Endotoxin contamination: a key element in the interpretation of nanosafety studies

The study of toxicity and potential risks of engineered nanoparticles is of particular importance in nanomedicine. Endotoxin, a common contaminant of bacterial origin, has biological effects that can mask the true biological effects of nanoparticles, if its presence is overlooked. In this review, we report the features of nanoparticle contamination by endotoxin, and the different biological effects of endotoxin-contaminated nanoparticles. We will describe different methods for endotoxin detection applied to nanoparticles, and discuss their pros and cons. Eventually, we describe various methods for eliminating endotoxin contamination in nanoparticles. Although there is no universal technique for efficiently removing endotoxin from nanoparticles, specific solutions can be found case by case, which can allow us to perform nanosafety studies in biologically relevant conditions.

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The effects of engineered nanomaterials on the biological processes and, consequently, their safety for human health and the environment are currently a topic of considerable interest. As for all novel nanomaterials, with particular regard to nanomedicines and health-related nanomaterials, immunological safety is a major aspect, given the key role of the immune system in defending the integrity of our body and maintaining its health. Several studies have shown that different kinds of nanomaterials can trigger inflammation in different *in vitro* and *in vivo* models, suggesting a possible risk for human health [1–3]. However, when considering these kind of studies, we should be aware of the fact that engineered nanomaterials might be contaminated by microbial components, such as bacterial endotoxin (also known as lipopolysaccharide [LPS]), a biologically active molecule with significant toxic and inflammatory effects. The innate immune

system is exquisitely able to recognize and react to foreign agents, particularly microbial products. The presence of endotoxin can generate misleading experimental results both in *in vitro* and *in vivo* assays aiming at evaluating the toxic and inflammatory effects of nanomaterials. Therefore, it is important to discriminate between the biological effects from the unrecognized contaminants from the *bona fide* nanoeffects, when assessing the possible inflammatory/toxic effects of nanomaterials [4,5].

Bacterial endotoxin

Endotoxin or LPS is a large molecule (molecular weight 200–1000 kDa) found on the outer membrane of Gram-negative bacteria. Endotoxin consists of a polysaccharide region and a lipid region (lipid A), which is responsible for inducing toxicity. In mammals, including humans, the toxic/inflammatory effects of LPS are mediated by the TLR4 sig-

Yang Li^{*1} & Diana Boraschi¹

¹Laboratory of Innate Immunity & Cytokines, Institute of Protein Biochemistry, National Research Council, 80131 Naples, Italy

*Author for correspondence:
yang.li.nano@gmail.com

naling pathway. LPS binds to the LPS-binding protein, and then engages the TLR4 receptor with the support of the co-receptors CD14 and MD2, thereby initiating cell activation. TLR4 is expressed by many cell types, which are therefore responsive to LPS challenge. Among these, activation of innate immune cells such as monocytes and macrophages by LPS through TLR4 is a major step of the defensive inflammatory response against bacteria [6–8]. LPS-stimulated cells produce a variety of inflammatory factors, including the cytokines IL-1 β , TNF- α and IL-6. Our previous research has shown that as low as 0.1 EU/ml of LPS/endotoxin can upregulate the expression of inflammatory genes in primary human monocytes [9].

Endotoxin is very toxic to humans and could cause various life-threatening diseases. Endotoxin acquired from the environment can cause respiratory symptoms, pulmonary inflammation and asthma [10–16]. High levels of endotoxin in blood circulation (called endotoxemia) can occur in some systemic infections or severe trauma, causing a systemic response such as fever, hypotensive shock, impaired organ function and eventually multiple organ failure and death [17,18]. Therefore, intravenously (iv.) administered drugs and surgical instruments must be endotoxin-free, to avoid severe effects due to contaminating LPS. The maximum permissible endotoxin levels in iv. drugs have been declared and regulated by the US FDA as early as 1985 and updated thereafter [19]. This regulation obliges pharmaceutical companies to follow and control their iv. drugs for bacterial endotoxin/pyrogen contaminations.

Endotoxin contamination is common in nanomaterials

Endotoxin is a heat-stable ubiquitous contaminant. It is present in almost all chemicals and glassware used in laboratories, unless specific precautions are taken [20]. It is important to be aware that endotoxin cannot be eliminated by sterilization methods [21]. Our evaluation on a number of commercially available nanomaterials showed that most of them are contaminated by endotoxin [UNPUBLISHED DATA]. A previous study has shown that different batches of the same nanomaterial synthesized in the same laboratory have different levels of endotoxin contamination depending on the variability in the cleanliness during the synthetic procedure (Figure 1) [9]. These findings underline the notion that endotoxin contamination commonly occurs in nanomaterial samples if no precautions are taken during their synthesis [22].

Given the highly reactive nature of their surface, nanomaterials can adsorb a wide range of molecules from the surrounding milieu, to reduce their surface-

free energy. Hence, unintentional contaminants can be accumulated on the surface of nanomaterials by adsorption of air- or solution-borne species [23]. Endotoxin is one of the molecules that can attach to nanomaterials. Endotoxin is likely to adhere to hydrophobic surfaces through its lipid moiety, while its phosphate group allows it to bind to positively charged surfaces. Therefore, endotoxin is in theory able to attach to all kinds of surfaces [20,23]. Because of these features, endotoxin is not only a contaminant in nanomaterials, but an extensive contaminant in all kinds of biomaterials [20,24]. This situation suggests caution in the interpretation of data showing the toxic/inflammatory effects of nanomaterials, since the unrecognized presence of endotoxin in nanomaterials/biomaterials may actually account for most of the observed effects or synergize with nanomaterials to induce cell activation or toxicity. Thus, the reliability of nanotoxicological data, either *in vitro* or *in vivo*, depends on discriminating true nanoeffects from the effects of biologically active contaminants, of which bacterial endotoxin is the most common.

