Chapter 12
Understanding Endotoxin and β-Glucan Contamination in Nanotechnology-Based Drug Products

Barry W. Neun and Marina A. Dobrovolskaia

Contents

Abstract Nanotechnology is increasingly used to formulate small molecules, biologics, and nucleic acid-based therapeutics. The attention to this technology is drawn by a variety of benefits including but not limited to the improved circulation time, reduced toxicity and the ability to target tissues and cells of interest. Clinical translation of nanotechnology-based drug products requires, among other investigations, the evaluation for the potential contamination with bacterial endotoxins. In the process of evaluating the safety of nanotechnology-based drug products, screening for additional microbial contaminants, such as beta-glucans, is an emerging new field. Herein, we will provide a general overview of the nanotechnology field and review challenges with estimating endotoxin and beta-glucan contamination in nanoparticle-based drug products.

12.1 Nanotechnology Overview

Nanotechnology does not have a universal definition. According to the National Nanotechnology Initiative, it is defined as "research and technology development at the atomic, molecular or macromolecular scale leading to the controlled creation

B. W. Neun · M. A. Dobrovolskaia (✉)
Nanotechnology Characterization Laboratory, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD, USA
e-mail: marina@mail.nih.gov

K. L. Williams (ed.), *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems*, https://doi.org/10.1007/978-3-030-17148-3_12

and use of structures, devices, and systems with a length scale of approximately 1–100 nanometers (nm)" [1]. In contrast, the US Food and Drug Administration states that for the purposes of regulatory approval process, a product will be considered as a nanotechnology when such product "is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1000 nm)" [2]. Over eight hundred products from over four hundred companies in over twenty countries are declared by manufacturers as those including nanotechnology. Clothing, wound dressings, washing machine liners, lens coatings in sunglasses, sporting equipment, food packaging, translucent sunscreens, cosmetic products to name the few, contain one or another type of nanoscale materials [3–9]. As such, global human exposure to nanomaterials is not an emerging trend, but rather a well-established fact. Therefore, consumer and occupational safety are one of the active areas of nanotoxicology.

Besides environmental and consumer products, nanotechnology has a unique niche in drug delivery, especially in the areas related to the targeted interaction with the immune system such as vaccines and immunotherapies. The rapid growth of this field is highlighted by business reports estimating the change of the global nanotechnology market from $39.2 billion in 2016 and to $90.5 billion by 2021 [10]. While in 2015 the main categories of products containing nanomaterials were environmental, and consumer applications, the use of nanotechnology in the biomedical field is predicted as the main category by 2021 [10]. Biomedical applications of nanotechnology include drugs, imaging agents, devices, immunotherapies, and vaccines. Evaluation of the current nanomedicine landscape revealed several common characteristics: (1) the major indication is cancer; (2) the average size is within 350 nm; (3) the main route of administration is intra-venous (i.v.); (4) the most common shape is spherical, and (5) the surface is neutral and hydrophilic [11]. According to the recent report by the US FDA, the majority of nanomaterials submitted to the FDA for regulatory approval are liposomes, nanocrystals, and emulsions [12]. Several examples of nanomedicines which are in current clinical use are listed in Table 12.1.

Traditionally, nanoparticles were engineered to evade the immune recognition to improve the drug delivery to the target sites [13–15]. Various strategies have been investigated to prevent nanoparticle recognition by the immune system. For example, the addition of a hydrophilic coating of poly(ethylene glycol) (PEG), using membranes of host cells [16], or host peptides preventing phagocytosis (e.g., CD47 peptides [17]) were tried to increase the nanoparticle circulation time and prevent the uptake by phagocytic cells. However, over the past decade of research in this field, it became clear that regardless of their structures, all nanoparticles eventually undergo clearance by the mononuclear phagocytic system (MPS). While the stealth properties are essential for drug delivery to non-immune cells, the non-stealth particles warrant immediate delivery of drugs, adjuvants, and imaging agents to the immune cells, which are the target for vaccines and immunotherapies. Therefore, targeting the immune system represents a unique niche for nanotechnology products. In the attempt to bring the nanotechnology to the market, evaluation of the

Table 12.1 Examples of nanomaterials approved for clinical use. Several nanoformulations used in clinic are summarized in this table. The brand name of each formulation is shown in the "Formulation" column. After patent expiration, a generic formulation of some of these nano drugs have been prepared and are also approved for clinical use. For example, Doxil and Caelyx formulations are currently available from two generics manufacturers under the name "PEGylated liposomal doxorubicin"

Formulation	Active ingredient	Indication	Nanocarrier
Doxil, Caelyx	Doxorubicin	Ovarian cancer, Kaposi sarcoma, myeloma	Liposomes
Myocet	Doxorubicin	Cancer	Liposomes
Abelcet	Amphotericin B	Fungal infections	Solid microparticles
Ambisome	Amphotericin B	Fungal infections	Liposomes
Amphotec, Amphocye	Amphotericin B	Fungal infections	Solid nanoparticles
Visudyne	Verteporfin	Age-related macular degeneration	Multilamellar liposomes
Feraheme	None	Iron deficiency	Iron oxide nanoparticles

immunotoxicity of these materials represents an important area of research. The landscape of the immunotoxicity of nanotechnology-based drug product has been extensively studied and discussed elsewhere [13, 18].

12.2 Nanoparticles and Endotoxin

12.2.1 The Reason for Concern

One of the focus areas of preclinical research of nanomaterials is the estimation of contamination with bacterial endotoxins. Endotoxin contamination is a common issue for engineered nanomaterials in that on average from 30% to 50% of preclinical nanoformulations fail every year due to the excessive endotoxin levels [19]. Endotoxin in nanomaterials is undesirable because it is responsible for the generation of erroneous data, which leads to wrong conclusions, could confound efficacy studies, result in undesirable toxicity, lead to the exaggeration of endotoxin-mediated inflammation, and create potential problems with the immunogenicity of protein-based APIs or targeting ligands [19–22].

