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EVALUATION OF NANOPARTICLES PYROGENICITY

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ABSTRACT
Nanoparticles used in different applications, including drug carrier systems, imaging and other biomedical uses. The large surface areas and high reactivity of nanoparticles make them potential targets for contamination with bacterial endotoxins (Paul and Kreyling, 2004; Jain et al., 2015). In addition, the nanoparticle interactions with platelets, coagulation factors and endothelial cells may all contribute to undesirable coagulation-mediated toxicities (Anna and Marina, 2013). The compatibility of administrated nanoparticles with components of the coagulation system depends on their physicochemical properties. Hence, the immunocompatibility is an important safety parameter for biomedical nanomaterial. Moreover, the endotoxin contamination can confound there results of safety screens for pharmaceutical products, causing false-positives for immunotoxicity; therefore, ideally, endotoxin should be accurately quantified in nanotechnology based drugs and devices prior to other safety evaluations (Mayer et al., 2009; Nel et al., 2009; Geraci et al., 2015). One of the common challenges in preclinical development of engineered nanomaterials is their interference with traditional *in vitro* tests (Dobrovolskaia et al., 2009; Dobrovolskaia et al., 2010). The high surface: volume ratios of nanoparticles may lead to high reactivity with proteins, enzymes and other biomolecules under physiological conditions, and can cause interference with Limulus amebocyte lysate (LAL) tests. As an alternative bioassay, *in vitro* macrophage activation test (MAT) as this test is recognized by the current pyrogen and endotoxin testing guidance as an acceptable alternative to the LAL test (US FDA, 2012). However, the applicability of this test is limited to nanoformulations that do not contain cytotoxic agents, since these inhibit endotoxin detection via this assay. Even a few studies also revealed that there is a correlation exit between MAT and RPT for nanomaterials. However, the appropriate method for detection of endotoxin is depending upon type of nanoformulations (Marina et al., 2013). Moreover; there will be need to develop alternative methods that may reliably detect endotoxin in nanoparticle samples when traditional methods cannot be used.

KEYWORDS: Nanoparticels, Pyrogens, RPT, LAL, MAT.

INTRODUCTION
Nanoparticles are worldwide produced and used in various commercially available applications and predictions estimate that by 2014, more than 15% of all products on the global market will have some kind of nanotechnology incorporated into their manufacturing process (Dawson, 2008; Sharon, 2013). The pharmaceutical industry is developing increasing numbers of drugs and diagnostics based on nanoparticles, and evaluating the immune response to these diverse formulations has become a challenge for scientists and regulatory agencies alike (Marina et al., 2009). Besides their ubiquitous lucrative effects, also toxic effects have been reported (Oberdorster et al., 2005). It cannot be excluded that nanoparticles, especially when they were not kept sterile, can be contaminated with endotoxin during production or handling. Therefore, endotoxin contamination should be assessed when evaluating the potential toxicity, to distinguish specific nanoparticles toxicity from the endotoxin effects.

Nanotechnology, the term derived from the Greek word ‘nano’, meaning ‘dwarf’, applies the principles of

engineering, electronics, physical and material science, and manipulation and study of structures in the 'nanometre' size - the same basic size as small as biological entities (Riehemann et al., 2009). The area of nonmaterial has been increasingly expanding during the last decade, ranging from optical systems, electronic, chemical industries, to environmental engineering and medicine, which up to now has resulted in approximately 800 nanoparticles-containing (Riehemann et al., 2009). The reason why nanoparticles (NP) are attractive for such purposes is based on their important and unique features, such as their surface to mass ratio, which is much larger than that of other particles and materials, allowing for catalytic promotion of reactions, as well as their ability to adsorb and carry other compounds. The reactivity of the surface originates from quantum phenomena and can make NP unpredictable since, immediately after generation, NP may have their surface modified, depending on the presence of reactants and adsorbing compounds, which may instantaneously change with changing compounds and thermodynamic conditions. Therefore, on one hand, NP have a large (functional) surface, which is able to bind, adsorb and carry other compounds such as drugs, probes and proteins. On the other hand, NP has a surface that might be chemically more reactive compared to their fine analogues (Paul and Kreyling, 2004). In nanotechnology, a particle is defined as a small object that behaves as a whole unit in terms of its transport and properties. It is further classified according to size: in terms of diameter, fine particles cover a range between 100 and 2500 nanometres, while ultrafine particles, on the other hand, are sized between 1 and 100 manometers. Similar to ultrafine particles, nanoparticles are sized between 1 and 100 manometers. These particles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials (Cristina et al., 2007; ASTM, 2012). Although the size of most molecules would fit into the above outline, individual molecules are usually not referred to as nanoparticles. Nanoclusters have at least one dimension between 1 and 10 manometers and a narrow size distribution. Nano powders are agglomerates of ultrafine particles, nanoparticles, or nanoclusters. Nanometer-sized single crystals, or single-domain ultrafine particles, are often referred to as nanocrystals (Abhilash, 2010). Nanoparticles are prepared by following either top-down approach or bottom-up approach, which makes characteristic changes at the quantum level of polymers or atoms. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nanoscale size-dependent properties are often observed. Nanoparticles are not merely small crystals but an intermediate state of matter placed between bulk and molecular material. Independently of the particle size, two parameters play dominant role (i) the charges carried by the particle in contact with the cell membranes and (ii) the chemical reactivity of the nanoparticles (Chanchal et al., 2014).