Endotoxin effects in nanomaterials

As mentioned above, endotoxin binds easily on the surface of nanoparticles. More than a decade ago Darkow *et al.* have tested endotoxin binding to functionalized nanoparticles. They showed that the capacity of binding endotoxin depends on the nanoparticles' ability to form Coulomb and van der Waals interactions with the endotoxin molecule, which is influenced by the nature of the suspension medium [25]. Karl *et al.* have also shown that endotoxin could adsorb to nanoparticles' surface, and that such adsorption was reduced when the nanoparticle surface was pretreated with phosphate. This pretreatment might cause particle aggregation thereby reducing the surface area available for LPS adsorption [26]. A recent study tested the LPS adsorption on the surface of functionalized paramagnetic nanoparticles, showing strong interaction between nanoparticles and the lipid A portion of LPS [27]. Another study on the interaction between LPS and polystyrene latex particles' surfaces proved that hydrophobic lipid A strongly binds to the surface of polystyrene particles. The orientation of the LPS molecules on the particles' surface was shown to be similar to that observed in the outer membrane of bacteria [28]. A more recent study showed that the polysaccharidic chain of LPS could strongly bind to the surface of Al₂O₃ nanoparticles, and LPS formed a layer on the nanoparticle surface detectable by atomic force microscopy [29]. The same study also showed that high pH, ionic strength and sulfate concentration can decrease the adsorption of LPS molecules on nanoparticles [29].

Figure 1. Endotoxin contamination in freshly synthesized gold nanoparticles. Left panel: endotoxin contamination in four different batches of 4 nm spherical Au NPs synthesized in the same laboratory. Right panel: the biological response to endotoxin by human primary blood monocytes shows that as little as 0.1 EU/ml of endotoxin can induce a potent reaction (upregulation of the inflammatory *IL1B* gene).

AU: Arbitrary units; Au NPs: Gold nanoparticles.

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Eventually, our recent data show that endotoxin is rapidly and stably adsorbed to gold nanoparticles' surface in a dose-dependent manner [30]. Indeed, because of the high affinity of nanoparticles for endotoxin, several studies are aiming at using nanoparticles for removing endotoxin contamination (see below).

We thus know that endotoxin could bind to the nanomaterials surface, but what are the biological effects of this endotoxin contamination? We have already mentioned that LPS has potent inflammatory activity, which mainly goes through the activation of the TLR4 receptor on the surface of innate/inflammatory leukocytes. Activation of the NLRP3 inflammasome is a key element in inflammatory activation, as it leads to the production of the active form of the cytokine IL-1 β , which has a central role in both protective and pathological inflammation [31,32]. Endotoxin not only induces the upregulation of the gene encoding pro-IL-1 β , the inactive form of IL-1 β [33], but it also primes the NLRP3 inflammasome to respond to activating stimuli such as lysosomal cathepsins or ATP [34–36]. Thus, the presence of endotoxin in nanoparticles may actually take part in all phases of IL-1-dependent inflammation, by inducing gene upregulation and also inflammasome activation. Several studies have shown that nanoparticles can actually bind and activate the LPS receptor TLR4 [37–40], and other studies have shown that nanoparticles can

activate the NLRP3 inflammasome by diverse mechanisms [41,42]. The involvement of possible contamination with endotoxin should be thoroughly examined in all studies, like those mentioned above, in which LPS is known to be a major activating stimulus, in order to dissect the *bona fide* effects of nanoparticles from those due to the presence of endotoxin.

Some researchers have already addressed the effect of endotoxin adsorbed on nanoparticles (Table 1). Cho *et al.* have investigated the interaction of orthopaedic wear particles with endotoxin, and found that the presence of endotoxin adsorbed on the particles can markedly enhance their inflammatory activity and affect implant loosening by inducing localized inflammation [43]. Ashwood *et al.* showed that 200 nm titanium dioxide nanoparticles can strongly bind LPS bridged by calcium cations, and that the LPS-nanoparticle complexes can significantly increase the caspase-1-dependent IL-1 β secretion and apoptotic-like cell death as compared with clean nanoparticles and to LPS alone. These effects can be inhibited by blocking both phagocytosis and receptor-mediated uptake of nanoparticles [44]. A few studies have indicated that micro-sized particles containing LPS can cause a significant increase of inflammatory cytokine production in primary human monocytes [45,46] and murine RAW264.7 macrophage-like tumor cells [47]. Other data showed that co-administration of endo-

Table 1. The biological effects of endotoxin in nanoparticles.

Particle type	Size	Cell or animal	Endotoxin	Particle concentration	Effects	Depyrogenation methods	Ref.
<i>In vitro</i>							
Four different orthopedic wear particles	Micro	RAW 264.7	1 mg/ml LPS preincubated with particles	10–100 particles/cell	LPS-treated particles significantly increase the IL-1 α , IL-1 β TNF- α production compared with clean particles	Ethanol or acetic acid washing or heating at 175°C for 3 h	[43]
Dietary grade titanium dioxide	200 nm	Human peripheral blood mononuclear cells	1 ng/ml LPS preincubated with particles	5 μ g/ml	LPS-precoated particles induce apoptosis-like cell death and significantly increase IL-1 β , IL-6, and TNF- α production compared with particles alone. These effects are inhibited by blocking scavenger receptor-dependent phagocytosis	No information	[44]
BaSO ₄ , ZrO ₂ and CMW3 prosthetic particles	1.3 μ m microparticles	Human monocytes	Present as contaminant	3 $\times$ 10 ⁸ or 6 $\times$ 10 ⁷ particles/ml	Endotoxin-contaminated particles induce TNF- α production. Such production is due to the presence of endotoxin onto the surface of particles	Extensive washing in endotoxin-free water, dried and γ -irradiated (25 kGy)	[45]
Air pollution particulates from indoor and outdoor	PM2.5 and PM10-2.5, microparticles	Human monocytes	Present as contaminant	1 $\times$ 10 ⁶ cells with 100 μ l of particle extract, total volume 500 μ l	Only endotoxin-contaminated outdoor air pollution particulates significant induce inflammatory effects. Endotoxin inhibition with lipopolysaccharide binding protein could inhibit the inflammatory effects	Endotoxin removal affinity resin from associates of Cape Cod	[46]
Polytetrafluoroethylene particles	Micro	Mouse L929	0.5 ng/ml LPS added together with particles	1–5 mg/ml	Treatment with LPS and particles significantly increases the TNF- α production compared with particles alone	No information	[47]
Amorphous silica nanoparticles	10–20 nm	Lung epithelial cell line A549	100 ng/ml LPS added together with particles	5, 50, 100, 200 μ g/ml	LPS enhance the low levels of oxidative stress and cytotoxicity induced by silica nanoparticles	Not relevant (originally endotoxin free)	[49]
Titanium particles	1–3 μ m	IC-21 Macrophages	7.8–780 ng/ml LPS preincubated with particles	1:1–1:1000 cell to particle ratios	LPS-coated particles stimulate the release of IL-6 and TNF- α more than particle alone	Combination of 1N HCl + ethanol + 10% Triton X-100 + 160°C for 24 h	[53]
Gold nanoparticles	10–15 nm	RAW 264.7	100 ng/ml LPS preincubated with particles	10–40 μ g/ml	Nanoparticles inhibit NO production and IFN- β expression induced by LPS	No information	[54]
LPS: Lipopolysaccharide; NO: Nitric oxide; ROS: Reactive oxygen species.							

