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Published in:
Journal of Nanoparticle Research

DOI:
10.1007/s11051-015-3110-6

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

J Nanopart Res (2015) 17:317
DOI 10.1007/s11051-015-3110-6

RESEARCH PAPER

The euglobulin clot lysis time to assess the impact of nanoparticles on fibrinolysis

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Received: 1 September 2014 / Accepted: 6 July 2015 / Published online: 21 July 2015

Abstract Nanoparticles (NPs) are developed for many applications in various fields, including nanomedicine. The NPs used in nanomedicine may disturb homeostasis in blood. Secondary hemostasis (blood coagulation) and fibrinolysis are complex physiological processes regulated by activators and inhibitors. An imbalance of this system can either lead to the development of hemorrhages or thrombosis. No data are currently available on the impact of NPs on fibrinolysis. The objectives of this study are (1) to select a screening test to study ex vivo the impact of NPs on fibrinolysis and (2) to test NPs with different physicochemical properties. Euglobulin clot lysis time test was selected to screen the impact of some NPs on fibrinolysis using normal pooled plasma. A dose-dependent decrease in the lysis time was observed with silicon dioxide and silver NPs without disturbing the fibrin network. Carbon black, silicon carbide, and copper oxide did not affect the lysis time at the tested concentrations.

Keywords Nanoparticles · Fibrinolysis · Hemocompatibility · Blood · Nanomedicine

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Introduction

Nanotechnology is a promising area of growing scientific developments. The development of novel nanomaterials (NMs) and their use in biomedicine has received much attention in recent years. Nanoparticles (NPs) offer many opportunities for medical devices as therapeutics, diagnostics, imaging, and drug delivery (De Jong and Borm 2008; Moghimi et al. 2005).

All these various applications may lead to an increase of human exposure. Due to the reduced particle size and increased surface area, NPs can enter the body and translocate into systemic blood flow after inhalation, ingestion, dermal contact, or systemic administration (Oberdorster et al. 2005). Besides this

risk, information on potential side effects is still lacking (Ahamed et al. 2010; You et al. 2011, 2012). Although the question about the potential toxicity of NPs on human health is considerable and of increasing concern worldwide, it remains a complex issue with currently very limited answers.

New therapeutic and diagnostic agents using nanotechnologies require being hemocompatible (Dobrovolskaia et al. 2008a; Simak 2009). Indeed, NPs in close contact with blood components may impact cells (red blood cells, platelets, leucocytes,…) or physiological processes such as complement, coagulation and fibrinolysis (Simak 2009). Evaluating the blood compatibility (or hemocompatibility) of potential nanomedical products is of primary concern in the context that these products are intended to be administered through the bloodstream (Moghimi et al. 2005). Especially, the evaluation of the thrombogenic potential of a NM aims at exploring how this material may interact with or affect functions of these hemostatic system (Simak 2009). In particular, fibrinolysis is a complex physiological process resulting in the degradation of the fibrin scaffold to avoid thrombosis and to supply oxygen to the surrounding tissue (Flemmig and Melzig 2012). The plasmin, the main enzyme responsible for this degradation, is present in the plasma in its inactive form called plasminogen. Fibrinolysis is highly regulated by activators (i.e., tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA)) and inhibitors (i.e., plasminogen activator inhibitor-1 (PAI-1), α2-antiplasmin, thrombin-activatable fibrinolysis inhibitor (TAFI)) (Collen and Lijnen 1991). Nanoparticles may interact with activators and inhibitors at different stages of the fibrinolytic pathway. A dysregulation of hemostatic balance leads to a disorder with either thrombotic or hemorrhagic symptoms prevailing (Boudjeltia et al. 2002). Based on in vivo adverse effects, thrombogenicity refers to the trend of a material to induce or promote clot or thrombus formation in the bloodstream, thereby possibly leading to thrombotic symptoms. Consequently, preclinical evaluation of NMs on the multifaceted blood system is an essential step to ensure their design as non-toxic agents with optimum hemocompatibility (Dobrovolskaia et al. 2008a).