The measurement of pyrogens is an important safety measure for parenterally applied drugs. Pyrogens (greek pyros: fire) can cause fever, hypotension and shock with organ failure and ensuing death. The severity of the adverse reaction depends on the concentration and biological activity of the respective Pyrogen. They have been measured with the so-called *in vivo* rabbit pyrogen test, where drugs are injected into at least three rabbits and a fever reaction is measured by a rectal probe, or *in vitro* like LAL (Limulus amoebocyte lysate test) based on a coagulation reaction of the amoebocytes of Limulus polyphemus when brought into contact with endotoxins.Pyrogens can be divided into two classes i.e. exogenous pyrogens, such as endotoxin from Gram-negative bacteria that induce fever when applied intravenously; and endogenous pyrogens that are induced inside the body as a reaction to the contact with exogenous pyrogens and which cause an elevation in body temperature (endogenous pyrogens have potent pyrogenic and inflammatory activities and include interleukin 1-a (IL-1a), interleukin-1b (IL-1b), tumor necrosis factor a (TNF-a) andinterleukin-6 (IL-6) (Tim Sandle, 2015).

The most potent pyrogens are the endotoxins produced from the cell walls of the Gram-negative bacteria (lipopolysaccharide). Generally, pyrogens have a high molecular weight, often, more than 1,000,000 Daltons (Tim Sandle, 2015). In terms of the nature of pyrogenicity, pyrogenic reactions and shock are induced in mammals upon intravenous injection of endotoxin at low concentrations (from 1 ng/mL) (Fiske et al., 2001; Pardo-Ruiz et al., 2016). The maximum level of endotoxin for intravenous applications of pharmaceutical and biologic products is set at 5 endotoxin units (EU) per kg of body weight per hour by each of the major pharmacopoeias (Daneshiam et al., 2006). The term EU describes the biological activity of an endotoxin. For example, 100 pg of the standard endotoxin EC-5 and 120 pg of endotoxin from *Escherichia coli* O111:B4 have activity of 1 EU (Hirayama and Sakata, 2002).Owing to its high reactivity and stability, the US FDA has established limits on endotoxin in pharmaceutical products and medical devices. For all routes of administration, except intrathecal, the thresholds are 5 EU/kg/hand 0.5 EU/ml for drugs and medical devices, respectively (USP, 2007). For intrathecally administered drugs and devices, the allowed endotoxin levels are lower: 0.2 EU/kg/h and 0.02 EU/ml for drugs and devices, respectively (USP, 2007), since theseproducts contact cerebrospinal fluid.

METHOD TO DETERMINE PYROGENCITY

There are two methods traditionally used in industry to detect and quantify endotoxin in pharmaceutical products and medical devices: the *in vitro* limulus amoebocyte lysate (LAL) assay and the *in vivo* rabbit pyrogen test (RPT) (US FDA, 2012). The most sensitive rabbit strains

