

Immunological properties of engineered nanomaterials

Most research on the toxicology of nanomaterials has focused on the effects of nanoparticles that enter the body accidentally. There has been much less research on the toxicology of nanoparticles that are used for biomedical applications, such as drug delivery or imaging, in which the nanoparticles are deliberately placed in the body. Moreover, there are no harmonized standards for assessing the toxicity of nanoparticles to the immune system (immunotoxicity). Here we review recent research on immunotoxicity, along with data on a range of nanotechnology-based drugs that are at different stages in the approval process. Research shows that nanoparticles can stimulate and/or suppress the immune responses, and that their compatibility with the immune system is largely determined by their surface chemistry. Modifying these factors can significantly reduce the immunotoxicity of nanoparticles and make them useful platforms for drug delivery.

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A number of pharmaceutical products that involve nanotechnology have already been approved for clinical use, and many others are at different stages of preclinical development. Indeed, it is estimated that approximately 240 nano-enabled products entered pharmaceutical 'pipelines' in 2006 (ref. 1) (Table 1). At present, as the use of nanomaterials in biomedical products is relatively new and no formal evaluation guidelines have been established, the strategies adopted for preclinical studies on these products resemble those undertaken for other pharmaceuticals, including the way immunotoxicity is assessed^{2–7}.

Nanoparticles can have many different shapes and chemical compositions, which means that their properties — such as solubility and surface chemistry — can be engineered to make them suitable for a specific biomedical application. Research has shown that nanoparticles can stimulate and/or suppress immune responses by binding to proteins in the blood. Bound proteins determine particle uptake by various cells of the immune system and influence how nanoparticles interact with other blood components^{8–10}. Assessing the immunotoxicity of nanobased biomedical products involves the evaluation of all these properties.

The influence of size, solubility and surface modification on the biocompatibility of nanoparticles and their use in biological applications is well known¹¹. However, the effects of nanoparticle properties on the immune system are still being explored, and immunological studies of various nanopreparations generally fall into one of two categories: (1) responses to nanoparticles that are specifically modified to stimulate the immune system (for example, vaccine carriers) or (2) undesirable side-effects of other nanoparticles.

(For this review, we will follow the nanoparticle definition endorsed by the ASTM International¹² (Table 2).)

Available preclinical safety data indicates that nanoparticles are not intrinsically more immunotoxic than 'conventional' drugs (Table 3). Furthermore, reformulation of conventional drugs to include nanotechnology-derived carriers often leads to reduced systemic (whole body) immunotoxicity compared with that of unmodified formulations. For example, nanoparticles may keep drugs away from blood cells and normal tissues, releasing them only at targeted sites. Nanoparticles may also decrease the immunotoxicity of a drug by improving their solubility. Research suggests that the physico-chemical properties (size, shape, charge, surface groups, and so on) of the nanoparticles determine their immunotoxicity.

Numerous studies have attempted to address the toxicity issues associated with different nanoparticle administration routes. However, few definite trends can be established. This is probably because of the lack of a comprehensive evaluation of: (1) the composition, size, architecture and zeta potential (surface charge) of the nanoparticles; (2) the density, thickness and stability of the surface coating under physiological conditions; (3) a thorough comparison of (1) and (2) across various animal models; (4) variability in raw materials used to make these particles and differences in manufacturing procedures; and (5) thorough immunological characterization.

Nanoparticles can be administered via nasal, oral, intraocular, pulmonary (lungs) and other routes. Here, we will focus on the systemic route, which is common for a broad variety of engineered nanoparticles, and attempt to summarize their interaction with the immune system. We outline key considerations for preclinical immunological characterization of nanomaterials intended for biomedical applications.

BLOOD CONTACT PROPERTIES

Early studies show that modifying the surface of nanomaterials^{13–17} alters the nanoparticle/biological fluid interface and influences the

Table 1 Nanomaterials in the pharmaceutical pipeline. The products described here were summarized from ref. 1 and from the public domains of the relevant company's website as indicated.