For NPs, no guidelines are currently available for the evaluation of their hemocompatiblity. Some studies report the hemocompatibility of NPs, in particular on red blood cells, platelets, and coagulation process (Dobrovolskaia et al. 2008b; Krajewski et al. 2013; Laloy et al. 2012, 2014a; Mocan 2013). More specifically, our team assessed and validated some specific techniques for the study of NPs on red blood cell hemolysis, primary hemostasis (platelet adhesion, activation, and aggregation), and secondary hemostasis (blood coagulation) (Laloy et al. 2012, 2014a, b). No data are currently available on the impact of NPs on fibrinolysis. The primary objective of this study is to select an ex vivo test to study the impact of NPs on fibrinolysis, available as a preclinical screening assay. NPs with different physicochemical properties were then tested on the most appropriate selected assay.

Methods

Nanoparticles characterization

Carbon black (CB, Printex, 21 nm) and silicon dioxide (SiO_2, 15 nm), silicon carbide (SiC, 60 nm), and copper oxide (CuO) were obtained, respectively, from Evonik Degussa Corporation (USA), Sigma-Aldrich (USA), and Io-Li-Tec (Germany) as nanopowders. Silver NPs were obtained from partner of the European project NanoValid. They were suspended in water at a stock concentration of 40 mg/mL, stabilized by polyvinyl pyrolidone (PVP), and stored at 4 °C. No trace of endotoxin was found in these NMs using the endotoxin Limulus Amebocyte Lysate kit (Lonza, Switzerland).

The nanopowders were suspended in Tyrode buffer [10 mM Hepes, 6 mM $NaHCO_3$, 137 mM NaCl, 2.7 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, and 0.8 mM NaH_2PO_4, diluted in ultrapure water (milliQ 18.2 MW.cm), pH 7.4]. Tyrode buffer was also used for the dilution of Ag NPs suspension. The NPs stock suspensions were prepared at a concentration of 1 mg/mL for the characterization of NPs in suspension, at 10 mg/mL for the fibrinolysis experiments and were stirred for 30 min.

The physicochemical characterization of the nanopowders was previously reported (Laloy et al. 2012). The average diameter measured by FEG-SEM/TEM, the specific surface area by BET, the bulk composition by EDX, and the surface composition by XPS are presented in Table 1. These experimental data were compared with the information supplied by the manufacturers. The results were generally in agreement with the supplied data. All the studied

Table 1 NPs characterization

NP	Properties	Average diameter by FEG-SEM/ TEM (nm)[a]	Specific surface area by BET (m^2/g)	Bulk composition by EDX (%)[b]	Surface composition by XPS (%)[b]
CB	Measured	36.4 ± 1.3	124	86.6 % C 13.4 % O	87.3 % C 12.7 % O
	Supplied[c]	21	115	NA[d]	NA[d]
SiO_2	Measured	19.9 ± 3.5	68	29.2 % Si 70.8 % O	28.8 % Si 71.2 % O
	Supplied[c]	10–20	140–180	99.5 %[e]	NA[d]
Sic	Measured	53.4 ± 12.4	30	65.6 % C 7.5 % O 26.9 % Si	71.8 % C 26.4 % O 1.8 % Si
	Supplied[c]	60	80	99.5 %[e]	NA[d]
CuO	Measured	11.71 ± 4.6/59.1 ± 28.0[f]	18.4	56.7 % Cu 43.3 % O	49.8 % Cu 50.2 % O
	Supplied[c]	12	NA[d]	99.5 %[e]	NA[d]
Ag	Measured	16 (primary particles) 71 (agglomerates)	NF[g]	4.71 % Ag, 34.22 % O, 60.78 % C, 0.29 % Na	5 % Ag, 23.8 % O, 61 % C, 9.2 % N, 1 % Na
	Supplied[c]	NA[d]	NA[d]	NA[d]	NA[d]

[a] Results are expressed as mean ± 1 SD

[b] Results are expressed as % of atomic element

[c] Information supplied by the manufacturers

[d] NA not available

[e] Product purity

[f] Average length of fiber particles

[g] NF not feasible

NMs have a spheroid shape except CuO which has a fiber shape. For CuO, the average length was also measured (59.1 ± 28.0 nm). Regarding the specific surface area, discrepancies with manufacturer data were observed for most of the NPs confirming the need of a complete physicochemical characterization in studies. The nanopowder surface composition was not similar to their bulk composition for most of the NPs (SiC, CuO and Ag) with a greater content of oxygen in surface. For Ag, the other elements are attributed to traces present in the suspension solution.