Interestingly, some nanomaterials are not pro-inflammatory *per se* but exaggerate endotoxin-mediated inflammation [19, 20, 23]. Such effects were described for fibrous nanomaterials (e.g., titanium nanobelt and nanocellulose), silica nanoparticles and dendrimers [19, 23–28]. The mechanisms are not always understood. However, one study from our group demonstrated that cationic dendrimers interfere with the negative regulators of inflammation, and suggested that such an effect may be responsible for the exaggeration effect [23] (Fig. 12.1).

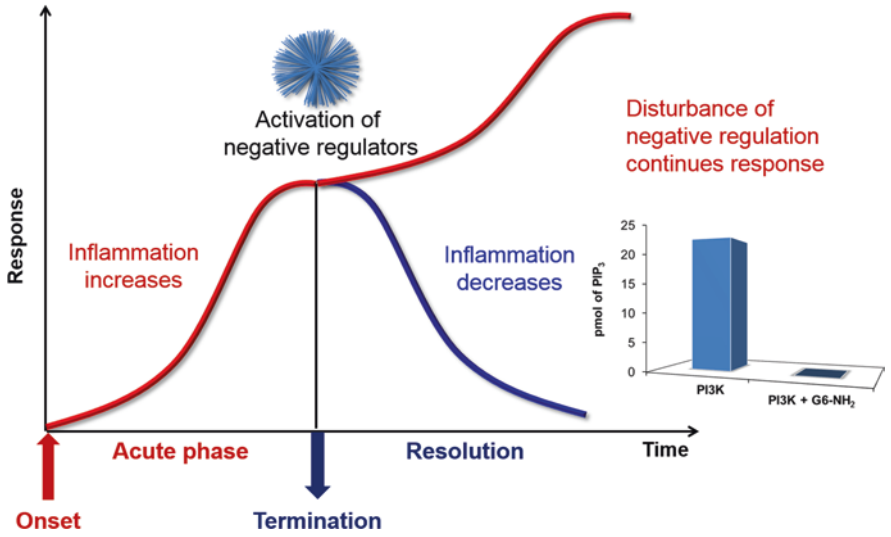


Fig. 12.1 Nanoparticles may exaggerate pro-inflammatory properties of endotoxin by affecting negative regulation of inflammation. Shown is the schematic of the inflammatory response. Under normal conditions, the inflammation increases during the acute phase of the response to eliminate a pathogen. Then, to resolve the inflammation, the cells rely on the negative regulators which decrease the inflammation and restore the healthy balance. Some nanoparticles may inactivate the negative regulators, thereby leading to the continuation of the inflammatory response. In a recent study [23], the exaggeration of the leukocyte procoagulant activity by cationic PAMAM dendrimers was attributed to the inhibition of PI3K, which serves as a negative regulator of PCA response

12.2.2 Common Sources of Contamination

Conventional sources of endotoxin contamination in nanoformulations include in-process contamination (Fig. 12.2) and starting materials. Water appears to be the primary source of endotoxin among both process-based and starting materials mediated sources. In some cases, bacterial contamination serves as the source of endotoxin in nanoformulations. In the experience of our laboratory, the following aquatic and environmental bacteria were identified in water and contaminated nanomaterials: *Phreatobacter oligotrophus, Ralstonia pickettii, Citrobacter freundii, Ochrobactrum anthropi, Achromobacter marplatensis, Pseudomonas beteli, Sphingomonas aeria, Shphingomonas zeae, Burkholderia contaminans.* This list points to two critical messages. First, dust in labs, quality of air, bacteria colonizing pipes and filters, create a particular point of concern and require serious consideration, monitoring, and control to avoid contamination in nanoformulations. Second, since chemical structure of endotoxin from various bacterial strains may differ substantially, quantification of endotoxin by some methods against the standard endotoxin derived from *E.coli* may not be accurate. Specifically, endotoxins from various

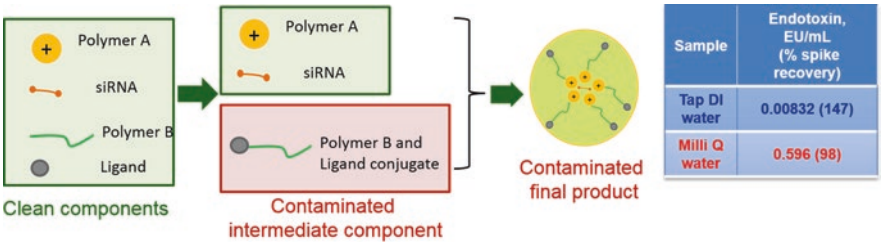


Fig. 12.2 In-process contamination is a common reason for the excessive endotoxin levels in nanomaterials. Shown is the schematic of a synthetic procedure of a nanoparticle used for the delivery of siRNA. All starting materials were sterile and did not contain endotoxin. However, the contamination was introduced during the synthesis of an intermediate component. The contaminated intermediate component served as the source of endotoxin contamination in the final product. Further evaluation revealed that purification of the intermediate component from unreacted chemicals and synthesis byproducts involved large volumes of MilliQ water. The amount of endotoxin in MilliQ water, as determined by the LAL assay, was higher than the amount of endotoxin in tap water from which the MilliQ system sourced the water for purification. It was further revealed that bacteria colonized the filter, which resulted in an excessive level of endotoxin in the MilliQ water

bacteria differ by the number and types of fatty acids, types, and quantity of monosaccharides, and the number of phosphates. The differences between rough, semi-rough, smooth and other types of endotoxins were discussed earlier [21]. Some methods, such as mass spectrometry rely on quantifying derivatized fatty acids [29–36]. If the fatty acids in the standard endotoxin and that contaminating a formulation are different, the mass spectrometry method will not generate an accurate result. The challenge in correcting this situation is that the chemical structure of bacteria commonly found in nanomaterials is not yet determined.

As shared in Fig. 12.2, the distilled water is not a guarantee of endotoxin-free quality, because bacteria may colonize filters. Therefore, it is highly recommended to verify the endotoxin level in water used for the synthesis of nanomaterials and to avoid the use of contaminated water.

