic counterparts activated platelets and induced their aggregation through the classical pathway, involving the upregulation of adhesion receptors [25]. Likewise, amine-modified nanoparticles decreased thrombin generation through depletion of factors VII and IX [16]. The effect was more prominent for small nanoparticles than for their large counterparts [16]. In contrast to the cationic nanoparticles, anionic ones activated the intrinsic coagulation pathway, which was also size-dependent, but more prominent for large particles [16]. It was hypothesized that surface curvature of the small particles was too high to provide enough room for the assembly of the multicomponent complex that initiates the intrinsic pathway [16].

Liposomes

Liposomal effects on coagulation are not as prominent as those reported for other nanomaterials, and are mainly related to agonist-induced coagulation. Surface charge is considered to be the key factor for this group of nanomaterials. For example, anionic liposomes inhibited agonist-induced platelet activation in buffer, while their cationic counterparts only had effects in plasma. The authors concluded that anionic liposomes interacted with platelets directly, while their cationic counterparts acted indirectly through interaction with plasma coagulation factors [26]. Likewise, another study demonstrated that anionic lipid-based nanoparticles inhibit agonist-induced platelet aggregation. This phenomenon was observed independently of the type of agonist applied and required high concentrations of nanoparticles [27]. However, another study contradicted these findings, in that anionic, but not cationic or neutral liposomes, shortened coagulation time *in vitro* and induced reversible aggregation of platelets both *in vivo* and *in vitro* [28]. These effects were attributed to the interaction between the anionic liposomes and coagulation factors XII and XI [28]. The reason for the discrepancy between the results of these studies is unclear, and may be attributed to the different composition of nanoparticles (cetyl alcohol/polysorbate and phosphatidylcholine, respectively). Although both studies used the same type of reagent to achieve a certain surface charge, the detailed particle characterization (e.g., size, ζ -potential, stability and purity), which could have helped in interpretation, were not provided.

Direct interaction of liposomes with blood cells, including platelets, was observed for different types of liposomes, and was shown to be dose-dependent [29]. The observed interactions may result from internalization of liposomes, or fusion of cellular and liposome membranes [30–32]. It is believed that interactions between liposomes and platelets are responsible for the transient thrombocytopenia observed after administration of negatively charged liposomes [33]. Electrostatic interactions between cationic liposomes and anionic cell membranes has been considered as an exploitable mechanism for drug delivery into endothelial cells. For example, it has been demonstrated that endothelial cells can take up cationic liposomes and accumulate them in the perinuclear region [34–36]. The most positively charged liposomes had the highest levels of uptake. This property was used for the delivery of chemotherapeutic agents into endothelial cells forming neovasculature. Liposomes loaded with cytotoxic agents were more toxic to endothelial cells than the liposome-free drug [34]. Although this strategy can aid in destroying tumor vasculature,

damage of neovascular endothelial cells is accompanied by the release of procoagulant signals and may lead to undesirable thrombotic outcomes.

Nanoemulsions

Knowledge regarding nanoemulsions' interactions with the coagulation system comes from preclinical and clinical experience with perfluorocarbon (PFC)-based emulsions used as artificial blood substitutes and propofol formulation used in anesthesia. Due to their rapid distribution into organs of the mononuclear-phagocytic system [37,38] and activation of the complement system [39], administration of PFC nanoemulsions resulted in transient changes in platelet counts, which were restored in patients 30 min after injection [38]. It was not associated with bleeding complications and is sometimes referred to as 'pseudothrombocytopenia'. A report about PFC interfering with platelet count suggests that these studies may need to be reconsidered [40]. Clinical data regarding propofol are controversial in that both prothrombotic [41] and antithrombotic [42] effects have been reported in patients. This discrepancy is most likely a result of the distinct underlying pathologies in patients to whom the propofol emulsion was administered.