Preparation of human normal pooled plasma

Normal pooled plasma (NPP) was prepared with blood from 40 healthy volunteers who were free from any medication for at least 2 weeks. The exclusion criteria were thrombotic and/or hemorrhagic events, antiplatelet and/or anticoagulant medication, pregnancy, and uptake of drugs potentially affecting the platelet functions and/or coagulation factor during the 2 weeks prior to blood draw. A written informed consent was obtained from each donor. The study population displayed the following characteristics: 24 females and 16 males aged from 19 to 48 years (mean age = 25 years) with body mass index (BMI) ranging from 16.8 to 34.6 kg/m^2 (mean BMI = 22.3 kg/m^2).

Blood was collected by venipuncture into tubes containing buffered sodium citrate (109 mM, nine parts blood to one part of sodium citrate solution) (BD Vacutainer®). The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the Centre Hospitalier Universitaire Dinant-Godinne UCL Namur (Yvoir, Belgium).

After collection of blood, the platelet-poor plasma (PPP) was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15 min at 1500 *g* at room temperature. It was immediately frozen at −80 °C after centrifugation.

Preparation of human platelet poor plasma from individual donors

PPP was prepared with blood from seven healthy volunteers who were free from any medication for at

least 2 weeks [3 females and 4 males aged from 23 to 51 years (mean age = 28 years)]. The exclusion criteria and the protocol of centrifugation are similar to the protocol used for NPP.

Euglobulin clot lysis time (ECLT)

It is the most common test used in laboratories to estimate plasma fibrinolytic capacity (Boudjeltia et al. 2002). This method is applied in clinic to normal healthy and pathological people. A shortened lysis time indicates a hyperfibrinolytic state with bleeding risk and an elongated lysis time indicates trend to thrombogenicity (Boudjeltia et al. 2002).

0.5 % of acetic acid was added to 500 µL of plasma in a 19:1 proportion and was incubated at 4 °C for 15 min. Fraction containing plasminogen, plasminogen activators [u-PA, t-PA], and fibrinogen is defined as euglobulin precipitate. The solution is then centrifuged at 2500×*g* for 15 min at 4 °C. The supernatant containing inhibitors of fibrinolysis is discarded. The pellet is redissolved in 500 µM Na_2HPO_4 0.05 M. Once dissolved, 500 µL of solution is placed in a cuvette in the machine thermostatized at 37 °C. 30 µL of NPs dispersed in tyrode buffer or tyrode buffer alone (negative control) is added in the cuvette. 470 µL of euglobulin solution is added. Then, 100 µL of thrombin 20 U/mL [Fibrinogen 5 (STAGO) diluted fourfold] is added to generate the fibrin scaffold. The detection is based on spectrophotometric (890 nm) microtiter plate reader which measures the optical density changes in euglobulin clot of plasma over time (Smith et al. 2003). The reference time for physiological fibrinolysis is between 210 and 540 min. If fibrinolysis occurs between 10 and 30 min, the risk of hemorrhage is high, between 30 and 60 min, the risk is low and between 60 and 120 min, the risk is limited.

The results were expressed as means ± 1 SEM ($n \geq 2$). Statistical analyses were performed with GraphPad Prism software using the unpaired t test.

Scanning electron microscopy

50 µL of tyrode buffer (negative control) or SiO_2 or Ag NPs (final concentration: 100 µg/mL) and 100 µL of $CaCl_2$ (final concentration: 25 mM) are added to 850 µL of NPP. The solution is then incubated in a bath at 37 °C. A fibrin clot is formed within 5 min. 500 µL of 2.5 % glutaraldehyde in cacodylate buffer ($Na(CH_3)_2 \cdot AsO_2 \cdot 3H_2O$ in distilled water at pH 7.4, final concentration : 0.1 M) was added and incubated for 90 min. The glutaraldehyde solution was removed and 400 µL of 0.2 M cacodylate buffer was added. The next step is the dehydration of the sample with successive baths of alcohol from 30° to 100°. A critical point drying was performed with the Balzers Critical Point Dryer (CPD) 030 (BAL-TEC Gmbh®, Germany). Then, a thin layer of platinum (11.6 nm) was deposited under argon atmosphere on the sample with the metallizer Balzers union (BAL-TEC Gmbh®, Germany). The samples were observed with FEG-SEM (Jeol, Japan; resolution of 0.6 nm at 20 keV).