develop a significant temperature increase upon exposure to 500 pg (i.e.5 IU) of reference LPS/kg. If the highest permitted volume (10 ml/kg body weight, depending on the drug characteristics) is injected, the resulting detection limit is 500 pg per 10 ml or 50 pg/ml (Hasiwa et al., 2003), while the human fever threshold lies around 30 pg/ml (Greisman and Hornick, 1969). Currently, the LAL test is the assay of choice for the determination of endotoxin in medicines, biological products and medical devices (Hurley, 1995). In general, three different LAL assays are used worldwide: gel clot, turbidimetric (increase in turbidity) and chromogenic (color formation) assay. A good overview of the different assays, along with their advantages and disadvantages, can be found in the review article of Hurley (Hurley, 1995). The LAL test exists in three main formats: gel-clot, chromogenic and turbidimetric. Theturbidimetric and chromogenic LAL can be performed in either end-point or kinetic mode. Although the FDA accepts all three LAL formats, historically, it was common practice in industry to use the gel-clot LAL to resolve a discrepancyof results between different LAL formats. This approach was recently incorporated into a FDA recommendation as part of a guidance document (ISO/FDIS, 2010). This is in contrast with the FDA's recently retired guidance on the LAL test, which dates back to 1987, and did not include guidance on how to resolve disparities between LAL formats (Dobrovolskaia et al., 2010). The new guidance recognizes two methods (ISO/FDIS, 2010) the recombinant factor C assay and the macrophage activation-type pyrogen test – as LAL alternatives and reviews considerations regarding validation of the alternative methods for a given product. Moreover, In spite of the new ISO norm published in 2010 on endotoxin test on nanomaterial samples for *in vitro* systems (ISO/FDIS, 2010), not much is known in which way nanoparticles interfere with the different types of LAL assays (Dobrovolskaia et al., 2010).

CHALLENGES TO ANALYZE ENDOTOXIN CONTAMINATION OF NANOMATERIALS

Drug formulations using nanotechnology are finding increasing application in many areas of medicine, especially for the treatment of cancer. Many nanomedicines for targeted drug delivery of chemotherapeutics have matured beyond the discovery phase of research, and a few such products are US FDA approved and on markets. Such targeted nano drug delivery systems can minimize dosages, reduce systemic toxicity and reduce adverse side effects of chemotherapeutics, increasing the overall therapeutic efficacy. Endotoxin contamination can be a significant hurdle to the preclinical development of nanoparticle formulations (US FDA, 2012).The large surface areas and high reactivity of nanoparticles make them potential targets for contamination with bacterial endotoxins (Vallhov et al., 2006). These factors, along with the fact that nanoparticles are frequently synthesized on equipment that may be novel to the pharmaceutical industry, causes endotoxin contamination to be common among many nanoparticle formulations undergoing preclinical characterization (Vallhov et al., 2006). Such contamination may cause misleading results in toxicity screens (nanoformulations that are not inherently toxic may appear so due to contamination) and in efficacy tests for certain applications (e.g., endotoxin *per se* has shown anticancer efficacy). The endotoxins contamination is often difficult to identify due to nanoparticle interference with traditional assays (Hall et al., 2007). Many conventional (i.e., not nano) pharmaceutical formulations also interfere with tests for endotoxin detection, but the large variation in nanoparticle physicochemical properties causes the spectrum of nanoparticle interferences to be quite wide, making an individual instance of interference difficult to detect. Moreover, In spite of the new ISO norm published in 2010 on endotoxin test on nanomaterial samples for *in vitro* systems (ISO/FDIS, 2010), not much is known in which way nanoparticles interfere with the different types of LAL assays. Nanoparticle interactions with platelets, coagulation factors and endothelial cells may all contribute to undesirable coagulation-mediated toxicities. 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 (Mayer et al., 2009; Nel et al., 2009), which, in turn, determines particle interaction with proteins (Twohy et al., 1984; United States Pharmacopeia-National Formulary, 2005; European Pharmacopoeia, 2005). Proteins involved in blood coagulation (e.g., fibrinogen) are common components of the 'protein corona' (European Pharmacopoeia, 2005). 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 (Nel et al., 2009; Sperling et al., 2009).

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 (Sperling et al., 2009). Likewise, anionic polystyrene nanoparticles activate intrinsic

coagulation (Oslakovic et al., 2012). 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.