Table 1. The biological effects of endotoxin in nanoparticles (cont.).						
Particle type	Size	Cell or animal	Endotoxin	Particle concentration	Effects	Depyrogenation methods Ref.
<i>In vitro</i> (cont.)						
Platinum nanoparticles	2.4 nm	RAW 264.7	10 µg/ml LPS added together with particles	100 µM	Nanoparticles significantly reduce the LPS-induced intracellular ROS production and the production of inflammatory cytokines (IL-1β, IL-6, TNF-α) induced by LPS	No information [56]
Single-walled carbon nanotubes	Over 150 nm long	RAW 264.7	1 ng/ml LPS preincubated with particles	10 µg/ml	LPS-induced inflammation is inhibited by serum-coated nanotubes, and inhibition is lost when serum protein binding was altered with an emulsifying agent (Pluronic F127)	No information [57]
PEGylated gold nanoparticles	28 nm	RAW 264.7	100 ng/ml LPS added together with particles	40 µg/ml	Treatment with LPS and particles significantly increases the inflammatory effect compared with particles and LPS alone	No information [58]
PVP-coated silver nanoparticles	116 nm	A549/monocyte-derived dendritic cells and macrophage co-culture	1 µg/ml LPS added together with particles	0.03, 0.3 and 3 µg Ag/cm ² and 10, 20 and 30 µg Ag/ml	Both air-liquid interface and submerged cultures used. No synergistic inflammatory effects were observed upon co-exposure to particles and LPS	No information [59]
Single-/multi-walled carbon nanotubes, carbon black, quartz	1–2 nm diameter single-walled carbon nanotube, 10–20 and 30–50 nm diameter multiwalled carbon nanotube, 14 nm carbon black, <5 µm quartz	A549 and rat alveolar macrophage cell line NR8383	100 ng/ml LPS added together with particles	5, 10, 50, 100 µg/ml	Carbon nanotubes do not alter the LPS-induced inflammation (TNF-α and IL-8); Carbon black and quartz slightly increase LPS-induced IL-8	Acid treatment during purification from metal impurities [61]
Silica, titania, zirconia, cobalt nanoparticles	15–200 nm	PMA-differentiated human myelomonocytic cell line U-937	100 ng/ml LPS preincubated with nanoparticles	50 to 400 µg/10 ⁶ cells	Different effects depending on the type of particle and the end point cytokine (TNF-α, IL-1β, IL-1Ra)	180°C for 4 h [62]
LPS: Lipopolysaccharide; NO: Nitric oxide; ROS: Reactive oxygen species.						

Table 1. The biological effects of endotoxin in nanoparticles (cont.).

Particle type	Size	Cell or animal	Endotoxin	Particle concentration	Effects	Depyrogenation methods	Ref.
<i>In vivo</i>							
Carbon black nanoparticles	14 and 56 nm	ICR male mice, 6-week-old	2.5 mg/kg LPS, alone or together with particles intratracheal 24 h before experiment	4 mg/kg, intratracheal 24 h before experiment	Nanoparticles have little inflammatory effect, co-administration of LPS and nanoparticles significantly increases the LPS-induced lung inflammation, this effect being more evident with smaller particles	250°C for 2 h	[48]
Diesel engine-derived nanoparticles	10–100 nm	ICR male mice, 6-week-old	125 µg/kg LPS intratracheal 12 h before experiment	15, 36 or 169 µg/m ³ intratracheal 5 h before experiment	Nanoparticles alone do not induce lung inflammation, but exacerbate LPS-induced lung inflammation in a dose-dependent manner	No information	[50]
Gold nanoparticles	30 nm	300 to 350 g male Wistar rats	1 mg/ml LPS 2 h before test, subcutaneous	40 mg/ml, subcutaneous	Nanoparticles decrease LPS-induced inflammation and oxidative damage in rat's uveitis	No information. Particles suspended in endotoxin-free water for inoculum	[55]
Latex nanomaterial	20, 50 and 100 nm	29 to 33 g ICR male mice, 6-week-old	75 µg/animal alone or together with nanoparticles, intratracheal 24 h before experiment	125 or 250 µg/animal, intratracheal 24 h before experiment	Nanoparticles have little effect on lung inflammation, but increase the LPS-induced lung inflammation. Smaller particles have more serious effects	No information	[51]
Single-/multi-walled carbon nanotubes	Not clear	ICR male mice	33 mg/kg together with carbon nanotubes	4 mg/kg	Nanotubes alone induce lung inflammation and enhance expression of inflammatory cytokines, but do not synergistically exacerbate LPS-induced inflammation	No information	[60]
Titanium dioxide particles	15, 50, 100 nm	ICR male mice	2.5 mg/kg together with nanoparticles, intratracheal 24 h before experiment	8 mg/kg, intratracheal 24 h before experiment	Nanoparticles increase the LPS-induced lung and systemic inflammation, the effect being more evident with smaller particles	No information	[52]

LPS: Lipopolysaccharide, NO: Nitric oxide, ROS: Reactive oxygen species.