Results

Fibrinolysis assays

Two types of biological laboratory assays to explore fibrinolysis are available: (1) specific assays exploring specific agents of the fibrinolysis process; (2) semi-global fibrinolysis assays studying the semi-global process of fibrinolysis.

Specific fibrinolysis assays do not provide information about the fibrinolysis process but measure the amount or activity of isolate components of the fibrinolytic system. Several assays exist: plasminogen, fibrinogen, alpha-2-antiplasmin (Alpha-2-Plasmin Inhibitor), plasminogen/antiplasmin, tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI) (Bertelmann et al. 2013; Meltzer et al. 2010; Montes et al. 1996; Tait et al. 1992). Another assay, the D-dimers assay, is useful for clinical application since it aims at measuring the fibrin D-dimer levels which is a sign of a recent fibrinolysis and considered as a clinical marker of thrombogenesis (Mullier et al. 2013; Schouten et al. 2013). These specific assays, based on enzyme-linked immunosorbent assays, cannot be applied ex vivo to study the impact of NPs on the fibrinolysis process without resorting to animal experiments. Consequently, we recommend to use semi-global fibrinolysis assay to screen the impact of NMs on the fibrinolysis process.

Among semi-global fibrinolysis assays, thromboelastometry is a viscoelastic whole-blood assay evaluating the hemostatic capacity of blood (Wikkelsoe

et al. 2011). The advantage of this technique is the use of whole blood which is closer to reality than assays performed in plasma. Thromboelastometry is a suitable technique for detection of highly pathological hyperfibrinolysis to evaluate the guide transfusion therapy for diagnosis and goal-directed therapy of perioperative massive hemorrhage (Lier et al. 2013). However, its limited running time of 8 h does not allow applying this technique as a screening assay to evaluate the potential of NMs to disbalance the fibrinolytic process. Indeed, its limited running time of 8 h affords only to detect highly pathological hyperfibrinolysis with no reference to negative control.

Euglobulin clot lysis time (ECLT) is an assay measuring the time required for clot dissolution. Plasma fibrinolytic activity has been assessed by ECLT since late 1950s. The detection is based on spectrophotometric (890 nm) microtiter plate reader which measures the optical density changes in euglobulin clot of plasma over time (Smith et al. 2003).

The ECLT assay was first performed in duplicates with the blood of seven different healthy donors and NPP from 40 healthy volunteers at seven different times. Values of lysis time from healthy donors showed a high inter-individual variability with a minimum value of 63.20 min, a maximum value of 692.1 min, and a coefficient of variation of 89.39 % (Fig. 1). With NPP, we observed a minimum value of 385.3 min, a maximum value of 628.2 min, and a coefficient of variation of 14.68 % (Fig. 1). The data from the individual donors were separated into two groups: 4 individual donors had a lysis time < 200 min (3 females aged 23–24 years and 1 male aged 51 years) and 3 individual donors had a lysis time $>$ 400 min (3 males aged 24–26 years). Consequently, to avoid high inter-donor variability, we decided to perform our experiments using only NPP.

To avoid the inter-experiment negative control value fluctuation, we decided to express the result as absolute value (average of the lysis time of the sample/average of the lysis time of the negative control).

Five different NPs were tested on ECLT using NPP at three final concentrations (1, 10, and 100 µg/mL). SiC and CB NPs had no impact on the fibrinolysis time at the tested concentrations (Fig. 2). CuO NPs demonstrated a dose-dependent decrease in lysis time although not statistically significant. SiO_2 and Ag

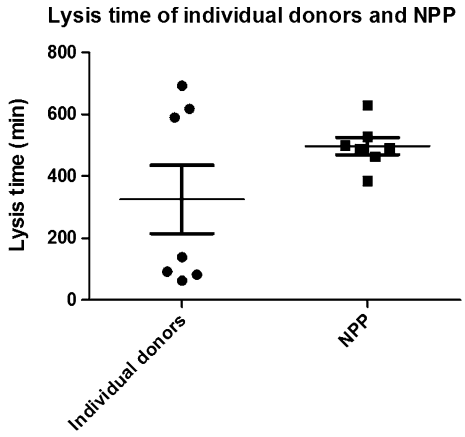

Fig. 1 Lysis time of seven different individual donors and lysis time of NPP from 40 healthy volunteers performed at seven different times ($n = 2$)