12.2.3 Challenges with Detection

While a concern regarding endotoxin contamination is not unique to nanomaterials and is also broadly applicable to other types of drug products, the contamination issue comes with a particular challenge to nanomedicine. This is because these materials have complex structures and undergo a complex regulatory approval process. In addition, many nanoparticles interfere with one or more of the assays traditionally used in the drug development industry for the detection of endotoxin. Nanoparticle interference with LAL assay is considered as a grand challenge of nanomedicine [19, 37–41]. Cationic nanoparticles, unfunctionalized carbon nanotubes, anionic metal colloids among other materials commonly result in an inhibition of the LAL assay

[19]. The inhibition is characterized by the spike recovery less than 50% [42]. Mechanisms of interference include but are not limited to adsorption of endotoxin to nanoparticle surface or binding of LAL proteins to the particles. Polymeric nanomaterials are notorious for the enhancement effect. The enhancement is evidenced by the spike recover above 200% and is commonly difficult to distinguish from the endotoxin contamination [42]. Presence of serine proteases, protease-like activities as well as beta-glucan contamination is among the mechanism for the false-positive LAL results for these types of nanomaterials [19]. The inhibition enhancement controls or positive product controls, therefore, are instrumental in estimating the validity of LAL results [19]. Variety of methods are available for overcoming nanoparticle interference with the LAL assays and have been described elsewhere [19, 22, 38–40, 43]. We discuss few such methods further below.

The addition of glucan blocking reagents (e.g., Glucashiled buffer from Associates of Cape Code) is commonly used to prevent false-positive results originating from nanoparticle contamination with beta-glucans [40]. Endotoxin-free protease treatments of protein containing or protein-based nanomaterials help to eliminate the interference coming from high protein concentration. The use of detergents (e.g., SDS or CHAPS) has been proposed to overcome the inhibitory effects of cationic liposomes on endotoxin detection by LAL methods [44, 45]. Nanomaterials with hollow cavities represent yet another challenge in that endotoxin can be entrapped in the particle cavities and, thereby, escape accurate detection by the LAL [19]. This type of interference cannot be detected by the inhibition-enhancement controls [19], and therefore require additional consideration. Some liposomes can be successfully destroyed by heat/cool procedure and release endotoxin from their cavities [19, 39, 40]. In many other cases, however, it is very challenging to destroy the particle without destroying endotoxin. The procedures for endotoxin release are product specific and require validation using control standard endotoxin.

Frequently, when traditional methods provide invalid results, FDA recommends considering alternative procedures [42]. In vitro monocyte activation test, recombinant Factor C assay, EndoLISA, TLR4-HEK Blue cells are few such tests [46–50]. In addition, several mass-spectrometry and gel-electrophoresis methods have also been described in the literature [30–36]. The example of some gel-based methods is shown in Fig. 12.3. The common limitations of these methods are that they are either less sensitive than LAL or not specific to endotoxin and can detect glycosylated proteins as well [51].

In our laboratory, we follow a decision tree developed by the first international workshop on the immunotoxicity of nanotechnology-based drugs and published elsewhere [13]. According to this decision tree, two different LAL methods are conducted for each formulation, and the results are compared. In the case when the results are in agreement (i.e., the EU/mL values obtained from one LAL methods are within 50–200% of the values obtained by another LAL method), the LAL results are reported. However, if two LAL formats show different levels of endotoxin in the same formulation, additional follow up studies are warranted. We described some examples of such discrepancies earlier [40, 43, 52]. Here, we will

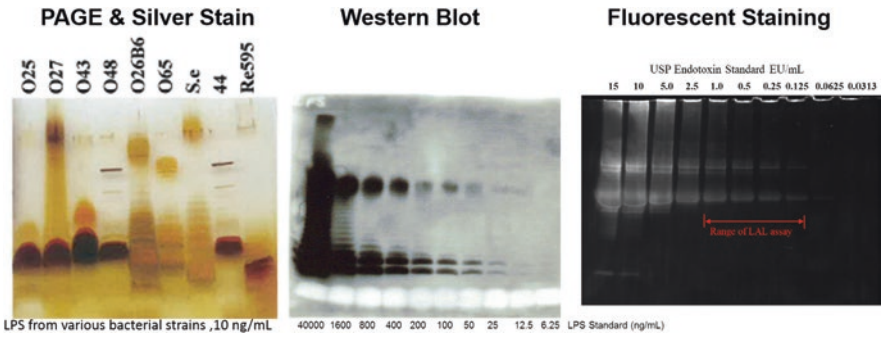
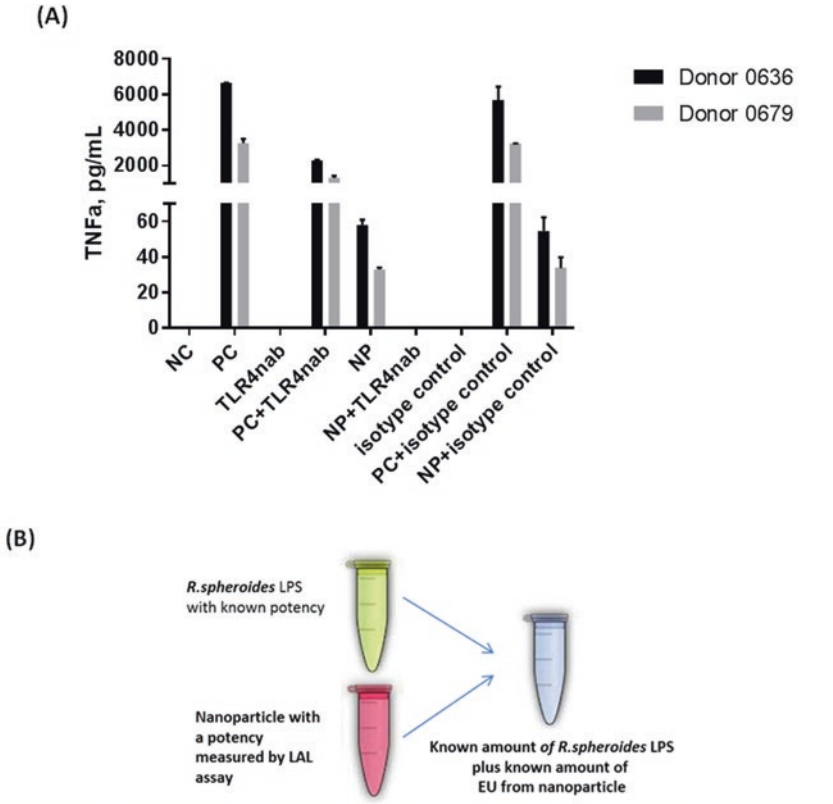