Class of Nanomaterial	Application	Examples
Dendrimers	Drug delivery, imaging agents	VivaGel (SPL7013)
Fullerenes	Drug delivery, therapeutics	Antioxidant fullerene
Liposomes	Drug delivery	Doxil*
Superparamagnetic particles	Drug delivery, imaging agents	Ferumoxitol, Ferumoxtran-10, TNT-Anti-Ep-CAM†
Colloidal gold particles	Drug delivery, therapeutics	Aurimune, Aurothol
Polymeric Nanoparticles	Drug delivery, therapeutics, imaging	Basulin, Contramid, MRX-951
Gold nanoshells	Photo-thermal tumour ablation	AuroLase‡
Quantum dots	Imaging, diagnostics	QDot-800 conjugate
Self-assembling peptide/protein particles	Drug delivery, therapeutics	Abraxane

*www.orthobiotech.com; †www.tritonbiosystems.com; ‡www.nanospectra.com.

way nanoparticles interact with blood constituents. Furthermore, plasma protein binding is important in determining the *in vivo* organ distribution and clearance of certain nanotechnology-derived drug carriers^{8,10}. Nanoparticles whose surfaces are not modified to prevent adsorption of opsonins (blood serum proteins that signal cells to ingest the particles) were reportedly removed from the bloodstream within seconds by macrophages — cells that stimulate other immune cells and remove foreign materials by ingesting them through a process known as a phagocytosis⁹.

Protein binding to the surface of nanoparticles depends on particle surface characteristics^{18–21}, composition and the method of preparation^{22,23}. When polymeric nanoparticles, iron oxide particles, liposomes and carbon nanotubes were examined for bound proteins, the most abundant ones were albumin, apolipoprotein, immunoglobulins, complement and fibrinogen^{19–21,24–30}. It has been suggested that adsorption of plasma proteins depends primarily on nanoparticle surface hydrophobicity or charge^{19,28}. For example, when polyhexadecylcyanoacrylate nanoparticles were more densely coated with polyethylene glycol (PEG), less proteins were bound²⁸. Whereas it has been reported that protein adsorption decreases with increasing surface charge for several polymer-based nanoparticles³¹, the opposite trend has also been shown¹⁹. This disparity probably reflects the difficulty in making isolated changes to one physico-chemical parameter. For example, altering surface charge, which often involves addition of different functional groups on the particle surface, may also affect the surface hydrophobicity. More exact studies, which vary only one parameter at a time, are required to pin down the crucial structural factor(s) for protein binding.

Another common preclinical safety assessment examines the interaction of nanoparticles with red blood cells and platelets — cells involved in blood coagulation. Below are examples of how surface modification can improve nanoparticle–blood

compatibility. *In vitro* exposure of human whole blood to polyvinyl chloride resin particles increased blood coagulation time, aggregation and adhesion of platelets to these particles³². However, the same particles coated with PEG affected neither platelet counts nor coagulation³². Similarly, folate-coated and PEG-coated gadolinium nanoparticles did not cause platelet aggregation or activate neutrophils (a type of white blood cell)³³. When PEGylated and non-PEGylated cetyl alcohol/polysorbate nanoparticles were compared, haemolysis (disruption of red blood cells), platelet aggregation and coagulation were found to be time- and dose-dependant and were greater for non-PEGylated particles³⁴. Not all surface modifications aimed at increasing particle solubility can improve their compatibility with plasma coagulation. Water-soluble fullerene derivatives, for example, accelerated clotting times in tumour-bearing mice³⁵.