Polymeric nanomaterials

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

Dendrimers

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* (Malik et al., 2000), 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 polypropyleniminedendrimers 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 (Stasko et al., 2007). 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 (Greish et al., 2011). This toxicity was dose dependent and observed when dendrimers were injected at dose levels exceeding their maximum tolerated dose (Greish et al., 2011). 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 (Dobrovolskaia et al., 2011; Dobrovolskaia et al., 2012; Jones et al., 2012). In agreement with their DIC-like toxicity *in vivo*, only cationic PAMAM dendrimers, but not their anionic and neutral counterparts, induce platelet aggregation (Dobrovolskaia et al., 2011; Jones et al., 2012) and PCA in HL-60 cells (Dobrovolskaia et al., 2012) 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 (Dobrovolskaia et al., 2011; Dobrovolskaia et al., 2012; Jones et al., 2012). 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 (Dobrovolskaia et al., 2011). *Invitro* 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 (Dobrovolskaia et al., 2012) of interest is the finding that cationic PAMAM dendrimers do not induce PCA in normal leukocytes, but exaggerate endotoxin-induced PCA (Dobrovolskaia et al., 2012). 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 (Inoue and Takano, 2011). 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, non-reacted 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 (Mayer et al., 2009). In contrast to theoretical expectations, activation of platelets was independent of nanoparticle surface charge (Mayer et al., 2009). 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 (McGuinnes et al., 2011). Likewise, amine-modified nanoparticles decreased thrombin generation through depletion of factors VII and IX (Oslakovic et al., 2012). The effect was more prominent for small nanoparticles than for their large counterparts (Oslakovic et al., 2012). In contrast to the cationic nanoparticles, anionic ones activated the intrinsic coagulation pathway, which was also size-dependent, but more prominent for large particles (Oslakovic et al., 2012). 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 (Oslakovic et al., 2012).

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 (Juliano et al., 1983). 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 (Koziara et al., 2005). 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 (Zbinden et al., 1989). These effects were attributed to the interaction between the anionic liposomes and coagulation factors XII and XI (Zbinden et al., 1989). 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 (Constantinescu et al., 2003). The observed interactions may result from internalization of liposomes, or fusion of cellular and liposome membranes (Male et al., 1992; Nishiya et al., 1995; Nishiya and Sloan, 1996). It is believed that interactions between liposomes and platelets are responsible for the transient thrombocytopenia observed after administration of negatively charged liposomes (Reinish et al., 1988). 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 (Campbell et al., 2002; Krasnici et al., 2003; Dabbas et al., 2008). 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 (Dabbas et al., 2008). 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 (Mitsuno and Ohyanagi, 1985; Lowe and Bentley, 1992) and activation of the complement system (Vercellotti et al., 1982), administration of PFC nanoemulsions resulted in transient changes in platelet counts, which were restored in patients 30 min after injection (Mitsuno and Ohyanagi, 1985). 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 (Cuignet et al., 2000). Clinical data regarding propofol are controversial in that both prothrombotic (Thomson et al., 2008) and antithrombotic (Beule et al., 2007) 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 (Nemmar et al., 2004; Barregard et al., 2008; Li et al., 2008; Ravichandran et al., 2011). The latter triggers production of proinflammatory cytokines (TNF-α, IL-1β and IL-8) and contributes to the development of inflammation (Salvi et al., 1999; Salvi et al., 2000; Baulig et al., 2003; Pourazar et al., 2005; Alfaro-Moreno et al., 2007; Erdely et al., 2009). Inflammation is a very potent procoagulant as many factors inducing inflammation can also trigger coagulation (Levi et al., 2010). 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 (Ghio et al., 2003; Liao et al., 2005). Inhalation studies in mice revealed elevated plasma levels of PAI-1 after exposure to multiwalled carbon nanotubes (MWCNTs) and single-walled carbon nanotube (SWCNTs) (Erdely et al., 2009). 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 (Nemmar et al., 1999; Nemmar et al., 2004). In addition, ultrafine particles were shown to penetrate lung tissue and distribute into the systemic circulation (Nemmar et al., 1999; Nemmar et al., 2004). The presence of inhaled radioactively labeled ultrafine carbon particles in the circulation of volunteers was detected within the first 10–20 min (Nemmar et al., 2002). 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 (Mills et al., 2006). Although several animal studies have confirmed cardiovascular, coagulation and pulmonary toxicities associated with the inhalation of carbon nanomaterials (Nemmar et al., 2003; Warheit et al., 2004; Lam et al., 2004; Inoue et al., 2005; Silva et al., 2005; Radomski et al., 2005), 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 (Nemmar et al., 2003). 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 (Nemmar et al., 1999; Nemmar et al., 2003;). 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 (Silva et al., 2004). 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 (Radomski et al., 2005). 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 (Radomski et al., 2005). 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. collagen-induced activation of platelets depended on the protein kinase C signaling pathway, platelet activation by MCN, SWNT and MWNT was protein C independent (Radomski et al., 2005). 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. (Bihari et al., 2010). These original findings were further verified and explored in more detail by Simak's laboratory (Semberova et al., 2009; Lacerda et al., 2011). In several elegant studies, this group has demonstrated that, unlike water-soluble fullerene derivatives, $C_{60}(OH)_{24}$ and nC60, MWCNT induce activation and aggregation of platelets. Activation of platelets by MWNT was accompanied by elevated levels of extracellular Ca^{2+} and could not be simply attributed to damage of cellular membranes because it was downregulated by calcium (Ca^{2+}) 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 Ca^{2+}. This study also proposed that intracellular membranes differ from plasma membranes; they are dense tubular systems that are probably more sensitive to nanotube perforation (Lacerda et al., 2011). Depletion of stored intracellular Ca^{2+} induced influx of Ca^{2+} from the outside and activated store-operated Ca^{2+} entry. Interestingly, despite the fact that water-soluble fullerene derivatives were detected inside platelets, they did not induce depletion of intracellular Ca^{2+} stores. In addition to the changes in intracellular Ca^{2+} storage, MWNTs induced the release of platelet membrane microparticles, which are capable of stimulating platelets and other cells (Semberova et al., 2009; Lacerda et al., 2011). In line with these data are results demonstrating that platelet activation by carbon nanoparticles can occur in the absence of pulmonary or systemic inflammation (Khandoga et al., 2004; Khandoga et al., 2010). Carboxylated nanotubes have been demonstrated to induce coagulation in vitro through the intrinsic pathway dependent on coagulation factor IX (Burke et al., 2011). 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 (Burke et al., 2011). Despite the differences observed in vitro, the thrombogenic properties of carboxylated or amidated MWNTs are comparable in vivo (Burke et al., 2011). 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 (Walker et al., 2009). Another study confirmed that exposure to carbon black particles caused membrane leakage and accumulation of particles inside cells (Yamawaki and Iwai, 2006). 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