Fig. 12.3 Alternative gel-electrophoresis based methods for detection of endotoxin. Three methods are shown and have also been discussed elsewhere [51]. All of them involve separation of endotoxin in polyacrylamide gels. Left to right shows the images of the gels analyzed by silver staining, western blot using KDO-specific antibody and fluorescent stain. The typical limitation of these alternative methods is that they are either less sensitive than LAL (e.g., silver stain and western blot) or not specific to endotoxin (e.g., a fluorescent stain which also detects proteins)

present a few additional case studies. In one of them, cytokine secretion by peripheral blood mononuclear cells (PBMC) with and without TLR4 neutralizing antibodies was used to verify that pyrogenicity observed in monocyte activation test (MAT) is indeed due to the endotoxin and not material mediated response (Fig. 12.4a). In the second case, we used endotoxin from *Rodobacter spheroides* to distinguish between material and endotoxin-mediated responses in the LAL method (Fig. 12.4b). We would like to note, however, that *R.spheroides* method worked well for nucleic acid-based nanoparticles (NANPs) based products (Fig. 12.4b), but was not efficient for liposomes (data not shown). In the experience of our laboratory, each nanoparticle product is unique and may require additional methods for verification of endotoxin contamination in situations when traditional methods, such as LAL, do not show consistent results.

12.2.4 Sterilization and Depyrogenation

The amounts of endotoxin in drugs and devices is carefully monitored and regulated. The levels of this biological contaminants is required to be below threshold pyrogenic dose, which is 5 EU/kg/h [55]. As such, the levels of endotoxin in individual nanoformulation are considered in the context of the intended dose and route of administration. If at the intended dose the amount of endotoxin in the given nanoformulation exceeds 5 EU/kg/h limit for all routes of administration except for the intrathecal route, an action is required to remove it before the use in veterinary and human patients. The removal of endotoxin from nanoformulation represents yet another challenge in the field of nanomedicines. A variety of standard sterilization methods also capable of reducing endotoxins are available, such as, for example,



Samples	*R.spheroides* LPS Unspiked EU/mL	*R.spheroides* LPS spiked with Nanoparticle EU/mL	Spiked-Unspiked EU/mL	Theoretical spike conc. of Nanoparticle EU/mL	Spike recovery,%
Mean Result EU/mL (N=2)	0.4435	0.4805	0.037	0.159	23.3

Fig. 12.4 Verification of endotoxin contamination by additional methods. A variety of study designs can be developed to verify the presence of endotoxin detected by traditional (e.g., LAL) and not traditional (e.g., monocyte activation test) methods. Two such examples are presented in this figure. (**a**) Human peripheral blood mononuclear cells from two healthy donors (0636 and 0679) were treated with a nanoparticle formulation (NP) without or in the presence of 1 μg/mL TLR4-neutralizing antibodies (TLR4nab). The secretion of a pro-inflammatory cytokine TNFα in the culture medium was measured by ELISA 24 hours after the initial treatment. The antibody but not its relevant isotype control suppressed the TNF induction in the nanoparticle-treated sample, thereby verifying that this response is mediated by endotoxin. Ultrapure *E.coli K12* LPS at a concentration of 20 ng/mL was used as a positive control (PC); incomplete inhibition of the cytokine in this sample is observed due to the high potency of the ligand. Blocking of this response would require a higher concentration of TLR4nab. It was not used in this experiment due to the high cost of these antibodies. The inhibition in the nanoparticle sample was complete, because the level of endotoxin in nanoparticle was lower, and the tested TLR4nab concentration was sufficient to block this response. (**b**) LAL method was conducted to measure endotoxin in RNA-nanoparticle; the positive response in this sample was verified by the addition to *R.shperoides* LPS, which acts as

gamma-irradiation or ethylene oxide sterilization. The case study summarized in Fig. 12.5a, shows two nanoformulations and demonstrates successful extraction of endotoxin from polymeric nanoparticle and the reduction of endotoxin level by gamma-irradiation in a metal oxide nanoparticle. The extraction of endotoxin from polymeric nanoparticle formulation using Triton X-114 has been described in details elsewhere [43]. Endotoxin removal and sterilization of nanoparticles represa ent yet another significant challenge in nanomedicine. This problem arises from the inability of many nanoformulations to resist standard sterilization procedures. In the sample shown in Fig. 12.5b, two metal oxide nanoparticles were subjected to sterilization using gamma-irradiation. Citrate-stabilized gold colloids remained their integrity after the sterilization as evidenced by their appearance in the transmission electron microscopy (TEM). However, citrate-stabilized silver colloids did not resist the sterilization procedure as evident by a change in their morphology in the TEM image. The mechanism of destruction of silver nanoparticles by the gamma irradiation has been attributed to their ability to scavenge reactive oxygen radicals produced by the gamma irradiation [56]. Due to this problem, the use of depyrogenated materials and nanoparticle production using aseptic procedura es represent the preferred method to terminal sterilization. Examples of depyrogenation and aseptic production have been described elsewhere [57]. Likewise, many studies investigated the stability of various types of nanomaterials under commonly used sterilization procedures including but not limited to autoclaving, UV light, and gamma-irradiation [41, 44, 56, 58–63].