toxin could promote the adverse effects of engineered nanoparticles on lung functions both *in vitro* and *in vivo* [48–52]. Titanium particles, which deliberately contaminated with endotoxin, triggered inflammation in macrophages by inducing the production of inflammatory cytokines. However, these inflammatory effects of contaminated titanium particles seem to be quantitatively weaker than those obtained with endotoxin alone, implying that upon binding, endotoxin partly loses its activity [53]. However, the unclear study design does not allow a precise quantitative comparison of the concentrations of bound versus unbound endotoxin, and therefore the data reported cannot be considered conclusive. The possibility may also exist that the titanium particles had an anti-inflammatory effect that counteracted the endotoxin activity. In this direction, Ma *et al.* have proved that the gold nanoparticles can suppress the endotoxin-induced NO production by inhibiting the NF- κ B and IFN- β /STAT1 pathways in RAW264.7 cells [54]. Furthermore, Pereira *et al.* showed that gold nanoparticles could inhibit cell activation induced by endotoxin through the TLR4-NF κ B pathway, resulting in decreased inflammation and oxidative damage in rat's uveitis [55]. Platinum nanoparticles could inhibit the intracellular ROS production and inflammatory activities in murine macrophages exposed to endotoxin and nanoparticles [56]. Single walled carbon nanotubes coated with serum proteins (mainly albumin) could inhibit LPS-induced cyclooxygenase-2 activation in RAW264.7 cells, an effect that was lost when the albumin adsorption was blocked by treating the nanotube surface with a nonionic surfactant [57]. On the other hand, Liu and co-authors have proven that gold nanoparticles can enhance LPS-induced NO production and inflammatory effects in macrophage cell lines [58]. Our recent studies also show that endotoxin-treated gold nanoparticles are highly active in inducing the inflammatory response of human monocytes *in vitro* compared with the clean gold nanoparticles, which have no effect [30]. However, other studies show that LPS does not synergize with nanoparticles in inducing inflammatory effects when co-administered with silver nanoparticles *in vitro* and carbon nanotubes *in vivo* and *in vitro* [59–61]. Lucarelli and co-authors found that different types of ceramic and metallic nanoparticles had different abilities that modulate the inflammatory/anti-inflammatory responses of human macrophage-like cells *in vitro*, depending both on the type of nanoparticle and on the presence of LPS [62]. Thus, the interaction of endotoxin with nanoparticles may vary depending on the type, shape and surface charge of the nanoparticles, the type and amount of endotoxin, and the cell type or biological entity/environment under investigation. In any

case, there is little doubt that the presence of endotoxin may affect the impact of engineered nanoparticles on a biological system.

Endotoxin measurement methods

The biological effects of endotoxin could mask or interfere with the true biological effects of nanomaterials. Hence, reliably discriminating the endotoxin activity from the nanomaterials' intrinsic inflammatory activity is important for nanosafety studies. The rabbit pyrogen test (RPT) and the Limulus amoebocyte lysate (LAL) assay are the most commonly used endotoxin detection methods that are approved by FDA and EMA, and accepted by almost every country. FDA approved the RPT assay in 1943. However, due to the high cost and long turnaround time of the assay (and the need of using animals), RPT is now only applied in combination with the LAL test for analyzing the parenteral drugs during the earlier development phase. As the most popular endotoxin detection method, the LAL assay could supply fast, sensitive and reasonably specific endotoxin assessment. The LAL assay also measures the activity of β -glucan, which however can be inhibited by a specific buffer, thus making the assay specific for endotoxin. FDA issued the final guideline on LAL testing in 1987 [63]. In 2000, the US and the European Pharmacopoeias have issued harmonized documents for LAL testing. In July 2011, FDA has withdrawn the 1987 guideline on LAL assay, considering it obsolete and not reflecting the Agency's opinion on the topic anymore. Drug manufacturers should now refer to the US Pharmacopeia General Chapter 85 Bacterial Endotoxins Test, which provides information on the performance and acceptance criteria for endotoxin testing [64]. Similar documents can also be found in European, Chinese and Japanese Pharmacopoeias. The FDA published a new guidance recommendation in 2012, in which two new methods are discussed as alternative LAL methods, the recombinant Horseshoe Crab factor C assay (specific for endotoxin, not recognizing β -glucan) and the macrophage activation-type pyrogen test (very sensitive but not specific for endotoxin) [65]. However, the FDA recommends validating the alternative methods for each product.

In the LAL assay, an enzyme in the blood cells (amoebocytes) of the horseshoe crab *Limulus polyphemus* causes protein clotting when activated by endotoxin (Figure 2). There are three different variants of the LAL assay: gel-clot, chromogenic and turbidimetric, in which the reaction is measured by different means.

The LAL assays are designed for soluble molecules and are active within a certain range of ionic strength. Thus, each assay should be accurately validated when

Figure 2. Schematic illustration of the *Limulus amoebocyte lysate* reaction. The reactions at the basis of the different types of LAL assays are indicated.

LAL: *Limulus amoebocyte lysate*.

applied to endotoxin detection in nanomaterials. In fact, the nanoobjects' peculiar physico-chemical properties (including their optical density) could significantly interfere with the LAL readouts, thereby leading to false results (Figure 3) [9,66]. The major advantages and disadvantages of the different LAL assays for testing endotoxin in nanomaterials are listed in Table 2.