Carbon-based nanoparticles

Carbon-based nanoparticles are used in various industrial applications and are abundant in the environment and in urban pollution. Both urban particulate matter and engineered carbon-based nanoparticles induce oxidative stress [43–46]. The latter triggers production of proinflammatory cytokines (TNF- α , IL-1 β and IL-8) and contributes to the development of inflammation [47–52]. Inflammation is a very potent procoagulant as many factors inducing inflammation can also trigger coagulation [53].

Exposure to environmental pollution has been shown to result in elevated levels of fibrinogen and von Willebrand factor (vWF) in individuals with pre-existing pathogenic (cardiovascular diseases and diabetes) conditions [54,55]. Inhalation studies in mice revealed elevated plasma levels of PAI-1 after exposure to multiwalled carbon nanotubes (MWCNTs) and single-walled carbon nanotube (SWCNTs) [52]. The most profound effects of urban particulate matter on coagulation were attributed to nanoparticles with sizes smaller than 100 nm, which had a higher propensity for distributing to and accumulating in lung tissue, causing an inflammatory response [44,56]. In addition, ultrafine particles were shown to penetrate lung tissue and distribute into the systemic circulation [44,57]. The presence of inhaled radioactively labeled ultrafine carbon particles in the circulation of volunteers was detected within the first 10–20 min [58]. The approach used in these studies was called into question by another study reporting that radioactivity detected in the circulation was attributable to the free isotope, rather than the nanoparticle-bound isotope [59]. Although several animal studies have confirmed cardiovascular, coagulation and pulmonary toxicities associated with the inhalation of carbon nanomaterials [60–65], some controversy still exists. For example, intratracheal instillation of 20–50 nm of diesel exhaust particles enhanced venous and arterial thrombi formation in a dose-dependent manner. These data were confirmed *in vitro*, in that the addition of diesel exhaust particles to the blood of untreated animals resulted in platelet activation [60]. The size and charge of carbon nanoparticles are thought to be the key parameters that determine thrombogenic propensity. Intratracheal instillation of cationic polystyrene particles resulted in enhancement of free radical-induced thrombosis in hamsters, and was comparable to that of diesel exhaust particles at the same dose level [44,60]. Interestingly, negatively charged or unmodified polystyrene particles did not have such effects on the coagulation system, suggesting that the presence of the amino group on the particle surface may have been responsible for the observed thrombogenicity. Moreover, larger particles with the same surface coating, administered at the same dose levels did not affect thrombus generation, despite the

inflammation observed in the lungs of treated animals. These data suggest that particle size and surface area are also important factors in the thrombogenicity of cationic particles. In agreement with these results, i.v. administration of positively charged ultrafine particles in another study enhanced thrombus generation [62].

Studies aimed at understanding the mechanism of thrombogenicity of carbon nanoparticles revealed intriguing findings. Urban particular matter (SRM1648), MWCNT, SWCNT, fullerene derivative C60CS and mixed carbon nanoparticles (MCNs) were compared with respect to their tendency to induce activation and aggregation of platelets [9]. All tested materials, except for the fullerene derivative, induced platelet aggregation; however, the magnitude of the impact varied among the materials: MCN > SWCNT > MWNT > SRM1648; MCN resulted in the strongest and SRM1648 the weakest platelet aggregation [9]. Platelet aggregation by MCN was accompanied by degranulation, ATP release and P-selectin exposure on the platelet surface. Treatment of platelets with carbon nanoparticles was also associated with the activation of glycoprotein IIb/IIIa on the cell surfaces. The upregulation of glycoprotein IIb/IIIa correlated well with the level of platelet aggregation induced by a given carbon nanomaterial. Since the aggregation of platelets by MCNs was comparable to that induced by collagen, the authors further explored the signaling events leading to the carbon nanoparticle-mediated platelet aggregation and compared it with that induced by collagen. The results were striking – although collagen-induced activation of platelets depended on the protein kinase C signaling pathway, platelet activation by MCN, SWNT and MWNT was protein C independent [9]. In contrast to traditional activators of platelet aggregation, carbon nanoparticles activated platelets through a matrix metalloproteinase-dependent pathway. The same study has also proposed that carbon nanotubes mimic cell–cell bridges, causing activation of platelets. Activation of platelets by SWCNT was further confirmed by Bihari *et al.* [66].