12.3 Nanoparticles and Beta-Glucans

12.3.1 Reason for Concern

Beta-glucans are present in cell walls of variety of microbes including fungi, yeast, and some bacteria. These polysacchardies are made of D-glucose monomers which are connected by [1–3] beta-glycosidic bonds [64]. Low (10–40 pg/mL) levels of beta-glucans are also present in the blood of healthy individuals due to the dietary sources (e.g., seaweeds and cereals) and commensal flora [65]. The primary sources of beta-glucan contamination in pharmaceutical products are plant-based raw

Fig. 12.4 (continued) antagonist to enterobacterial LPS in mammalian, but not in CHO or horse cells [53, 54]. The design of the experiment is shown in the top panel and the result in the table below. The spike recovery of endotoxin units (EUs) in nanoparticle sample was expected to be suppressed in the presence of *R.spheroides* LPS, if these EUs were coming from endotoxin. However, if a nanoparticle behaved in the LAL assay as endotoxin, then the presence of *R.spheroides* LPS would not affect the spike recovery. Since the spike recovery was below 50%, we concluded that the EUs in the nanoparticle sample indeed come from endotoxin contamination. In a control experiment, *R.spheroides* LPS inhibited endotoxin spike recovery in a positive water control made of LAL grade water and control standard endotoxin (data not shown)

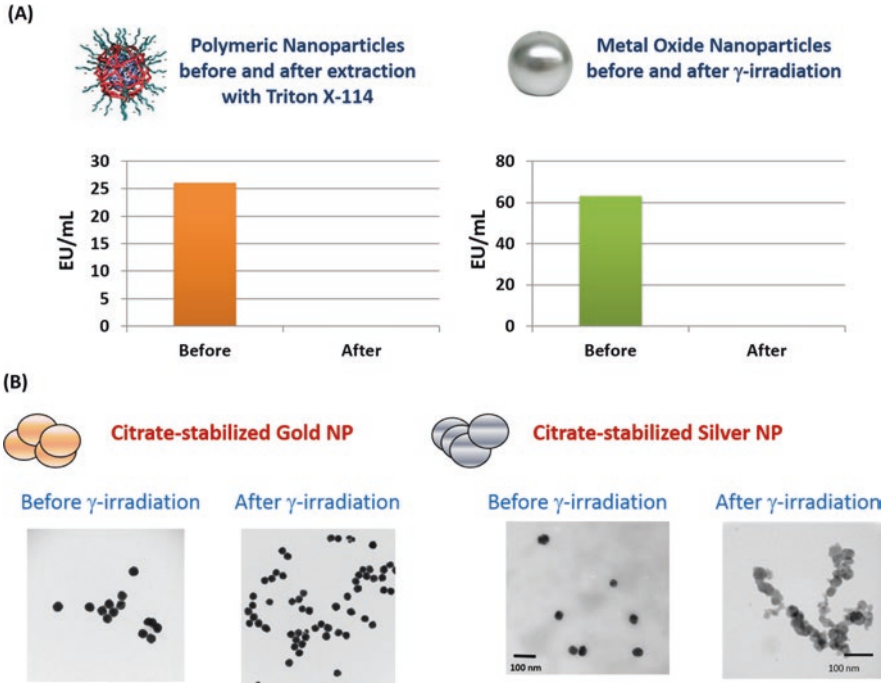


Fig. 12.5 Sterilization and nanoparticles. (**a**) Shown are two examples. In one case, endotoxin was removed in polymeric nanoparticles using Triton X-114 extraction procedure [43]. In another example, metal oxide nanoparticles were sterilized using gamma irradiation, and the sterilization also resulted in a reduction in endotoxin levels. In both of these cases, the physicochemical properties of nanoparticles were verified and confirmed to be unaffected by the sterilization or extraction procedure. (**b**) Not all nanoparticles can tolerate common sterilization methods. While some common sterilization procedures can be efficient at reducing endotoxin levels in nanoformulations, it is very important to confirm the particle integrity after the sterilization [56]. In the example shown in this figure, citrate-stabilized gold nanoparticles were sterilized by gamma irradiation without any changes in particle size. In contrast, citrate-stabilized silver nanoparticles were destroyed by the same sterilization method

materials (e.g., sucrose and cellulose) and cellulose-based filters [65]. Beta-glucans are immunostimulatory and activate the immune cells through a variety of receptors including but not limited to Toll-like receptors, Complement receptors, Dectins and combination thereof [66–73]. Although beta-glucans are less potent than endotoxin in stimulating the immune cells, they can both exaggerate endotoxin-mediated pyrogenicity and contribute to the immunogenicity of therapeutic protein formulations. As such, the US FDA recommends detecting beta-glucans as the innate immunity modulating impurities in therapeutic proteins and considers this data in the context of immunogenicity of the products [2]. When it comes to the pyrogenicity assessment, however, the main regulatory concern is the endotoxin, while beta-glucans remain a grey area. Particularly, threshold pyrogenic dose and limits for beta-glucans in drug products are unknown. In the current era of immunotherapies,

the presence of beta-glucan in nanoparticles intended for specific immunomodulation represents a special interest because these contaminants may confound the results of both safety and efficacy studies of these materials.

12.3.2 Challenges with Detection and Estimation of Safe Levels

Beta-glucans can be detected in vitro using modified LAL assay [74]. In the traditional LAL lysate, both Factor C (specific to endotoxin) and Factor G (specific to beta-glucans) are present [74]. Such co-presence creates a false-positive interference of beta-glucans during endotoxin detection [40]. However, when Factor C is extracted from the lysate, the remaining Factor G initiates a proteolytic cascade in response to the presence of beta-glucans [75]. An immunoassay similar to the LAL and known as Fungitell was approved the FDA in 2004 for the diagnosis of fungal infections (https://www.fungitell.com/). The same assay is also marketed in Europe since 2008. A research grade assay is available commercially under brand name Glucatell (http://www.acciusa.com/pdfs/accProduct/inserts/Glucatell_Kit.pdf).

The applicability of the Glucatell assay to engineered nanomaterials has not been studied before. In Table 12.2 we present the results of a study in which we tested a variety of research- and clinical grade nanomaterials using commercial kit (http://www.acciusa.com/pdfs/accProduct/inserts/Glucatell_Kit.pdf). Each nanoparticle was tested at several dilutions (5, 50 and 500). The spike recovery and inhibition/enhancement control requirements applied to the detection of endotoxin by LAL assays were also applied to evaluate the performance of the Fungitell assay. Two of six screened formulations (Doxil and Feraheme) interfered with the

Table 12.2 Evaluation of beta-glucan levels in clinical- and research-grade nanoformulations using in vitro Glucatell assay. Each formulation was tested at three dilutions (5, 50 and 500-fold). The data were reported from the lowest dilution not interfering with the commercial Glucatell assay. The interference was estimated as a spike recovery outside of the 50–200% range

Formulation	β-glucan conc., pg/mL	Spike recovery, %	Lowest Dilution with acceptable spike recovery
Citrate-stabilized gold colloids	BLOQ*	109	5
Citrate stabilized Silver colloids	91.3	90	5
Doxil	307	120	50
Abraxane	29.2	123	5
Ambisome	85.01	142	5
Feraheme	306	133	50

*BLOQ=below low limit of quantification

assay at the lowest tested dilution 5. Therefore, for these two formulations, we reported the results from the lowest not interfering dilutions, which was 50. Other formulations (colloidal silver, colloidal gold, Abraxane and Ambisome) did not interfere with the assay at the dilution 5. Levels of beta-glucans in tested formulations varied from undetectable to 306 pg/mL. To evaluate whether these levels may represent any safety concerns, we converted the detected levels of beta-glucans from pg/mL to pg/dose.