NPs were profibrinolytic. Ag NPs induced a significant decrease of the lysis time at 10 µg/mL. At 100 µg/mL, SiO_2 NPs and Ag NPs induced a 1.2-fold and 1.5-fold decrease, respectively, but raw values remained in the physiological range (mean = 377.68 min for SiO_2 NPs and 351.57 min for Ag NPs).

Scanning electron microscopy (SEM) was used as a qualitative technique to study the fibrin fiber morphology as already done in the literature (Swanepoel et al. 2014). Because of a profibrinolytic potential of SiO_2 NPs and Ag NPs on the ECLT assay, we decided to investigate if ultrastructural changes occurred in the presence of these NPs compared to our control, potentially inducing a loose fibrin network explaining the shortened lysis time. SEM micrographs showed no structural change in the fibrin scaffold in the presence of SiO_2 or Ag NPs compared to the control (Fig. 3). In Fig. 3e, a white arrow shows an SiO_2 NPs agglomerate (maximal length: 5.8 µm and minimal width: 2.5 µm).

Discussion

In this work, we evaluated the ECLT to study ex vivo the impact on fibrinolysis of manufactured NMs. This is the first scientific paper assessing methods to study the impact of NMs on fibrinolysis. Five NPs (SiO_2, SiC, CB, CuO, and Ag) with different physicochemical profiles, most of them currently developed in nanomedicine were tested (Chen et al. 2013; de Mel et al. 2012; Pelgrift and Friedman 2013). Their