(Walker et al., 2009) was 37 nm, while it was 248 ± 161.4 nm in the second study (Yamawaki and Iwai, 2006). 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 (Yamawaki and Iwai, 2006; Walker et al., 2009); 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 (Vallhov et al., 2006; Pulskamp et al., 2007). Carbon black particles were also shown to reduce eNOS expression, which has an impact on the development of prothrombogenic condition (Walker et al., 2009).

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 (Pericleous et al., 2012), 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 (Geys et al., 2008), 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 (Geys et al., 2008). 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 (Ramot et al., 2010). 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 (Wang et al., 2010; Yan et al., 2011).

Metal nanoparticles

Gold nanoparticles

There is no evidence that gold nanoparticles (GNPs) are thrombogenic; although several studies have reported that GNPs bind fibrinogen (Dobrovolskaia et al., 2009; Chen et al., 2011; Deng et al., 2013) and the cysteine residues of fibrinogen are responsible for the formation of sulfur–gold bonds (Chen et al., 2011). 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 (Dobrovolskaia et al., 2009). 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 (Deb et al., 2011). 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 (Deb et al., 2011; Ilinskaya and Dobrovolskaia, 2013). A few studies have demonstrated the uptake of GNPs by endothelial cells (Pan et al., 2009; Alkilany and Murphy, 2010; Bartczak et al., 2012; Shilo et al., 2015). It is believed that the GNP's core is inert (Alkilany and Murphy, 2010) and, therefore, unimportant/inactive for cytotoxicity, whereas size and surface chemistry determine reactivity (Pan et al., 2009; Shilo et al., 2015). 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 (Bartczak et al., 2012).

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 (Shrivastava et al., 2009; Ragaseema et al., 2012; Huang et al., 2016). 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 (Ragaseema et al., 2012; Huang et al., 2016). Since thrombin and arachidonic acid activate platelets via different pathways, the inhibition by SNP was attributed to the step the agonists had in common (Ragaseema et al., 2012; Huang et al., 2016). 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 (Shrivastava et al., 2009). These observations led to the hypothesis that SNPs affect the glycoprotein–fibrinogen interaction, probably through the induction of conformational changes in integrins (Shrivastava et al., 2009; Huang et al., 2016). 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 (Shrivastava et al., 2011).