Next, we estimated how much beta-glucans would be injected into a 70 kg adult with each dose of nanomaterials. We estimated that the blood volume of such adult is 5.6 L (or 8% of the body weight), and subsequently converted the injected amounts of beta-glucans per one milliliter of blood. Finally, we compared these estimated concentrations to what is considered in the clinical diagnostic assay as an abnormal level of beta-glucans (70 pg/mL). In all cases, the amounts of beta-glucans injected with an intended clinical dose did not exceed 70 pg/mL of blood. We emphasize that this approach relies on the logic assuming that the levels of beta-glucans above 70 pg/mL may represent a safety concern. However, there is no formal limit against which this data could be compared. The results of this study demonstrate that the Fungitell assay is not immune to nanoparticle interference, and suggests that thorough studies aiming at the evaluation of safe levels of beta-glucans are warranted.

12.4 Conclusions and Future Directions

The data and literature discussed above demonstrate that the detection of endotoxins and beta-glucans in nanotechnology formulations is not a trivial task and requires thorough planning and relevant controls. We further emphasize that more research is needed to understand both the evaluation procedure and safety levels of beta-glucans in nanotechnology-based drug products.

Acknowledgments The study was supported in part by federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. National-Nanotechnology-Initiative. Defition of Nanotechnology. https://www.nano.gov/nanotech-101/what/definition.
2. US FDA. Considering whether an FDA-regulated product involves the application of nanotechnology. 2014.
3. Gao Y, et al. China and the United States–global partners, competitors and collaborators in nanotechnology development. Nanomedicine. 2016;12(1):13–9. https://doi.org/10.1016/j.nano.2015.09.007.

4. He X, Hwang HM. Nanotechnology in food science: functionality, applicability, and safety assessment. J Food Drug Anal. 2016;24(4):671–81. https://doi.org/10.1016/j.jfda.2016.06.001.
5. Hofmann-Amtenbrink M, Hofmann H, Hool A, Roubert F. Nanotechnology in medicine: European research and its implications. Swiss Med Wkly. 2014;144:w14044. https://doi.org/10.4414/smw.2014.14044.
6. Padovani GC, et al. Advances in dental materials through nanotechnology: facts, perspectives and toxicological aspects. Trends Biotechnol. 2015;33(11):621–36. https://doi.org/10.1016/j.tibtech.2015.09.005.
7. Panahi Y, et al. Recent advances on liposomal nanoparticles: synthesis, characterization and biomedical applications. Artif Cells Nanomed Biotechnol. 2017;45(4):788–99. https://doi.org/10.1080/21691401.2017.1282496.
8. Sonkaria S, Ahn SH, Khare V. Nanotechnology and its impact on food and nutrition: a review. Recent Pat Food Nutr Agric. 2012;4(1):8–18.
9. Tapia-Hernandez JA, et al. Micro- and nanoparticles by electrospray: advances and applications in foods. J Agric Food Chem. 2015;63(19):4699–707. https://doi.org/10.1021/acs.jafc.5b01403.
10. Cumming S. Nanotechnology sees big growth in products and applications, Reports BCC Research. BCC Research. 2017. https://globenewswire.com/news-release/2017/01/17/906164/0/en/Nanotechnology-Sees-Big-Growth-in-Products-and-Applications-Reports-BCC-Research.html.
11. Etheridge ML, et al. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomedicine. 2013;9(1):1–14. https://doi.org/10.1016/j.nano.2012.05.013.
12. D'Mello SR, et al. The evolving landscape of drug products containing nanomaterials in the United States. Nat Nanotechnol. 2017;12(6):523–9. https://doi.org/10.1038/nnano.2017.67.
13. Dobrovolskaia MA, Germolec DR, Weaver JL. Evaluation of nanoparticle immunotoxicity. Nat Nanotechnol. 2009;4(7):411–4. https://doi.org/10.1038/nnano.2009.175.
14. Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. Nat Nanotechnol. 2007;2(8):469–78. https://doi.org/10.1038/nnano.2007.223.
15. Deci MB, Liu M, Dinh QT, Nguyen J. Precision engineering of targeted nanocarriers. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2018. https://doi.org/10.1002/wnan.1511.
16. Parodi A, et al. Synthetic nanoparticles functionalized with biomimetic leukocyte membranes possess cell-like functions. Nat Nanotechnol. 2013;8(1):61–8. https://doi.org/10.1038/nnano.2012.212.
17. Rodriguez PL, et al. Minimal "Self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles. Science. 2013;339(6122):971–5. https://doi.org/10.1126/science.1229568.
18. Brand W, et al. Nanomedicinal products: a survey on specific toxicity and side effects. Int J Nanomedicine. 2017;12:6107–29. https://doi.org/10.2147/ijn.s139687.
19. Dobrovolskaia MA. Pre-clinical immunotoxicity studies of nanotechnology-formulated drugs: challenges, considerations and strategy. J Control Release. 2015;220(Pt B):571–83. https://doi.org/10.1016/j.jconrel.2015.08.056.
20. Dobrovolskaia MA, et al. Dendrimer-induced leukocyte procoagulant activity depends on particle size and surface charge. Nanomedicine (Lond). 2012;7(2):245–56. https://doi.org/10.2217/nnm.11.105.
21. Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect. 2002;4(9):903–14.
22. Donnell ML, Lyon AJ, Mormile MR, Barua S. Endotoxin hitchhiking on polymer nanoparticles. Nanotechnology. 2016;27(28):285601. https://doi.org/10.1088/0957-4484/27/28/285601.
23. Ilinskaya AN, et al. Inhibition of phosphoinositol 3 kinase contributes to nanoparticle-mediated exaggeration of endotoxin-induced leukocyte procoagulant activity. Nanomedicine (Lond). 2014;9(9):1311–26. https://doi.org/10.2217/nnm.13.137.
24. Hamilton RF, et al. Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity. Part Fibre Toxicol. 2009;6:35. https://doi.org/10.1186/1743-8977-6-35.