These original findings were further verified and explored in more detail by Simak's laboratory [67,68]. In several elegant studies, this group has demonstrated that, unlike water-soluble fullerene derivatives, C₆₀(OH)₂₄ and nC₆₀, MWCNT induce activation and aggregation of platelets. Activation of platelets by MWNT was accompanied by elevated levels of extracellular Ca²⁺ and could not be simply attributed to damage of cellular membranes because it was downregulated by calcium (Ca²⁺) entry blockers SKF96365 and 2-aminoethoxy-diphenyl borate. To further elaborate on the mechanism, Simak's group employed electron microscopy and demonstrated that MWNT perforated cellular membranes leading to the release of intracellular Ca²⁺. This study also proposed that intracellular membranes differ from plasma membranes; they are dense tubular systems that are probably more sensitive to nanotube perforation [68]. Depletion of stored intracellular Ca²⁺ induced influx of Ca²⁺ from the outside, and activated store-operated Ca²⁺ entry. Interestingly, despite the fact that water-soluble fullerene derivatives were detected inside platelets, they did not induce depletion of intracellular Ca²⁺ stores. In addition to the changes in intracellular Ca²⁺ storage, MWNTs induced the release of platelet membrane microparticles, which are capable of stimulating platelets and other cells [67,68]. In line with these data are results demonstrating that platelet activation by carbon nanoparticles can occur in the absence of pulmonary or systemic inflammation [69,70].

Carboxylated nanotubes have been demonstrated to induce coagulation *in vitro* through the intrinsic pathway dependent on coagulation factor IX [71]. Moreover, factor IXa was found to be physically associated with functionalized MWNTs. Since under physiological conditions the binding of IXa to platelets significantly increases its enzymatic activity, it was suggested that MWNTs induce coagulation by creating a platform for factor IXa. MWNTs with amidated functional groups induce the extrinsic pathway of coagulation

through platelet activation [71]. Despite the differences observed *in vitro*, the thrombogenic properties of carboxylated or amidated MWNTs are comparable *in vivo* [71].

The direct impact of carbon nanotubes on the viability of endothelial cells has been evaluated in several studies. Distress and dysfunction of endothelial cells could increase the risk of thrombogenic complications. For example, exposure of human aortic endothelial cells to SWNTs and MWNTs is accompanied by membrane leakage, actin rearrangement and intercellular contact disruption [72]. Another study confirmed that exposure to carbon black particles causes membrane leakage and accumulation of particles inside cells [73]. However, the magnitude of the impact of carbon black particles on cells was different between these studies, despite the fact that particle concentrations and exposure time were comparable. The discrepancy between the two studies may be attributed to the presence of impurities in tested nanomaterials and to differences in particle size (e.g., the diameter of the carbon black particles in the first study [72] was 37 nm, while it was 248 ± 161.4 nm in the second study [73]). The difference in cell model (human aortic endothelial cells vs HUVEC) could also have contributed to disparate results. The exposure of endothelial cells to carbon nanotubes was reported to induce proinflammatory cytokine production [72,73]; however, it is unclear whether this effect is carbon nanotube mediated, the result of endotoxin contamination and/or the presence of an iron catalyst. The latter two factors are known to skew the results of inflammatory cytokine secretion studies of engineered nanomaterials [74,75]. Carbon black particles were also shown to reduce eNOS expression, which has an impact on the development of prothrombogenic condition [73].

Quantum dots

QDs are photo-stable semiconducting nanocrystals. Advanced fluorescence properties such as tunable emission and broad absorption spectrum make QDs superior candidates for many diagnostic imaging applications [76], while *in vivo* use of these materials is often tempered by concerns about their toxicity.