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 (Jun et al., 2011). 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. (2009). 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 (Ragaseema et al., 2012).

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 (Zhu et al., 2008; Sarti et al., 2015). 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 (Zhu et al., 2008). Functionalization of the iron core prevents induction of an acute inflammatory response and functionalized particles are, therefore, better tolerated (Herrmann et al., 2011). 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 (Herrmann et al., 2011). 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 (Simberg et al., 2009). 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 (Simberg et al., 2009; Wu, 2015).

Ceramic nanoparticles

Inorganic nanoporous silica particles are attractive drug carriers to achieve sustained and controlled drug release (Lu et al., 2007; Xia et al., 2009; Kwon et al., 2013). 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 (Merget et al., 2002; Karkhanis and Joshi, 2011). 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 (Tavano et al., 2010; Liu et al., 2012). 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 (Corbalan et al., 2012) 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− (Tavano et al., 2010). The same trends in NO and ONOO− release were observed after exposure of HUVECs to amorphous silica nanoparticles (Corbalan et al., 2011; Guo et al., 2015). 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-κB, 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) (Liu et al., 2010; Corbalan et al., 2011; Guo et al., 2015). 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 (Corbalan et al., 2011; Guo et al., 2015). This study is supported by the results of a study by Napierska et al. (2009), 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 (Bauer et al., 2011). 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 vWFultralarge fibers (Bauer et al., 2011).

APPROPRIATE METHOD FOR DETECTION OF ENDOTOXIN DEPEND UPON NANOFORMULATIONS

Nanoparticles and nanomaterials are increasingly being explored for use in drug delivery, imaging and other biomedical applications (Geraci et al., 2015). Immunocompatibility is an importantsafety parameter for biomedical nanomaterials. Endotoxin contamination can confound the results of safety screens for pharmaceutical products, causing false-positives for immunotoxicity; therefore, ideally, endotoxin should be accurately quantified in research-grade nanotechnologybased drugs and devices prior to other safety evaluations (Nel et al., 2009; Tenzer et al., 2011). One of the common challenges in preclinical development of engineered nanomaterials is their interference with traditional *in vitro* tests (Nel et al., 2009; Dobrovolskaia et al., 2010). The LAL test is no exception and many nanomaterials have been shown to interfere with one or more formats of the LAL test (Cooper, 1990; Cooper et al., 1997; Bohrer et al., 2001; Ochiai et al., 2001; Fujita et al., 2011). Many studies havealready previously reported that research-grade nanomedicine formulations show a greater rate of interference with the gel-clot LAL than with other LAL formats, and expressed a concern that for nanomedicines, relying on the gel-clot LAL may underestimate endotoxin levels and possibly lead to endotoxin overdose (Dobrovolskaia and McNeil, 2012). Recently, Smulders *et al.* (2012) reported similar LAL test interference from several types of environmental nanoparticles. A National Cancer Institute immunotoxicity workshop led us to develop a decision tree for selecting an appropriate LAL format (Dobrovolskaia et al., 2010). This decision tree recommended using the RPT to resolve a discrepancy between two or more LAL formats for a given nanoformulation. This approach proved reliable in distinguishing between real contamination and nanoparticle interference in research-grade nanoformulations (Nel et al., 2009). The recent study by Smulders *et al.* (2012) took a similar approach, but relied on an *in vitro* assayutilizing a TLR4-transfected reporter cell line to verify LAL results. Research-grade nanomedicine formulations and nanoparticles used for environmental studies are often created from materials and using equipment not traditionally used in biological applications and may be especially prone to endotoxin contamination. The high surface: volume ratios of nanoparticles may lead to high reactivity with proteins, enzymes and other biomolecules under physiological conditions, and can cause interference with LAL tests (US FDA, 2012). Sample dilution is one of the most common approaches used to resolve interference with LAL assays (US Department of Health and Human Services, 1987; USP, 2007; Dobrovolskaia et al., 2009), but care has to be taken not to simply dilute to the point that endotoxin is undetectable. The maximum valid dilution (MVD) is determined by the sample's initial concentration and the LAL format sensitivity, with gel-clot LAL having the lowest sensitivity (0.03 EU/ml),

followed bychromogenic (0.001 EU/ml) and turbidity LAL (0.001 EU/ml), which have comparable sensitivities. Research-grade materials are often tested *in vitro* and in animals at doses that are higher than what eventually becomes the clinical dose. Because of this, one may speculate that some of the observed interference with the gel-clot LAL in research-grade formulations is simply due to insufficient dilution. Once the clinical dose is determined, the MVD calculated from that (presumably lower) dose, may be free from interference. The study was conducted to test clinical-grade nanoformulations, which have optimized physicochemical properties and established dose levels, in the LAL assays and to verify the applicability of alternative bioassays in instances of discrepancy among different LAL formats.