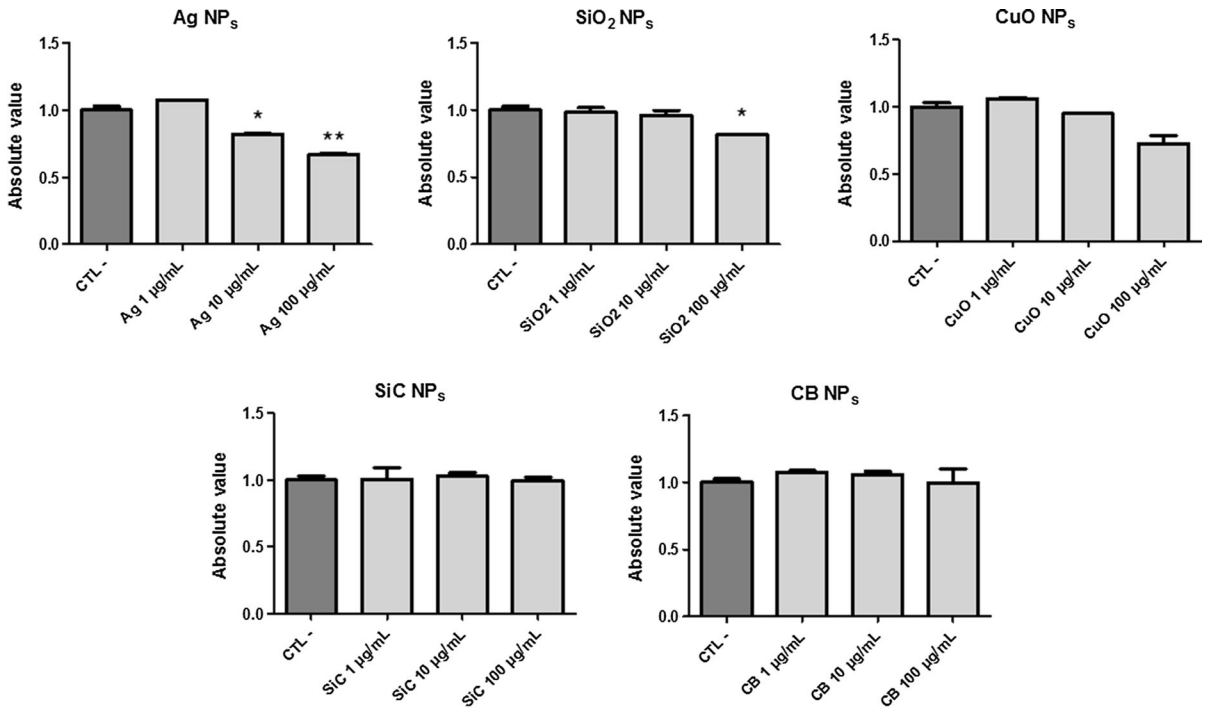

Fig. 2 Impact of different concentrations of NPs reported to negative control in absolute values. The results were expressed as means ± 1 SEM ($n \geq 3$). Statistical analyses were performed with GraphPad Prism software using the unpaired *t* test

physicochemical properties were widely characterized and their potential pro- or anti-fibrinolytic activity was evaluated with the selected assay, the ECLT.

We identified ECLT as the most suitable assay to assess the impact of NPs on fibrinolysis. A spectrophotometric (890 nm) microtiter plate reader measures the optical density changes in euglobulin clot of plasma over time. It is known that NPs interfere with methods using light absorbance detection (Laloy et al. 2012). Although ECLT is based on an optical density, there is no light interference with this assay. Indeed, the informatics system records the absorbance over time, and the time at which there is sudden decrease in absorbance due to clot lysis is recorded which corresponds to the lysis time. Interestingly, even if the tested NPs absorb at the wavelength, they do not mask the detection point, i.e., the change of absorbance at the lysis time. This assay suffers from some limitations that need to be taken into consideration. Because of specific precipitation of euglobulins, it does not measure "natural" fibrinolysis. In fact, during the sample preparation, a part of activators and the majority of inhibitors of fibrinolysis are removed. Because of redissolution of the euglobulin clot in Na_2HPO_4 in the protocol, interactions between NPs and other blood components (RBC, WBC…) or plasma proteins are not taken into account. The potential corona effect induced by plasmatic protein coating on NPs may therefore be underestimated (Dobrovolskaia et al. 2014). Still, ECLT is the most suitable assay to evaluate ex vivo the impact of NPs on fibrinolysis especially for a screening study.

Fibrinolysis time depends on many variables such as the circadian rhythm, the stress, the venous stasis, the weight… (Laffan and Manning 2006) High inter-individual variabilities in the lysis time were demonstrated with the plasma of seven healthy donors during the pre-test. To avoid this high variability, we decided to work with NPP of 40 healthy donors.

No change in the lysis time was observed with SiC and CB NPs. CuO NPs suggested a dose-dependent decrease in the lysis time although not statistically significant. SiO_2 and Ag NPs showed a decrease in the lysis time. However, this decrease in the population of 40 healthy donors was not pathological because raw values of lysis time with SiO_2 and Ag NPs were in the reference range for physiological fibrinolysis