The ISO 29701:2010 regulation mentions the possibility that nanomaterials can interfere with the optical readouts of LAL assays and recommends to run appropriate controls when measuring endotoxin in nanomaterial samples [67]. Despite this recommendation, little is known of the potential interference of nanomaterials with LAL assays. Thus, evaluating the interference of nanomaterials with the LAL assays' components and readouts is a necessary requirement in order to reliably detecting endotoxin contamination in nanomaterial preparations [4]. Several years ago, Brooks *et al.* tested the endotoxin contamination in microparticles by gel-clot and chromogenic LAL assays. They found that the gel-clot assay is not suitable and suggested to use the chromogenic LAL method instead, given its high sensitivity and lack of interference by particles [45]. Dobrovolskaia *et al.* obtained contradictory results when using different LAL formats to test the same kind of nanoparticles [68]. They suggested that at least two LAL formats should be used to evaluate the pres-

ence of endotoxin in nanomaterials. Moreover, when the results from two LAL assays differ by more than 25%, the LAL results should be verified with RPT before the nanoparticle could enter the *in vivo* testing phase [4,68]. The Nanotechnology Characterization Laboratory of NCI (NIH, USA) has used this strategy to characterize nanoparticles for the presence of endotoxin and claimed to have solved the above-mentioned conflicting LAL results [68,69]. Smulders *et al.* compared different endotoxin detection assays (gel-clot LAL assay, chromogenic LAL assay and endotoxin detection method with TLR4 reporter cells) for an accurate evaluation of endotoxin contamination in nanomaterials [70]. They stated that the gel-clot LAL is not adequate, while the other methods were not significantly perturbed. However, they did not evaluate the interference of nanomaterials with the assays' readouts. Our experimental results with the chromogenic assays showed that interference by metal and metal oxide nanoparticles could occur not only at the level of the final readout (optical density of the nanoparticles at 405 or 540 nm) but also by adsorption and subsequent quenching of the colored product released from the substrate (p-nitroaniline), thereby resulting in underestimating the endotoxin levels [66]. In a recent study, various types of silica nanoparticles were found able to interfere with the gel-clot LAL assay [71]. In another

Figure 3. Interference of nanoparticles with the readouts of three different *Limulus amoebocyte lysate* assays.

Interference by Au nanoparticles (A, C & E) and by Ag nanoparticles (B, D & F) was measured on the reading of pNA at 405 nm (A & B), azo dye (pNA plus diazo reagents) at 540 nm (C & D) and fluorescence at excitation/emission 380/440 nm (E & F). In (E & F), the dotted lines are the positive fluorescence control (generated by 1 EU/ml endotoxin in the fluorogenic assay), and shaded areas represent the standard deviation above and below the mean. Data are the mean $\pm$ standard deviation of triplicate determination within one test representative of three performed.

* $p < 0.05$ vs control; ** $p < 0.01$ vs control.

FU: Fluorescence unit; OD: Optical density; pNA: Para-nitroaniline.

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Table 2. Strengths, weaknesses, opportunities, threats analysis of different Limulus amoebocyte lysate assays for use with nanoparticles.

Parameter	Gel-clot	Turbidimetric	Chromogenic	Fluorogenic	EndoLISA
Strengths	Easy, no special equipment needed, cheap	Quantitative, highest sensitivity, cheaper vs chromogenic	Quantitative, high sensitivity (highest for kinetic test), kinetic test is automatic, one step, easy, end point test can use two different wavelengths	Very specific, high sensitivity	Washing steps can eliminate interfering substances compared with other Limulus amoebocyte lysate assays, very specific, high sensitivity, wide endotoxin detection range could eliminate dilution of samples, easy performance (ELISA-like)
Weaknesses	Semi-quantitative, low sensitivity, low specificity, variability, subjectivity	Low specificity, it may be interfered by nanoparticles at high optical density, end point test is not commercially available, kinetic test needs special equipment and longer time	High cost, low specificity, likely to be interfered with by nanoparticles with absorbance at or close to 405 or 540 nm, end point test is less sensitive than kinetic, kinetic test needs special equipment and longer time	High cost, requires special equipment	High cost, requires special equipment (fluorescence microplate reader)
Opportunities	In the case of doubt or dispute with other tests, the final decision is usually made based on the gel-clot technique, although not clear if this may also apply to nanoparticles	Can be applied to nanoparticles that interfere with the chromogenic assay, by using them at low optical density. High sensitivity allows to increase nanoparticle maximal dilution, which will also decrease possible interference with the assay	Can be used with nanoparticles having high optical density, after appropriate controls. High sensitivity allows to increase nanoparticle maximal dilution, which will also decrease possible interference with the assay	May be used for most nanoparticles that do not have autofluorescence	May be used for all nanoparticles, because of extensive washing procedure (which could eliminate all the nanoparticles in the wells)
Threats	Not precise, prone to mistakes due to subjective performance, easy to obtain misleading results with nanoparticles	May be interfered by most nanoparticles, in particular at high concentrations, due to their turbidity	Specificity and nanoparticle interference must be controlled	Nanoparticles may significantly interfere with enzyme reaction, or quench fluorescence	Nanoparticles may interfere with the step of LPS binding to the antibody pre-coated wells. Not clear if washing could detach LPS (bound to the wells) from particles (which would be washed off), remove LPS from wells together with particles (on which surface it is tightly bound) or leave particles in the wells (because attached to LPS). Residual particles may significantly interfere with enzyme reaction or quench fluorescence if not washed out from the wells

CAUTION: Nanoparticles may adsorb and modify enzyme and substrate, thereby interfering with the assay development. Nanoparticles can also adsorb endotoxin and alter its detection in the assay.

LPS: Lipopolysaccharide.

study, Dobrovolskaia *et al.* tested various clinical-grade nanoparticles with different LAL assays [69], and again found that the gel-clot LAL assay is not suitable. They eventually stated that no LAL format was optimal for all the tested nanoparticles. In industry, the gel-clot LAL is commonly used to resolve discrepancy of results between different LAL formats. This approach was recently incorporated into a FDA recommendation as part of a guidance document [65]. However, as reported above, for the specific case of nanomaterials, the gel-clot LAL seems largely inappropriate. This evidence also challenges the guidance documents for bacterial endotoxin testing in nanomedicine products.