When thrombogenic properties of QDs (CdSe/ZnS core/shell) with cationic and anionic surface coatings were compared *in vivo* [77], pulmonary thrombogenicity was more prominent in the carboxy-terminated QD group and could be overcome by heparin treatment. Animals treated with carboxy-terminated QDs developed thrombocytopenia. Neither carboxy- nor amine-terminated QDs could induce murine platelet aggregation *per se*, but carboxy-terminated QDs enhanced ADP-induced platelet aggregation *in vitro*. In agreement with the *in vivo* study, the carboxy-terminated QDs were more potent. These data, combined with the observed massive fibrin fibers in thrombi induced by anionic QDs, supported the study's conclusion that carboxy-terminated QDs caused contact activation of the coagulation cascade [77]. At first glance, these results may suggest that the relationship between nanoparticle charge and thrombogenic properties differs among different classes of nanoparticles; for example, dendrimers (for which cationic charge was attributed to thrombogenicity) versus QDs (for which anionic charge seems to be the leading factor). However, a closer look at the characterization data from the QD study suggests that the composition of the analyzed particles does not exactly match their theoretical design, since the ζ -potential of the amine-terminated QDs was negative (-14.2 mV). Further studies with thorough synthesis and nanoparticle characterization are required to verify the role of QD surface charge in their thrombogenicity.

Another study indicates that thrombi form in the pulmonary arteries of NOD/SCID mice 1 week after i.v. administration of human embryonic palatal mesenchymal cells labeled with commercially available QD525 (Invitrogen, CA, USA). However, it is unclear whether the observed thrombogenic effect was associated with the QD *per se* or is a result of the procedure used to load human embryonic palatal mesenchymal cells with the QDs [78].

Several studies reported QD-mediated damage of endothelial cells, which is considered a prothrombogenic event since it leads to the exposure of tissue factor and extravascular matrix. For example, Cd/Te QDs caused dose-dependent decreases in the viability of endothelial cells *in vitro*. Elevated levels of ROS were detected in the presence of QDs, which was associated with DNA and mitochondrial damage [79,80].

Metal nanoparticles

Gold nanoparticles—There is no evidence that gold nanoparticles (GNPs) are thrombogenic; although several studies have reported that GNPs bind fibrinogen [81,82] and the cysteine residues of fibrinogen are responsible for the formation of sulfur–gold bonds [81]. Fibrinogen is one of the most abundant proteins in plasma and many other nanoparticles have also been shown to interact with this protein. Fibrinogen binding to nanoparticle surfaces increases particle size, but does not usually cause coagulation [82]. GNP-potentiated ADP-induced platelet activation occurs in a size-dependent manner; in other words, smaller nanoparticles (in combination with the agonist) caused stronger platelet activation than their larger counterparts. This may be due to different routes of cellular entry, since small GNPs are internalized via the canalicular system, whereas larger GNPs are not [83]. Citrate-stabilized GNPs with hydrodynamic sizes greater than 60 nm did not potentiate the agonist-induced activation of platelets. Some reports suggested that GNPs alter platelet degranulation [83]. A few studies have demonstrated the uptake of GNPs by endothelial cells [84–86]. It is believed that the GNP's core is inert [85] and, therefore, unimportant/inactive for cytotoxicity, whereas size and surface chemistry determine reactivity [86]. The role of nanoparticle shape in the interaction with endothelial cells was studied using spherical, rod-like, hollow gold spheres and silica/gold (core/shell; mono-carboxy(1-mercapto dec-11 ye) hexylene glycol) oligo(ethylene glycol)-coated GNPs; and it was demonstrated that the shape did not correlate with toxicity to endothelial cells [84].