The biochemical cascade that removes pathogens, known as the complement system, is divided into two pathways. The classical complement pathway is activated by antigen–antibody complexes, whereas the alternative pathway is antibody independent. Nanoliposomes (Table 3) and engineered carbon nanotubes can activate the complement system²⁹. Intriguingly, both single-walled (SWNTs) and double-walled (DWNTs) carbon nanotubes stimulate the classical pathway but only DWNTs have been reported to trigger the alternative pathway. Despite a clear difference in protein adsorption by SWNTs and DWNTs, the mechanism of this selective complement activation remains unknown²⁹.

In summary, the above data suggest that nanomaterial structure and composition may be engineered to minimize effects on biochemical and cellular components of the blood. Understanding particle structure–activity relationships at the level of interaction with blood cellular and protein components will facilitate the development of a new generation of biocompatible drug carriers.

Table 2 Definitions. The terms summarized in this table are widely applicable in the literature, yet their use is sometimes controversial. The definitions provided here have been summarized from references 12,72–74.

Terminology	Definition
Ultrafine particle Nanoparticle	A particle ranging in size from approximately 0.1 μm (100 nm) to 0.001 μm (1 nm). A sub-classification of ultrafine particle with lengths in two or three dimensions greater than 0.001 μm (1 nm) and smaller than about 0.1 μm (100 nm) and which may or may not exhibit a size-related intensive property.
Endocytosis	A complex and highly regulated process of macromolecule and particle internalization by cells; includes two subcategories: phagocytosis and pinocytosis.
Phagocytosis	Actin-dependent endocytic mechanism restricted to 'professional' phagocytes: macrophages, dendritic cells and neutrophils; also defined as 'cell eating', uptake of large particles; subcategories include Fc γ receptor-, complement receptor- and mannose receptor-mediated phagocytosis.
Pinocytosis	Endocytic mechanism for the cellular uptake of fluids and solutes, which is further sub-categorized into macropinocytosis (endocytic vesicle (EV) size >1 μm); clathrin-mediated endocytosis (EV size ~120 nm); caveolin-mediated endocytosis (EV size ~60 nm) and clathrin- and caveolin-independent endocytosis (EV size ~90 nm); only macropinocytosis is actin-dependent, the three other pathways are actin-independent.

Table 3 Products in the drug development pipeline. These nanoparticle formulations were selected from ref. 1 with preference given to late-stage preclinical, clinical, and approved products (many are from the Nanotechnology Characterization Lab database). The selected conventional pharmaceuticals have long clinical-use records. This is not an exhaustive list, but many nanotechnology-based drugs are associated with fewer adverse immune reactions than conventional drugs. NA: not applicable; NAD: no available data; NSC: non-small cell; i.v.: intravenous; subc.: subcutaneous; i.m.: intramuscular; NDA: new drug application; TNF: tumour necrosis factor; RBC: red blood cell; BM: bone marrow; NK: natural killer cell; EPO: erythropoietin.