As an alternative bioassay, *in vitro* macrophage activation test (MAT) as this test is recognized by the current pyrogen and endotoxin testing guidance as an acceptable alternative to the LAL test (Dobrovolskaia et al., 2009). Many studies revealed that a correlation between MAT and RPT for nanomaterials (Dobrovolskaia et al., 2010). Nanoparticle interference with the limulus amoebocyte lysate (LAL) test for endotoxin levels has been previously reported for research- grade and environmental nanoparticles. This study tests commercial clinical-grade nanoformulations for interference with the LAL was established by using six clinical-grade formulations: Abraxane, Abelcet, Amphotec, Depocyts, Feraheme and Visudyn. All formulations were measured using dynamic light scattering to determine their hydrodynamic diameter, by laser Doppler velocimetry to determine their zeta potential and by gel-clot LAL, end point chromogenic LAL and kinetic turbidimetric LAL to determine endotoxin levels. The interference with one or more LAL formats was observed for some formulations at stock concentrations, but sample dilutions below the maximum valid dilution eliminated this interference. All formulations tested negative for endotoxin in the gel-clot LAL test. For 50% of the tested formulations, all three LAL formats resulted in endotoxin measurements, which were in agreement. For 83% of the tested formulations, there was agreement between two LAL formats. All three LAL formats produced different endotoxin values for one formulation. These data suggest that relying on the gel-clot LAL alone may lead to a higher likelihood of underestimating endotoxin values than applying a decision tree, in which at least two LAL formats are used for each formulation and discrepancies between LAL results are resolved by bioassay. Hence nanoparticle interference with the LAL test has been reported for metal colloids (Smulders et al., 2012), polymeric nanoparticles (Nel et al., 2009; Dobrovolskaia et al., 2010), nanocrystals (Dobrovolskaia et al., 2010) and liposomes (Harmon et al., 1997; Sakai et al., 2004). The interference is not unique to only research-grade formulations and but it was also observed with clinical-grade nanomaterials. Moreover, the bioassays such as the MAT, are useful for verifying LAL data, however, the applicability of these tests is limited to nanoformulations that do not contain cytotoxic agents, since these inhibit endotoxin detection via this assay. Moreover, in situations where test results from two different LAL assays are more than 25% different, verification by some other method is needed, unless interference with one of the test formats can be conclusively demonstrated. Traditionally, the RPT was used by industry to exclude pyrogenic products from pharmaceutical pipelines. In recent years, *in vitro* tests have gained greater application, due to their lower costs and to ethical issues associated with animal testing. As such, in the last two decades, LAL testing has almost completely replaced the RPT for drug testing. However, the medical device industry still utilizes both the LAL and RPT tests. Therefore the use of the RPT to verify the LAL data still is used in these situations.

CONCLUSION

Nanoparticles have been used broadly for applications in medical field, drug discovery, drug delivery and diagnostic. The detection of pyrogenicity in NP is a rapidly emerging concern because of its vast use in research and clinical formulations. The endotoxins contamination is often challenging to detect due to nanoparticle interference with traditional assays. In addition, the nanoparticle interactions with platelets, coagulation factors and endothelial cells may all contribute to undesirable coagulation-mediated toxicities. Moreover, In spite of the new ISO norm published in 2010 on endotoxin test on nanomaterial samples for *in vitro* systems, not much is known in which way nanoparticles interfere with the different types of LAL assays and it will require decades to sort outwhat is true and what is relevant. The additional testing demand for NP adds to the urgency of developing new advanced approaches to check pyrogenicity in nanoformulations. Taken together, it appears that nanopyrogenicity, to a large extent, is dependent on the use and further development of alternative approaches. The more we know what we are looking for, the better we can target our testing. If we have no hypothesis, screening in many models and black-box types of animal tests might be the only way forward. Last but not least though NPs are different, but they are not so different that we should expect completely new hazards.

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