Silver nanoparticles—Studies reporting the interaction of silver nanoparticles (SNPs) with the blood coagulation system are controversial. Several studies utilizing similar experimental approaches and assessing similar end points have reached opposite conclusions. For example, one set of papers concluded that SNPs have innate antiplatelet properties [87,88]. PEG-coated and uncoated SNPs with core sizes of 20 nm and 10–15 nm reduced agonist-induced platelet aggregation in a dose-dependent manner. Various agonists (thrombin, ADP, collagen and arachidonic acid) were used to induce platelet activation, and inhibition of platelet activation by SNPs was observed in each case. However, the degree of the observed inhibition depended on the agonist used and peaked when thrombin and arachidonic acid were used to induce platelet activation [87]. Since thrombin and arachidonic acid activate platelets via different pathways, the inhibition by SNP was attributed to the step the agonists had in common [88]. Cytotoxicity and interruption of membrane integrity were ruled out, since no phosphatidylserine exposure and LDH leakage were observed. Pretreatment of platelets with SNPs resulted in a decrease in the cytosolic Ca^{2+} levels. Microscopy analysis revealed accumulation of SNPs in vacuole spaces inside platelets. Another interesting observation is that SNPs reduced the adhesion of platelets to fibrinogen, but not to polylysine. In addition, SNPs interfered with fibrin retraction [88]. These observations led to the hypothesis that SNPs affect the glycoprotein–fibrinogen interaction, probably through the induction of conformational changes in integrins [87,88]. Another study demonstrated that small (10–15 nm) SNPs can interact with fibrin and induce changes in its conformation. Changes in the secondary structure of the protein caused retardation of fibrin polymerization and clot formation *in vitro*. However, when experiments were performed in plasma, much higher concentrations of the SNPs were required to delay clotting. These data suggest that SNP binding to fibrinogen is not specific, and is easily

outcompeted by other proteins in the plasma, thus decreasing the amount of bound fibrinogen and making the effect on its function less pronounced [89].

In contrast to these results, Jun *et al.* demonstrated that silver nanopowder induced platelet aggregation and PCA in a size- and dose-dependent manner *in vitro*, and enhanced thrombus formation *in vivo* in a rat model of venous thrombosis [90]. Increased intracellular Ca^{2+} levels and thrombogenicity caused by these particles could be counteracted by the presence of the Ca^{2+} chelator EGTA. Platelet aggregation and fibrin polymerization in response to silver colloids were demonstrated in another study by Guildford *et al.* [91].

SNPs' size and charge, as well as solubility, presence or lack of hydrophilic polymers on the surface, and various impurities, may contribute to the differences in results reported by different laboratories.

There are only a few studies investigating SNP effects on endothelial cells. It has been shown that SNPs are not cytotoxic to HUVEC cells *in vitro* and do not affect expression of thrombogenic markers; however, they increase vWF and decrease tPA expression at high concentrations, suggesting that these particles can be prothrombogenic [87].

Iron oxides—Iron oxide nanoparticles are increasingly being used as diagnostic imaging and drug delivery agents, as well as in hyperthermia-mediated therapeutic approaches and cell homing studies. Iron nanoparticles are also found in urban pollution particulate matter due to the widespread use of iron in industrial processes [92].

Instillation of 22-nm iron oxide nanoparticles at dose levels of 0.8 mg/kg caused prolongation of prothrombin times and activated partial thromboplastin times 30 days after exposure. The alteration in plasma coagulation parameters was greater with small (22 nm) nanoparticles than with large (280 nm) ones. Changes in coagulation induced by iron oxide nanoparticles could be due to several reasons, including oxidative stress, inflammation and biodistribution [92]. Functionalization of the iron core prevents induction of an acute inflammatory response and functionalized particles are, therefore, better tolerated [93]. For example, incubation of blood samples with carbon-coated iron carbide nanoparticles with a mean diameter of 30 nm significantly shortened clotting times, while PEGylation greatly reduced this effect [93]. The importance of nanoparticle surface properties was demonstrated in a study of aminated dextran-coated superparamagnetic iron oxide (50 nm) nanoparticles that induced contact activation of kallikrein [94]. This feature inversely correlated with the level of amination of the nanoparticles in that non-/low-aminated particles were more active than their highly aminated counterparts. It is likely that kallikrein was activated through patches on the particle surface not covered by the coating and possibly through other mechanism(s), because nanoparticle-mediated thrombosis in tumors was observed in kininogen-deficient mice [94].