Product	Type of material	Size (nm)	Indication	Route of administration	Phase	Company	Interactions with immune system
Nanotechnology-based products							
Doxil	PEGylated liposome	75–80*	Metastatic ovarian cancer	i.v.	Approved	OrthoBiotech	Complement activation ¹⁰¹
Abraxane	Albumin-bound paclitaxel particles	130 ¹⁰²	NSC lung cancer, breast cancer, others	i.v.	Approved	Abraxis Oncology	Hypersensitivity reactions, weaker and less frequent than in paclitaxel ¹⁰¹
Basulin	Polyglutamate polymer dotted with insulin	20–50 [†]	Type I diabetes	subc.	Phase II	Flamel Technologies	NAD
Biovant	Nanosized calcium phosphate	NAD	Vaccine component	i.m.	Phase I completed	BioSante Pharmaceuticals	Adjuvant properties Elicits positive immune response to DNA-based and protein antigens stronger and with less hypersensitivity reactions than traditional aluminum hydroxide [†]
Cycloset-camptothecin INGN-401	Cyclodextrin nanoparticles	3–60 [†]	Metastatic solid tumours	i.v.	Phase I	Insert Therapeutics	NAD
	Liposome	NAD	Metastatic lung cancer	i.v.	Phase I	Introgen	NAD
Combidx (Ferumoxtran-10)	Iron oxide nanoparticles	17–20 ¹⁰³ 26.1–27.5 ¹⁰⁴	Tumour imaging	i.v.	NDA filed	Advanced Magnetix	No haematotoxicity, minor skin hypersensitivity reactions ¹⁰³
CYT-6091 (Aurimmune)	TNF α -bound PEGylated colloidal gold particles	33 ¹⁰⁵	Solid tumours	i.v.	Phase I	CytImmune Sciences	Decreases systemic TNF toxicity, targets tumours, but not healthy tissues [†]
MRX-951	Branching block copolymer self-assembling nanoparticles	40–70 ¹⁰⁶	Oncology	i.v.	Preclinical	ImaRx Therapeutics	No bone marrow toxicity even with repeated doses ¹⁰⁴
TNT-Anti-Ep-CAM	Polymer-coated iron oxide	NAD	Solid tumours	i.v.	Preclinical	Triton Biosystems	NAD
CYT-21001 (Auritol)	Taxol and TNF α -bound colloidal gold	26 [†]	Solid tumours	i.v.	Preclinical	CytImmune	NAD
AuroLase	Gold nanoshell	150*	Head and neck cancer	i.v.	Preclinical	Nanospectra Biosciences	Non toxic to RBC and platelets*
Dendrimer–Magnevist complex	PAMAM dendrimer	9.8 ¹⁰⁷	MRI imaging agent	i.v.	Preclinical	Dendritic Nanotechnologies	No interactions seen <i>in vitro</i> with RBCs, BM, platelets, coagulation, macrophage, NK and leukocyte functions ¹⁰⁷
Bioconjugated nanoparticles	Luminescent quantum dots encapsulated with ABC triblock copolymer	10–15 ¹⁰⁸	Cancer	subc.	Preclinical	Emory-Georgia Tech Nanotechnology Center	Accumulation in spleen; no data available about toxicity to immune cells ¹⁰⁶
DF1	Dendritic fullerene	4.7–4.9*	Chemoprotection	i.v.	Preclinical	Carbon Nanotechnology	Inhibit collagen-induced platelet aggregation; no toxicity to RBC and BM <i>in vitro</i> *
C3	Fullerene derivative	NAD	Chemoprotection	i.v.	Preclinical	Carbon Nanotechnology	Inhibit collagen-induced platelet aggregation; no toxicity to RBC and BM <i>in vitro</i> *
Biotechnology-derived and small molecule pharmaceuticals							
Infliximab	Neutralizing TNF α antibody	NA	Ankylosing spondylitis, i.v. arthritis, Crohn's disease,		Approved	Centocor	Reduces inflammation; side effects include tuberculosis, invasive fungal infections and rarely hepatosplenic T-cell lymphomas ¹⁰¹

Table 3 continued

Product	Type of material	Size (nm)	Indication	Route of administration	Phase	Company	Interactions with immune system
Epogen/Procrit	Recombinant human erythropoietin alpha	NA	Anaemia	i.v. subc.	Approved	Amgen/OrthoBiotech	Elevate and maintain RBC; side effects include pure red cell aplasia due to formation of EPO neutralizing antibodies ¹⁰¹
Roferon-A	Recombinant human interferon alpha2	NA	Chronic hepatitis C AIDS-related Kaposi sarcoma, melanoma	subc. i.m.	Approved	Hoffman La-Roche	Can cause or aggravate autoimmune or infectious disorders ¹⁰¹
Doxorubicin HCl/ Adriamycin RDF Paclitaxel/Taxol	Doxorubicin salt	NA	Cancer	i.v.	Approved	Bedford labs/ Pharmacia&Upjohn	Myelosuppression ¹⁰¹
		NA	Cancer	i.v.	Approved	SuperGen/BMS	Bone marrow suppression (neutropenia); anaphylaxis and severe hypersensitivity reactions ¹⁰¹
Neoprogen	Ibuprofen lysine	NA	Patent ductus arteriosus	i.v.	Approved	Ovation	Inhibit platelet aggregation ¹⁰¹

*Nanotechnology Characterization Lab, unpublished data.