25. Inoue K. Promoting effects of nanoparticles/materials on sensitive lung inflammatory diseases. Environ Health Prev Med. 2011;16(3):139–43. https://doi.org/10.1007/s12199-010-0177-7.
26. Inoue K, Takano H. Aggravating impact of nanoparticles on immune-mediated pulmonary inflammation. ScientificWorldJournal. 2011;11:382–90. https://doi.org/10.1100/tsw.2011.44.
27. Inoue K, et al. Effects of inhaled nanoparticles on acute lung injury induced by lipopolysaccharide in mice. Toxicology. 2007;238(2–3):99–110. https://doi.org/10.1016/j.tox.2007.05.022.
28. Inoue K, et al. Effects of airway exposure to nanoparticles on lung inflammation induced by bacterial endotoxin in mice. Environ Health Perspect. 2006;114(9):1325–30. https://doi.org/10.1289/ehp.8903.
29. Kilar A, Dornyei A, Kocsis B. Structural characterization of bacterial lipopolysaccharides with mass spectrometry and on- and off-line separation techniques. Mass Spectrom Rev. 2013;32(2):90–117. https://doi.org/10.1002/mas.21352.
30. Klein DR, Holden DD, Brodbelt JS. Shotgun analysis of rough-type lipopolysaccharides using ultraviolet photodissociation mass spectrometry. Anal Chem. 2016;88(1):1044–51. https://doi.org/10.1021/acs.analchem.5b04218.
31. Kocsis B, et al. Mass spectrometry for profiling LOS and lipid a structures from whole-cell lysates: directly from a few bacterial colonies or from liquid broth cultures. Methods Mol Biol. 2017;1600:187–98. https://doi.org/10.1007/978-1-4939-6958-6_17.
32. Larrouy-Maumus G, Clements A, Filloux A, McCarthy RR, Mostowy S. Direct detection of lipid A on intact Gram-negative bacteria by MALDI-TOF mass spectrometry. J Microbiol Methods. 2016;120:68–71. https://doi.org/10.1016/j.mimet.2015.12.004.
33. Li H, Hitchins VM, Wickramasekara S. Rapid detection of bacterial endotoxins in ophthalmic viscosurgical device materials by direct analysis in real time mass spectrometry. Anal Chim Acta. 2016;943:98–105. https://doi.org/10.1016/j.aca.2016.09.030.
34. Robert CB, et al. Mass spectrometry analysis of intact Francisella bacteria identifies lipid A structure remodeling in response to acidic pH stress. Biochimie. 2017;141:16–20. https://doi.org/10.1016/j.biochi.2017.08.008.
35. Uhlig S, et al. Profiling of 3-hydroxy fatty acids as environmental markers of endotoxin using liquid chromatography coupled to tandem mass spectrometry. J Chromatogr A. 2016;1434:119–26. https://doi.org/10.1016/j.chroma.2016.01.038.
36. Zaia J. Capillary electrophoresis-mass spectrometry of carbohydrates. Methods Mol Biol. 2016;984:13–25. https://doi.org/10.1007/978-1-62703-296-4_2.
37. Li Y, Boraschi D. Endotoxin contamination: a key element in the interpretation of nanosafety studies. Nanomedicine (Lond). 2016;11(3):269–87. https://doi.org/10.2217/nnm.15.196.
38. Li Y, et al. Bacterial endotoxin (lipopolysaccharide) binds to the surface of gold nanoparticles, interferes with biocorona formation and induces human monocyte inflammatory activation. Nanotoxicology. 2017;11(9–10):1157–75. https://doi.org/10.1080/17435390.2017.1401142.
39. Neun BW, Dobrovolskaia MA. Detection and quantitative evaluation of endotoxin contamination in nanoparticle formulations by LAL-based assays. Methods Mol Biol. 2011;697:121–30. https://doi.org/10.1007/978-1-60327-198-1_12.
40. Neun BW, Dobrovolskaia MA. Considerations and some practical solutions to overcome nanoparticle interference with LAL assays and to avoid endotoxin contamination in nanoformulations. Methods Mol Biol. 2018;1682:23–33. https://doi.org/10.1007/978-1-4939-7352-1_3.
41. Vetten MA, Yah CS, Singh T, Gulumian M. Challenges facing sterilization and depyrogenation of nanoparticles: effects on structural stability and biomedical applications. Nanomedicine. 2014;10(7):1391–9. https://doi.org/10.1016/j.nano.2014.03.017.
42. US FDA. Guidance for industry: pyrogen and endotoxins testing: questions and answers. 2012.
43. Dobrovolskaia MA, et al. Ambiguities in applying traditional Limulus amebocyte lysate tests to quantify endotoxin in nanoparticle formulations. Nanomedicine (Lond). 2010;5(4):555–62. https://doi.org/10.2217/nnm.10.29.
44. Dutz S, Wojahn S, Grafe C, Weidner A, Clement JH. Influence of sterilization and preservation procedures on the integrity of serum protein-coated magnetic nanoparticles. Nanomaterials (Basel). 2017;7(12). https://doi.org/10.3390/nano7120453.