In order to eliminate the interference with the LAL assay, dilution of nanoparticle samples is recommended. A study published in 1984 reports that, among 333 drug products tested, 236 (71%) interfered with the LAL test if not previously diluted or treated [72]. Thus, an accurate detection of endotoxin contamination can be achieved by diluting the nanoparticles to a concentration that shows no readout interference. Maximum valid dilution is the maximum dilution of the samples at which the presence of endotoxin is still detectable. The maximum valid dilution depends on the sensitivity of the LAL formats, the sample's initial concentration and the endotoxin limit under the clinical usage (0.2 EU/kg for intrathecal drug; 5 EU/kg for drugs delivered by other routes) [64]. These limits however do not apply to nanosafety studies, in which clinical doses are not defined. Therefore, sample dilution should be carried out until interference disappears. In the case that endotoxin is below the assay's detection limit at such dilution, the endotoxin contamination of the sample could not be appropriately determined. For instance, a nanoparticle sample interferes with the assay unless diluted 1/1000, and at this dilution the LPS detection is below the assay sensitivity limit (0.1 EU/ml). Thus, the endotoxin contamination of the sample cannot be determined, and we can only say that it is <100 EU/ml. In this case, either we select a different assay for determining the endotoxin content (less prone to interference by these particles), or we use the nanoparticles in nanosafety studies at concentrations that are for sure free of endotoxin (i.e., at least diluted 1/1000).

To eliminate the problem of nanoparticle interference with the LAL assay, an endotoxin extraction method is mentioned in the ISO 29701:2010 regulation. In fact, an extraction protocol could be found in the 'Guideline on LAL' as early as 1987 for medical devices [63]. Some extraction methods have been described for airborne dust samples and microparticles [45,73,74]. An extraction procedure has been developed for carbon nanomaterials in 2010 [75]. Smulders *et al.* performed

an extraction procedure on nanoparticle samples in the attempt of measuring the endotoxin contamination by assessing the levels of extracted LPS, thereby avoiding the problem of nanoparticle interference with the assay. However, no measurable endotoxin could be recovered, suggesting that the extraction method was not efficient [70]. In fact, a study performed in 1999 has reported a significant loss of the endotoxin recovered after extraction from commercial titanium nanoparticles, leading to underestimation of the real endotoxin contamination [76]. Brooks *et al.* found that increasing the particle soaking time and including various sonication steps for extraction could increase the endotoxin recovery, but none of the extraction procedures could recover endotoxin at the level that is measurable *in situ* [45].

The European Centre for the Validation of Alternative Methods (ECVAM) published various highly sensitive and reliable bioassays for assessing pyrogens, such as the human PBMC activation assay and the human monocyte activation assay (MAT assay). Another possible assay is based on reporter cell lines obtained by co-transfecting the human TLR4, MD-2 and CD14 genes (all participating to the LPS receptor complex), and an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP is controlled by an IL-12 p40 promoter that can be activated by NF- κ B and activator protein 1 (AP-1). TLR4 agonists (e.g., LPS) can induce SEAP production by activating NF- κ B and consequently activating the IL-12 p40 promoter. Thus, LPS contamination can be detected and quantitated in terms of SEAP production [70,77]. However, none of these bioassays is specific for endotoxin, because any pyrogen (theoretically including nanomaterials) can trigger the same biological effects (usually the NF- κ B-dependent production of inflammatory cytokines) in the PBMC and MAT assays, since different molecules besides endotoxin can activate TLR4 or can activate NF- κ B through other receptors. Thus, the pyrogen bioassays (also including the old RPT *in vivo*) are not adequate for specifically detecting the endotoxin contamination in nanomaterial samples. Dobrovolskaia *et al.* suggested to use the bioassays only for confirming LAL findings, limited to those nanomaterials that do not cause cytotoxicity [69]. Some reports showed that the TLR4 signaling pathway is involved in the uptake and inflammatory effects induced by titanium dioxide nanoparticles [78–80]. Some researchers have shown that functionalized gold nanoparticles and graphene oxide particles could induce TLR4-dependent activation, autophagy and macrophage death in the absence of endotoxin [37–39]. These results however, as in many other cases, could be considered valid only when the presence of endotoxin or of other TLR4 ligands will

be formally proven. Thus, the use of bioassays should be considered only for confirming LAL data, and in any case it should be subjected to the appropriate controls.

Eliminating the endotoxin contamination in nanomaterials

Nanomaterials that undergo health safety studies need to be sterilised, so as to avoid the presence and activity of contaminating bacteria. There are several sterilization methods that can be applied to nanomaterials, such as filtration [81,82], autoclaving [83], irradiation [84], the gas plasma method [85], and others (reviewed in [86]). These methods are effective in the removal or destruction of microbial contamination. However, all these sterilization techniques may, in most cases, alter the nanomaterials' physico-chemical properties, stability and toxicity/biological effects [85]. Therefore, for each nanomaterial a specific sterilization method should be applied that is validated as uninfluential on the physico-chemical and biological activities of the nanomaterials [85]. In any case, we should be aware that the sterilization processes are usually unable to removing endotoxin in nanomaterials' preparations. To date, still no efficient technique has been developed to remove endotoxin contamination in nanomaterials' samples. The difficulties in removing endotoxin from samples are due to the endotoxin's high variability in molecular weight, and to its pH and thermic stability. Endotoxin removal is a difficult issue not only for nanomaterials, but also for proteins for clinical use [87,88]. The endotoxin removal ability and the sample recovery rate of different endotoxin removal methods have been studied [89]. However, it should be noted that there is no gold standard for endotoxin removal, regardless of protein or nanoparticles. Therefore, the revalidation and recharacterization processes are always needed after endotoxin removal.

Several methods are used to remove endotoxin from samples, a process called 'depyrogenation' [90]. How much the depyrogenation methods can be applied to nanoparticles depends on the characteristics of the nanoparticles and, in many instances, they cannot be applied at all. A list of the most used methods and of their pros and cons is reported hereafter.

Ultrafiltration

Filtration through a 0.22 μm sterilizing membrane is very useful for eliminating bacteria from soluble samples. Endotoxin, which is a heterogeneous molecule found in different conformations and aggregation states over a wide range of molecular weights (from 1 to 1000 kD), usually cannot be removed by filtration on the 0.22 μm filter, although some aggregated forms could be removed by ultrafiltration. Some reports have

shown that endotoxin is quite effectively retained by a 0.025 μm filter, as endotoxin can adsorb to the filter membrane; the method has been used for removing endotoxin from water [91–93]. However, filtration of nanoparticle suspension may pose problems because the filter may also trap the particles and, if endotoxin is already adsorbed on the particle surface, the contaminated particles could go through the filter without losing the endotoxin.