[†]Information obtained from the public domain of the respective company's website.

IMMUNOSTIMULATORY PROPERTIES

Biological therapeutics are often immunostimulatory, that is, the immune system efficiently recognizes them as foreign substances and mounts a multilevel immune response against them. The molecular structure, architecture of folding motifs, degradation byproducts, formulation, package purity and stability of a biotechnology-derived pharmaceutical have all been shown to solicit an immune response on administration³⁶. Nanomaterials that trigger the immune response are expected to do so in an equally complex process. The immunostimulatory properties of nanoparticles discussed here include their antigenicity, adjuvant properties, inflammatory responses and the mechanisms through which nanoparticles are recognized by the immune system.

ANTIGENICITY

Biotechnology-derived pharmaceuticals can cause a specific antibody response (antigenicity). Antibodies, also called immunoglobulin, are specialized proteins produced by plasma B cells in response to an antigen (any foreign material). The immune response to a composite nanoparticle-based drug potentially involves antibodies for both the particles and the surface groups. Antibodies generated in response to the nanoparticles themselves will only affect the efficacy of particle-based products. However, the immune response to nanoparticles functionalized with growth factors, receptors or other biological molecules could include formation of neutralizing antibodies that will recognize both the particle and the endogenous (body's own) molecules, with clinical implications similar to those observed with biotechnology-derived therapeutics^{37,38}.

There are few studies on the antigenic properties of nanoparticles. An early study on polyamidoamine (PAMAM) dendrimers did not reveal overt antigenicity of generation 3, 5, and 7 amino-terminated dendrimers³⁹. Data on antigenicity of other dendrimers are not available. There are several studies on the immunogenic properties of nanosized gold for biomedical applications^{40,41} but none of them report particle-specific antibody generation. To date, only two studies describe specific anti-nanoparticle immunoglobulin

formation: the first describes polyclonal C₆₀-specific antibodies with a subpopulation cross-reacting with the C₇₀ fullerene⁴² and the second reports C₆₀-specific monoclonal antibodies⁴³. Two other studies did not observe anti-C₆₀ specific immunoglobulins even when used with complete Freund's adjuvant — an oil-in-water emulsion of killed mycobacteria that stimulates cell-mediated immunity in animals^{44,45}. This discrepancy in experimental results may be partly explained by the different choices of fullerene derivatives or by variation in the genetic background of the animal models. It is not currently clear if C₆₀, when used as a drug carrier, induces formation of C₆₀-specific antibodies. A variety of factors, including particle surface properties, functional groups and the genetic susceptibility and autoimmune disease history of patients receiving C₆₀ may ultimately affect the systemic antigenicity of this drug carrier.

ADJUVANT PROPERTIES

Adjuvants are agents added to a vaccine to augment immune responses toward antigens. A number of studies describe the use of nanoparticles as adjuvants. For example, polymethylmethacrylate nanoparticles induced long-lasting antibody titres (the quantity of substance required to produce a reaction) in HIV2 whole virus vaccine in mice. The antibody response was 100 times higher than when a traditional aluminium sulphate (alum) adjuvant was used⁴⁶. Similarly, when immunized with rabies glycoprotein, lipid-coated polysaccharide nanoparticles induced four times more immunoglobulin G than when immunized with alum adjuvant⁴⁷. Amino-terminated generation-5 PAMAM dendrimers also enhanced anti-ovalbumin immunoglobulin G and immunoglobulin M levels⁴⁸. Immunization of animals with both complete antigens and haptens — small molecules that can elicit an immune response only when attached to a large carrier such as a nanoparticle or a protein — conjugated to the surface of colloidal gold particles generated higher levels of specific antibodies than immunization of the same antigens with classical adjuvants⁴⁹. Furthermore, the amount of antigen required to achieve a high antibody response was an order of magnitude lower than for immunization with Freund's adjuvant⁴⁹. Injection of C₆₀

derivatives into mice resulted in an approximately 20-fold increase in anti-ovalbumin antibodies⁴⁵.