45. Piluso LG, Martinez MY. Resolving liposomal inhibition of quantitative LAL methods. PDA J Pharm Sci Technol. 1999;53(5):260–3.
46. Gaines Das RE, Brugger P, Patel M, Mistry Y, Poole S. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs: test design and data analysis. J Immunol Methods. 2004;288(1–2):165–77. https://doi.org/10.1016/j.jim.2004.03.002.
47. Gimenes I, Caldeira C, Presgrave OA, de Moura WC, Villas Boas MH. Assessment of pyrogenic response of lipoteichoic acid by the monocyte activation test and the rabbit pyrogen test. Regul Toxicol Pharmacol. 2015;73(1):356–60. https://doi.org/10.1016/j.yrtph.2015.07.025.
48. Solati S, Aarden L, Zeerleder S, Wouters D. An improved monocyte activation test using cryopreserved pooled human mononuclear cells. Innate Immun. 2015;21(7):677–84. https://doi.org/10.1177/1753425915583365.
49. Wunderlich C, Schumacher S, Kietzmann M. Pyrogen detection methods: comparison of bovine whole blood assay (bWBA) and monocyte activation test (MAT). BMC Pharmacol Toxicol. 2014;15:50. https://doi.org/10.1186/2050-6511-15-50.
50. Boratynski J, Szermer-Olearnik B. Endotoxin removal from Escherichia coli bacterial lysate using a biphasic liquid system. Methods Mol Biol. 2017;1600:107–12. https://doi.org/10.1007/978-1-4939-6958-6_10.
51. Dobrovolskaia MA, McNeil SE. In: Dobrovolskaia MA, McNeil SE, editors. Chapter 7, Immunological properties of engineered nanomaterials, vol. 1. New York: World Scientific Publishing Ltd; 2016. p. 143–86.
52. Dobrovolskaia MA, Neun BW, Clogston JD, Grossman JH, McNeil SE. Choice of method for endotoxin detection depends on nanoformulation. Nanomedicine (Lond). 2014;9(12):1847–56. https://doi.org/10.2217/nnm.13.157.
53. Oblak A, Jerala R. The molecular mechanism of species-specific recognition of lipopolysaccharides by the MD-2/TLR4 receptor complex. Mol Immunol. 2015;63(2):134–42. https://doi.org/10.1016/j.molimm.2014.06.034.
54. Soler-Rodriguez AM, et al. Neutrophil activation by bacterial lipoprotein versus lipopolysaccharide: differential requirements for serum and CD14. J Immunol. 2000;164(5):2674–83.
55. United States Pharmacopoeia. Bacterial endotoxin test 85. 2017.
56. Zheng J, Clogston JD, Patri AK, Dobrovolskaia MA, McNeil SE. Sterilization of silver nanoparticles using standard gamma irradiation procedure affects particle integrity and biocompatibility. J Nanomed Nanotechnol. 2011;2011(Suppl 5):001. https://doi.org/10.4172/2157-7439.s5-001.
57. Afonin KA, et al. Design and self-assembly of siRNA-functionalized RNA nanoparticles for use in automated nanomedicine. Nat Protoc. 2016;6(12):2022–34. https://doi.org/10.1038/nprot.2011.418.
58. El-Salamouni NS, Farid RM, El-Kamel AH, El-Gamal SS. Effect of sterilization on the physical stability of brimonidine-loaded solid lipid nanoparticles and nanostructured lipid carriers. Int J Pharm. 2015;496(2):976–83. https://doi.org/10.1016/j.ijpharm.2015.10.043.
59. Galante R, et al. About the sterilization of chitosan hydrogel nanoparticles. PLoS One. 2016;11(12):e0168862. https://doi.org/10.1371/journal.pone.0168862.
60. Montanari E, et al. One-step formation and sterilization of gellan and hyaluronan nanohydrogels using autoclave. J Mater Sci Mater Med. 2015;26(1):5362. https://doi.org/10.1007/s10856-014-5362-6.
61. Rowenczyk L, et al. Development of preservative-free nanoparticles-based emulsions: effects of NP surface properties and sterilization process. Int J Pharm. 2016;510(1):125–34. https://doi.org/10.1016/j.ijpharm.2016.06.014.
62. Sakar F, et al. Nano drug delivery systems and gamma radiation sterilization. Pharm Dev Technol. 2017;22(6):775–84. https://doi.org/10.3109/10837450.2016.1163393.
63. Shimojo AA, de Souza Brissac IC, Pina LM, Lambert CS, Santana MH. Sterilization of autocrosslinked hyaluronic acid scaffolds structured in microparticles and sponges. Biomed Mater Eng. 2015;26(3–4):183–91. https://doi.org/10.3233/bme-151558.
64. Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. J Hematol Oncol. 2009;2:25. https://doi.org/10.1186/1756-8722-2-25.

65. Barton C, et al. Beta-glucan contamination of pharmaceutical products: how much should we accept? Cancer Immunol Immunother. 2016;65(11):1289–301. https://doi.org/10.1007/s00262-016-1875-9.
66. Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature. 2001;413(6851):36–7. https://doi.org/10.1038/35092620.
67. Brown GD, Gordon S. Immune recognition of fungal beta-glucans. Cell Microbiol. 2005;7(4):471–9. https://doi.org/10.1111/j.1462-5822.2005.00505.x.
68. Gao D, Li W. Structures and recognition modes of toll-like receptors. Proteins. 2017;85(1):3–9. https://doi.org/10.1002/prot.25179.
69. Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. Immunol Rev. 2009;230(1):38–50. https://doi.org/10.1111/j.1600-065X.2009.00793.x.
70. Gow NA, et al. Immune recognition of Candida albicans beta-glucan by dectin-1. J Infect Dis. 2007;196(10):1565–71. https://doi.org/10.1086/523110.
71. Legentil L, et al. Molecular interactions of beta-(1-->3)-glucans with their receptors. Molecules. 2015;20(6):9745–66. https://doi.org/10.3390/molecules20069745.
72. Netea MG, et al. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest. 2006;116(6):1642–50. https://doi.org/10.1172/jci27114.
73. Sahasrabudhe NM, Dokter-Fokkens J, de Vos P. Particulate beta-glucans synergistically activate TLR4 and Dectin-1 in human dendritic cells. Mol Nutr Food Res. 2016;60(11):2514–22. https://doi.org/10.1002/mnfr.201600356.
74. Roslansky PF, Novitsky TJ. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. J Clin Microbiol. 1991;29(11):2477–83.
75. Tran T, Beal SG. Application of the 1,3-beta-D-glucan (Fungitell) assay in the diagnosis of invasive fungal infections. Arch Pathol Lab Med. 2016;140(2):181–5. https://doi.org/10.5858/arpa.2014-0230-RS.