Gel filtration chromatography

Size-exclusion chromatography (SEC) is a method that could be applied to eliminate endotoxin based on its size and molecular weight. SEC has been used for separating endotoxin from proteins and plasmid DNA [94,95]. However, as mentioned above, the wide range of the endotoxin size and molecular weight makes it difficult to remove all the endotoxin with this method (0.8 EU/mg still present in the purified sample; see [95]). In addition, there is no evidence showing the suitability of SEC for removing endotoxin from nanoparticles.

Two-phase extraction

By using detergents, endotoxin could be separated from sample solutions by a two-phase extraction method [96]. After mixing the detergent (e.g., Triton X-114) with sample solutions, endotoxin can be separated with centrifugation and a temperature increasing process from the water phase because of the nonpolar interaction of lipid A with the detergent. Normally, this process should be repeated until the endotoxin contamination falls below the threshold limit [87,96]. The two-phase extraction method is normally used to remove endotoxin from proteins, but it is also applied to nanoparticles. It has reported that gold nanoparticles became nonpyrogenic (tested by a RPT) after this two-phase depyrogenation [68]. The drawback is that a certain amount of detergent always remains in the sample solution, which asks for further procedures to remove it (adsorption or gel-filtration). All this implies a time-consuming procedure and a significant product loss [96].

Ion-exchange chromatography

Endotoxin is normally negatively charged, and can therefore easily bind to an anion exchanger. On this basis, anion-exchange chromatography may be effective in removing endotoxin, and in fact this method is normally used to purify proteins from endotoxin contamination [87]. Again, whether this method is also applicable for the depyrogenation of nanoparticles has not been assessed. Moreover, only positive-charged nanoparticles could be subjected to anion-exchange chromatography, thereby significantly limiting its possible applicability to nanoparticle decontamination.

Affinity adsorption chromatography

The affinity adsorption method is based on the use of an endotoxin-selective affinity sorbent that specifically binds and retains endotoxin. The antibiotic polymyxin B has high binding affinity to the lipid A part of endotoxin. Therefore, polymyxin B affinity columns are very effective in removing endotoxin contamination. However, since endotoxin is usually adsorbed to the surface of nanomaterials and is hardly released, the polymyxin B affinity columns are likely to remove the nanoparticles together with the endotoxin adsorbed on their surface, leading to a significant loss of nanoparticles [22]. Thus, the strategy of using affinity columns to remove endotoxin contamination from nanoparticles' solutions should be discarded if a loss of nanomaterials in the process cannot be accepted. Moreover, polymyxin B can stimulate human monocytes to produce IL-1 β [97], which may cause problems of uncontrolled activation/toxicity if/when the polymyxin B is released from the column. There are many other commercial high affinity chromatographic endotoxin removal systems (e.g., ActiClean Etox, ReductEtox, Detoxi-Gel, EndoTrap) based on different endotoxin-adsorbing substances. Also in this case, there is no information on the ability of these methods in removing endotoxin from the surface of contaminated nanoparticles.

Plasma discharge

The plasma discharge method is an excellent method for removing bacteria, immunostimulatory components and pyrogens from the surface of materials such as surgical equipment, dental instruments and long-term prosthetic implants. The plasma discharge method is effective for both sterilization and depyrogenation. The UV radiation from the plasma discharge process is allegedly responsible of the inactivation of spores. The etching/erosion by the radicals and active species (e.g., atomic oxygen, OH radicals) produced by the plasma discharge is considered the most potent and direct mechanism for killing spores and microorganisms by destruction of their membrane, and it also destroys other contaminants such as pyrogens/endotoxin [98,99]. Many studies have shown that the plasma discharge method can be used for depyrogenation [99–103], as it achieves a 3 log reduction of the initial pyrogen amount. PEG-coated gold nanoparticles treated by plasma discharge at 45°C for 50 min showed a strong alteration of the particles' characteristics (in particular alterations in the PEG coating) [85]. On the other hand, the plasma discharge treatment seems effective for tiopronin-coated gold nanoparticles, since the treatment has no effect on the physico-chemical properties and does not affect the particles' lack of toxicity, measured as induction of cell death and induction of ROS in the human U937 cell line [85]. Therefore, the

plasma discharge method can be successfully used for depyrogenating nanoparticles, but the method should be tested and validated for each type of sample.

Inactivation

Since endotoxin removal from the particle surface is clearly problematic, the choice of endotoxin-destroying/inactivating methods is apparently the most effective way of eliminating the problem of endotoxin bioactivity and toxicity. The common methods for pyrogen inactivation are acid-base hydrolysis, oxidation, treatment with sodium hydroxide and heating. However, all these are aggressive methods that normally cannot be applied to nanomaterials without changing their physico-chemical characteristics. Some reports have demonstrated that the endotoxin contamination of micro-sized particles could be removed by acid or sodium hydroxide treatment [43,76]. The endotoxin contamination in the micro-sized titanium particles on orthopedic implant decreased 100-times after treatment in alkali-ethanol and nitric acid [76]. Cho *et al.* also showed that micro-sized particles washed in acetic acid-ethanol were unable to stimulate the murine RAW264.7 cells to produce inflammatory cytokines [43]. In fact, the most efficient way of destroying endotoxin is incineration, which can be obtained, for instance, by dry heating at high temperatures (180°C for 3 h or 250°C for 30 min) and that can reduce endotoxin activity by 5 logs [90]. In general, the same effects can be obtained at higher temperatures for shorter times and at lower temperatures for longer times [90].