Adjuvant properties of another C₆₀ derivative were weaker than those of classical Freund's and alum adjuvants during the first immunization, but increased significantly after the sixth booster dose⁴⁴. Although the mechanisms of nanoparticle adjuvanticity are not completely understood, some studies suggest that nanoparticles can enhance antigen uptake and/or stimulate antigen-presenting cells. For example, poly-lactic-glycolic acid (PLGA) and modified lipid nanoparticles enhanced the immune response towards the lowly immunogenic cancer antigen MUC1 and plasmid DNA, respectively^{50,51}. In both cases, this was attributed to the particles' ability to induce the maturation of dendritic cells (DCs) — cells that detect and capture pathogens that enter the body. Other mechanisms and nanoparticle effects on vaccine efficacy are reviewed elsewhere⁵².

In summary, nanoparticles can be engineered to serve as vaccine carriers and adjuvants. Some studies suggest that nanoparticles can exacerbate allergic reactions⁴⁵ and, therefore, careful preclinical characterization is required. Comprehensive structure–activity relationship studies are necessary to further understand the critical parameters that determine the antigenic and adjuvant properties of nanoparticles.

INFLAMMATORY RESPONSES

In an inflammation, cells of the immune system are activated. The cells recognize the pathogens (or any invading foreign substance) and trigger an inflammatory response, which involves secretion of signalling molecules — known as cytokines — to attract more cells to destroy the foreign substance. Immune cells recognize nanoparticles by their surface properties and core composition and mount an inflammatory response in a similar way (Fig. 1a). However, many of the molecular events are still poorly understood. A systematic examination of a range of nanoparticle sizes and surface charges within each class of engineered nanomaterials is required to quantify how these parameters influence the inflammatory response.

In general, cationic (positively-charged) particles are more likely to induce inflammatory reactions than anionic (negatively-charged) and neutral species. For example, anionic generation-4.5 PAMAM dendrimers did not cause human leukocytes (white blood cells) to secrete cytokines⁵³ but cationic liposomes induced secretion of cytokines such as TNF, IL-12 and IFN γ (ref. 54). Systemic administration of another cationic nanoliposome alone or in combination with bacterial DNA did not induce cytokine production but increased the expression of DC surface markers, CD80/CD86 (ref. 51), which are important in the inflammatory response. Liposomes combined with bacterial DNA stimulated greater DC maturation than the DNA alone⁵¹. The immunostimulatory properties of nanoliposomes may benefit cancer treatment because they have shown anti-tumour effects in animal cancer models^{55,56}. Further, PLGA-based nanoparticles loaded with oligonucleotides induced greater cytokine production and T-cell proliferation than the oligonucleotide alone⁵⁷. Carboxyfullerene nanoparticles were seen to enhance the ability of neutrophils (another type of white blood cell) to destroy bacteria in a mouse model infected with *Streptococcus pyogenes*⁵⁸.

Trace impurities within the nanomaterial formulation can also frequently induce an inflammatory response. Early studies suggest that carbon nanotubes induce inflammatory reactions⁵⁹, but a more recent study shows that they don't when they are purified⁶⁰. Similarly, purified iron oxide and gold nanoparticles do not induce cytokine secretion^{61,62}. This shows that material purity and stability under physiological conditions can affect toxicity.