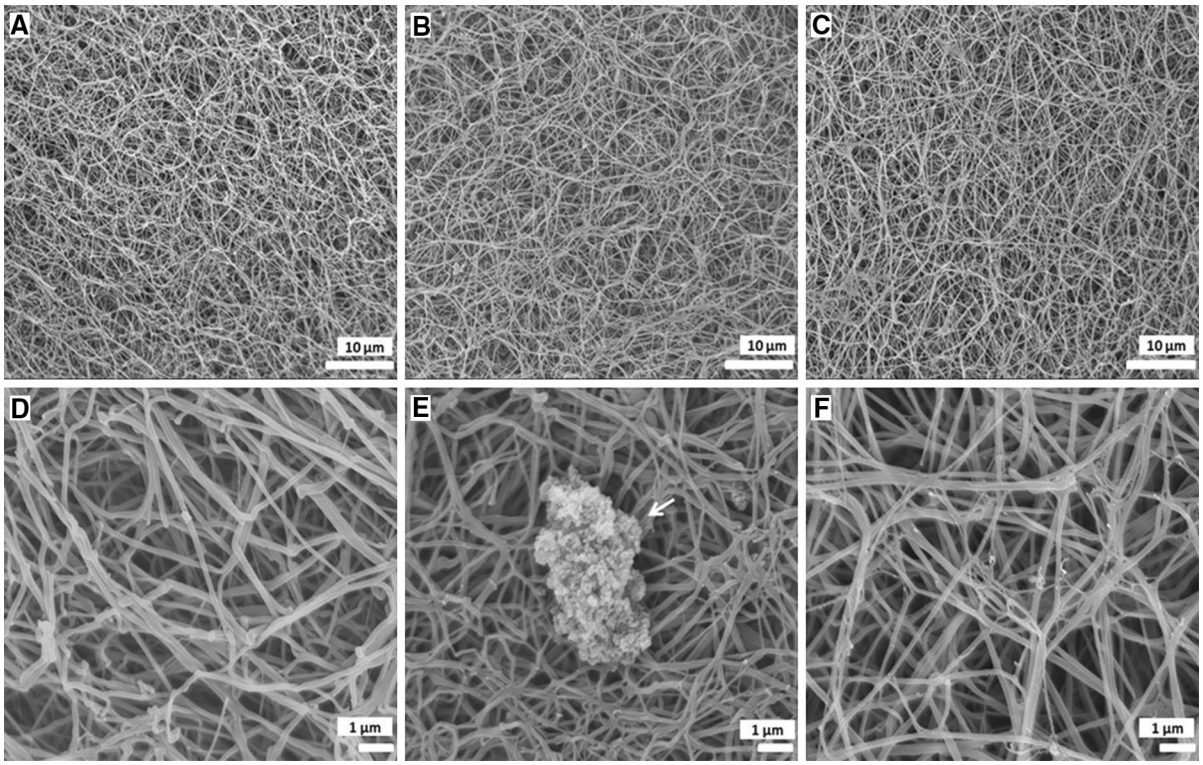

Fig. 3 SEM micrographs: control fibrin clot (**a, d**), fibrin clot in the presence of SiO_2 NPs 100 µg/mL (**b, e**) and in the presence of Ag NPs 100 µg/mL (**c, f**). *Scale bar* = 10 µm (**a–c**); 1 µm (**d–f**). *White arrow*: SiO_2 agglomerate

(between 210 and 540 min). We used SEM as a qualitative technique to study the impact on the fibrin network of NPs showing profibrinolytic potential with ECLT assay, i.e., SiO_2 and Ag NPs. SEM micrographs revealed no ultrastructural changes in the structure scaffold in the presence of SiO_2 or Ag NPs compared to the control. Consequently, these NPs did not prevent the formation of a solid fibrin scaffold as observed with the control. As demonstrated in previous studies, SiO_2 and Ag NPs are also procoagulants (Laloy et al. 2012, 2014a). One potential hypothesis of the underlying mechanism is that the charges of SiO_2 and Ag NPs induce a catalytic surface for coagulation and fibrinolytic factors, disturbing the hemostatic balance.

We observed an agglomerate of SiO_2 NPs in plasma on SEM micrographs with a length of 5.8 µm. The diameter range of capillaries is between 5 and 7 µm (Pries and Secomb 2003). Consequently, there is a concern about NP agglomerates getting trapped in capillary narrowings and disturbing capillary exchanges. The concentration of NPs in the blood stream influences the degree of agglomeration leading to modification of specific surface area that may have an impact on the toxicity. Inherent properties of NPs developed for nanomedicine, such as size and agglomeration potential, should be evaluated to ensure their design as non-toxic agents in the blood stream (Hock et al. 2011).

To promote safe and ethical development of new pharmaceuticals, we support that the nanodrugs have to be subjected to multifaceted blood system testing in early preclinical development prior to animal or human exposure. Indeed, ex vivo nonclinical hemocompatiblity safety studies characterize potential toxic effects and the information provided is helpful in guiding the selection of an initial maximum safe dose.

Conclusion

The development of reliable non clinical assays is essential to assess the hemocompatibility of NMs and to ensure their design as non-toxic agents with

optimum hemocompatibility (Dobrovolskaia et al. 2008b). This is the first scientific paper assessing several methods to explore the impact of NMs on fibrinolysis. Among the existing assays, the ECLT assay was found suitable. Using this method, SiO_2 and Ag NPs showed an impact on fibrinolysis at high concentrations. Taken together with our previous studies on hemolysis, primary hemostasis, and secondary hemostasis, this study provides the scientific knowledge for an international guidance/guideline document on hemocompatibility of NMs.

Acknowledgments The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under Grant Agreement No. 263147 (NanoValid - Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials).

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