Ceramic nanoparticles—Inorganic nanoporous silica particles are attractive drug carriers to achieve sustained and controlled drug release [95,96]. Silica is also used in industry to produce a wide variety of products, such as for rubber and tire manufacturing, paints, cosmetics, printer ink, and adhesives. Exposure to silica dust is accompanied by increased risk of pulmonary diseases such as silicosis and chronic bronchitis [97]. A recent study reported that synthetic silica nanoparticles induced coagulation through contact activation of Hageman factor XII, while organically modified silica nanoparticles caused the expression of tissue factor in monocytes [98]. PEGylation of these nanoparticles significantly reduced tissue factor-mediated PCA. The nonpolar vinyl groups of organically modified nanoparticles are thought to facilitate opsonization and increased uptake by monocytes. In addition to different surface groups, the particles under comparison also had

different ζ -potentials: synthetic silica nanoparticles were anionic (-25.9 mV), while modified nanoparticles were neutral (-6.2 mV).

The direct effect of anionic amorphous silica nanoparticles of various size (10, 50, 150 and 500 nm) on human platelets was described in the study, demonstrating nanoparticle-mediated activation of glycoprotein IIb/IIIa and surface expression of selectin P [99]. Interestingly, small (10 nm) nanoparticles resulted in stronger platelet activation than larger (50 nm) particles. The thrombogenicity of these nanoparticles was partially reduced by inhibitors of the ADP and MMP2 pathways, suggesting direct interaction between nanoparticles and cellular proteins (e.g., the Ca^{2+} ion channel) or the involvement of other pathways. Silica nanoparticles resulted in the release of both NO and ONOO⁻; however, the latter dominated. A low ratio of NO/ONOO⁻ is considered a marker of oxidative stress and of reduction in NO⁻ availability, which prevents platelet activation. The authors hypothesized that the silica nanoparticles produced this effect as a result of the flux of Ca^{2+} into the cytoplasm, which leads to the activation of eNOS and release of NO. Later, depletion of the substrate causes the uncoupling of eNOS and production of superoxide, which interacts with NO to form ONOO⁻ [99].

The same trends in NO and ONOO⁻ release were observed after exposure of HUVECs to amorphous silica nanoparticles [100]. In agreement with the platelet studies, the effects correlated with concentration and inversely correlated with particle size. The most prominent changes were observed for 10-nm silica nanoparticles. Nanoparticle uptake by HUVEC cells was confirmed by transmission electron microscopy. Oxidative stress was accompanied by the activation of NF- κ B, leading to the upregulation of expression of several proinflammatory genes (*ICAM1*, *VCAM1*, selectin E, *IL-8* and *IL-6*) and inducers of coagulation (tissue factor) [100,101]. In addition, exposure of HUVEC cells to small (10-nm) silica nanoparticles significantly reduced viability, and cell damage is often considered to be a procoagulant factor itself [100]. This study is supported by the results of a study by Napierska *et al.* [102], who demonstrated that smaller amorphous silica nanoparticles (14–16 nm) were significantly more cytotoxic to human endothelial cells (EAHY926) than larger (104 and 335 nm) particles. Cytotoxicity of silica nanoparticles of various sizes (16, 41, 80, 212 and 304 nm) correlated with both nanoparticle size and dose [103]. In addition, 24-h incubation of HUVEC cells with 310 nm particles resulted in exocytosis of Weibel–Palade bodies and release of vWF. Microscopy images demonstrated that the interaction of 310-nm silica nanoparticles with endothelial cells was accompanied by the formation of vWF ultralarge fibers [103].

Since there is no standard dose metric for engineered nanomaterials, the discrepancy between the studies may be due to the use of different dose metrics. For example, the earlier studies used the mass of administered nanoparticles as a measure of the dose [96–99], while the more recent study used the number of particles [103].