Another consideration in the inflammatory response is maintaining the Th1/Th2 response — the inflammatory reaction

triggered by Th cells that direct and activate other immune cells such as B and T cells and macrophages to secrete different cytokines. This response is important for protecting against cancer cells and pathogens and to avoid hypersensitivity (undesirable and exaggerated immune response) reactions. Several studies have addressed the influence of nanoparticles on Th1 and Th2 responses. Large (>1 μ m) industrialized particles induced the Th1 response, whereas smaller ones (<500 nm) were associated with Th2 (ref. 63). In contrast, some small engineered nanoparticles such as 500 nm PLGA^{57,64}, 270 nm PLGA⁶⁵, 80 nm and 100 nm nanoemulsions^{66,67}, 95 nm and 112 nm PEG–PHDA nanoparticles⁶⁸, and 123 nm dendrosome⁶⁹ induced the Th1 response. Other engineered particles (for example, 5-nm generation-5 PAMAM dendrimers) do not cause overt inflammatory reactions *in vivo*, but weakly induce Th2 cytokine production and enhanced immunoglobulin production^{48,70}. More structure–activity relationship studies are required to understand how size, surface modification and charge of engineered particles influence the Th1/Th2 balance. A detailed understanding of the properties that determine the Th response to biomedical nanoparticles will allow us to engineer nanotechnology-derived drug carriers that can protect the immune system and prevent hypersensitivity reactions.

Particle stimulation of adaptive (acquired) immunity has also been described. For example, small (<100 nm) polystyrene particles promoted CD8 and CD4 T-cell responses and were associated with higher antibody levels than larger (>500 nm) particles⁵². Understanding the mechanisms requires further investigation, and is important for nanovaccine formulation development⁵².

Using nanotechnology, particles can potentially be engineered to possess certain properties so they resemble pathogens and are dealt with in the body in a similar way. For example, key molecules that alert the immune system to pathogen infection are the Toll-like receptor (TLR) ligands. Modifying particles with TLR ligands essentially creates a pathogen-like particle that can be recognized by DCs. When TLR-modified nanoparticles are loaded with antigens, they can stimulate DCs to activate T cells that are antigen specific. Such regulated delivery of an antigen to the DCs can control the outcome of any immune response. In one study, DCs that received TLR-modified PLGA nanoparticles containing tetanus toxoid (a non-toxic derivative of a bacterial toxin) and MUC1 lipopeptide (a cancer vaccine candidate) induced significantly higher T-cell proliferation than control cells that were treated with the tetanus antigen alone or antigen and TLR ligands in solution⁵⁰. In another study, modifying the particle surface with mannose (a sugar molecule) stimulated uptake of the particle in macrophages by mannose receptors⁷¹, which are also specific for pathogen uptake and removal.

Liposome polycation plasmid DNA nanoparticles that stimulate the immune system is also thought to involve TLR-dependent signalling⁵¹. In contrast to Fc γ , mannose and complement receptors, which are discussed below, TLRs are not involved in phagocytosis (uptake of material), but rather regulate phagosome (membrane bound vesicle) maturation and initiation of phagosome-mediated inflammatory responses⁷². A better understanding of how modified particles stimulate TLR signals will allow drug designs that balance the intended immune responses (for example, improved vaccine efficacy) with exaggeration of inflammatory disorders.

UPTAKE MECHANISMS

Mammalian cells have five ways to internalize macromolecules and/or nanoparticles: phagocytosis (via mannose receptor-, complement receptor-, Fc γ receptor, and scavenger receptor-mediated pathways), macropinocytosis, clathrin-mediated, caveolin-mediated, and clathrin/caveolin-independent endocytosis^{72–74}