Matthews *et al.* showed that the micro-sized wear debris particles are endotoxin-free after treatment at 180°C for 3 h [104]. Another study showed that cobalt chrome particles (nano- and micro-sized) can be exposed to dry heating for depyrogenation (180°C for 4 h). Intentionally contaminated particles were endotoxin-free after such treatment (from 90 to <0.004 EU) [105]. Likewise, Cho *et al.* also have shown that micro-sized particles in powder can be depyrogenated by incineration (175°C for 3 h), and the treated particles cannot induce inflammatory cytokine production (both at the gene and protein level) in RAW264.7 cells. The dry heating depyrogenation method turned out to be much more effective than the acetic acid-ethanol washing [43]. Similar results were also obtained by Schwab *et al.* They found that dry heating (160°C for 24 h) was the most effective method to eliminate the endotoxin contamination in titanium micro-particles, compared with hydrochloric acid washing, ethanol washing and 10% Triton X-100 washing [53]. They found that repeated ethanol washings and dry heating was the most effective way to eliminate endotoxin in titanium particles, with a residual endotoxin contamination of 0.22 EU/ 2.5×10^6 particles. Unfortunately,

the authors did not assess the initial contamination of their particles, and therefore we cannot know the real efficacy of their methods [53]. Other nanoparticles in powder (silicon oxide, titanium oxide, zirconium oxide and cobalt nanoparticles) can be depyrogenated at 180°C for 4 h [62]. Carbon black nanoparticles can be submitted to depyrogenation at 250°C for 2 h without damage [48]. Other reports showed that treatment at 110°C for 12 h can depyrogenate hydroxyapatite particles used to coat prosthetic implants [106–108]. However, it should be noted that these hydroxyapatite particles were synthesized with sintering at 600°C or 1180°C, temperatures at which LPS would be destroyed anyway.

From the available evidence, the incineration method can be applied to nanoparticles in solid form (powder), such as carbon nanotubes or titanium dioxide nanoparticles, which are resistant to the high temperature treatment. In any case, nanoparticles need to be recharacterized after the depyrogenation process, in order to make sure they have maintained their original physico-chemical characteristics.

Other removal methods

The oldest and simplest method for depyrogenation consists in rinsing the contaminated material with endotoxin-free water or washing in ethanol [90]. These methods can be used to remove endotoxin from particles used in implants and for surgical materials. It has been found that implant particles lose the ability to induce NO production in murine RAW264.7 cells after washing with 70% ethanol [109]. However, other studies reported that the 70% ethanol washing was unable to depyrogenate the micro-particles, which still induced the production of inflammatory cytokines in RAW264.7 cells [43]. The overall low removal efficiency of these washing and rinsing methods limits their application to nanoparticles, to which surface endotoxin is generally strongly adsorbed. In addition, these methods cannot be applied to particles coming from wet synthesis, because the rinsing/washing process will dilute or remove the nanoparticle stabilizing agents, and aggregation is likely to occur.

Use of nanoparticles for depyrogenation

Since endotoxin binds efficiently to the nanoparticle surface, nanoparticles can be used to remove endotoxin from samples [26,110,111]. While this approach is likely to work with samples in solution, it is not obvious that it could work for depyrogenating other nanoparticles. The endotoxin-capturing nanoparticles should have a very strong affinity for endotoxin for being able to displace it from the surface of other particles, a situation that might be difficult to achieve and control. Subsequently, the endotoxin-capturing nanoparticles should

be removed from the sample (e.g., with a magnet if these are iron-based particles).

Preventing contamination

Because of the difficulty of endotoxin removal, preventing contamination during the nanoparticles synthesis would be the best way for obtaining endotoxin-free particles. This can be obtained by using only endotoxin-free reagents, instruments and glassware during the synthesis process [22]. It should be noted, however, that endotoxin-free conditions are not easy to achieve in chemical labs and therefore not everybody may be equipped for obtaining endotoxin-free particles. Moreover, the endotoxin-free condition of particles can be lost very easily because of the poor handling process, such as using endotoxin-presence containers, water and buffers [20].

Conclusion & future perspective

Several biologically active microbial compounds present in the environment can contaminate nanomaterials and confer to them inflammatory effects. Most of them are of protein or polysaccharidic nature, as for instance the exotoxins of gram-positive bacteria, and therefore are short-lived in the environment and easily removed from nanomaterials during sterilization procedures. On the other hand, gram-negative endotoxin is a ubiquitous and long-lived contaminant, resistant to sterilization and endowed with high inflammation-inducing capacity. It is known that endotoxin contamination can be responsible for many of the inflammatory effects obtained with micro-particles [45]. Despite this evidence, nanosafety studies have paid only recent and partial attention to the issue of endotoxin contamination of nanoparticles and to the possibility of obtaining misleading results if such contamination is not considered. It is of key importance that guidelines for nanosafety studies include a full characterization of the biologically active contaminants of the nanomaterials under test, to avoid attributing to the material toxic effects that are instead caused by the contaminating endotoxin [112]. Since the endotoxin detection methods in nanomaterials are still challenging, more robust strategies need to be developed and much more attention is required in evaluating the endotoxin contamination in nanomaterials. To identify and avoid biologically active contaminations such as that by LPS, not only the biologists/toxicologists should be aware of the danger and appropriately design and perform their experiments, but chemists should redesign their synthesis processes so as to avoid contamination.

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Executive summary

- Nanoparticles may be contaminated with endotoxin, which can generate misleading results when the toxic and inflammatory effects of nanomaterials are examined, if the presence of endotoxin is overlooked.

Endotoxin contamination in nanomaterials is common

- Endotoxin is a heat-stable ubiquitous contaminant.
- Endotoxin can adhere easily to both negatively and positively charged surfaces.

Biological effects of endotoxin contamination in nanomaterials are significant

- Endotoxin contamination can be responsible of effects such as induction of inflammation, cytotoxicity and apoptosis.

Endotoxin measurement methods can be interfered with by nanoparticles

- Limulus amoebocyte lysate (LAL) assays are the most commonly used endotoxin detection methods.
- LAL assays can be interfered by nanoparticles at the level of readout, enzyme and substrate.
- The validation of LAL assays with nanoparticles should be performed before any endotoxin detection test.
- No LAL format is optimal for all nanoparticle types.
- Bioassays for assessing pyrogens are not specific for endotoxin.

Endotoxin elimination is not easy & autoclaving is not suitable

- Autoclaving cannot eliminate endotoxin contamination.
- The physico-chemical characteristics of the nanomaterials can be changed after the sterilization/depyrogenation process.
- Endotoxin removal is difficult, thus preventing contamination during nanoparticle synthesis is the best way for obtaining endotoxin-free particles.

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