A correlation between nanoparticle size and toxicity was also reported by Greish *et al.*, who investigated aminopropyltriethoxysilane-coated silica nanoparticles [7]. CD-1 mice tolerated small (50-nm) particles better than their large (200-nm) counterparts. Although the study concluded that surface groups had no effect on the thrombogenicity, because the toxicity of 200-nm amine-terminated silica nanoparticles was comparable with that of hydroxy-terminated particles of the same size, close examination of the nanoparticle characterization data presented in this study suggest that the conclusions about nanoparticle surface charge may be misleading. The small (50-nm) amine-terminated particles used in this study had negative ζ -potentials of -17.6 mV, while their hydroxy-terminated counterparts were strongly anionic (with ζ -potentials of -61 mV). The large (200-nm) silica nanoparticles with amine-terminated surfaces had ζ -potentials of $+46$ mV, which is consistent with their

intended cationic surface, while their hydroxy-terminated counterparts were anionic (ζ -potential of -72 mV), rather than neutral. These data suggest that either the synthesis was not optimized, which resulted in particles with a different surface functionality from the intended one, or there was an error in their characterization. Taken together, the data warrant further investigation of the role of the surface charge of silica nanoparticles in their compatibility with the coagulation system. Carefully designed and thoroughly characterized materials are a prerequisite for such a study.

Conclusion & future perspective

The current data suggest that nanoparticle thrombogenic properties are largely determined by physicochemical properties: size, charge, density of surface groups, presence of targeting moieties, surface chemistry and composition. Nanoparticles may interact with and modulate the activity of various components of the coagulation system such as platelets, endothelial cells, leukocytes and plasma coagulation factors. Coagulopathies, such as DIC, are increasingly reported for cationic engineered nanomaterials. However, it is unclear whether these are caused by the nanomaterials *per se*, by traces of endotoxin (which is very common in nanoparticle formulations), or by a combination of nanoparticles and endotoxin. The current data also emphasize the importance of careful design and thorough physicochemical characterization of nanoparticles in order to understand coagulation-mediated toxicities and avoid complications associated with the *in vivo* use of engineered nanomaterials.

Since most coagulation disorders are multi-component in nature, a battery of specialized tests both *in vitro* and *in vivo* is needed to screen nanomaterials for their effects and to identify potential problems. One positive aspect of nanotechnology is that nanoparticle physicochemical properties can be tailored to avoid negative results such as thrombogenic complications. Future studies should focus on understanding the mechanisms of nanoparticle thrombogenicity by systematically looking at structure–activity relationships to identify critical parameters, as well as developing approaches to optimize the synthesis of nanomaterials with the aim of avoiding thrombogenicity.

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Executive summary

Background

- Benefits of using nanotechnology in various areas of industry, biology and medicine are often halted by the concerns regarding the safety of engineered nanomaterials.
- Identifying potential adverse effects of nanoparticles on the blood coagulation system and understanding their mechanisms is essential for establishing safety profiles of these new materials.

Undesirable interactions between nanoparticles & components of the coagulation system

- When nanoparticles not intended for the modulation of coagulation interact with components of the coagulation system they may cause adverse reactions.
- Nanoparticles may affect structure and appropriate function of coagulation factors, endothelial cells and platelets.
- Particle size, charge, hydrophobicity and presence of certain coating materials on the surface may influence their effects on components of the coagulation system.
- Each nanoparticle is unique, and their adverse effects and mechanisms may vary, even for nanoparticles of the same category.
- In general, charged and hydrophobic materials are more reactive, and often activation of coagulation occurs through noncanonical pathways.

Conclusion & future perspective

- Nanoparticles can be optimized to improve their compatibility with the coagulation system; understanding the role of nanoparticle physicochemical properties is important to fine tune these materials before their use in nanomedicine.
- Thorough characterization and standardized methods are critical to create an accurate knowledge base about nanomaterials' effects on the coagulation system.

Figure 1. Undesirable interactions between nanomaterials and the coagulation system
When nanoparticles not intended for the modulation of coagulation interact with components of the coagulation system, they may cause pathological processes such as thrombosis and hemorrhage.