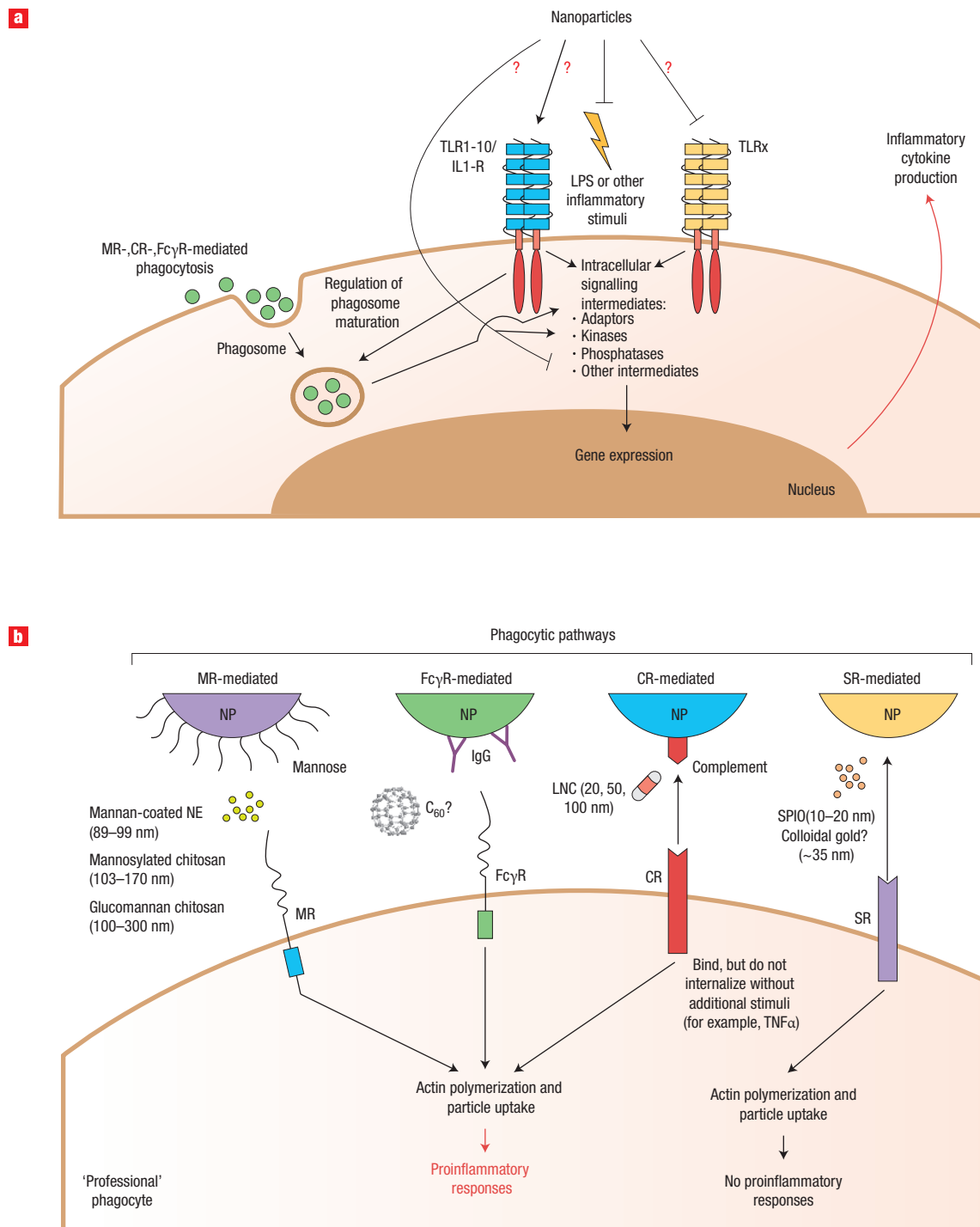


Figure 1 Cell uptake of materials and the different pathways. **a**, Cell uptake of nanoparticles by mannose receptor- (MR-), complement receptor- (CR-), and immunoglobulin Fc γ receptor- (Fc γ R)-mediated pathways elicit inflammatory responses and may affect toll-like receptor (TLR) signalling, which in turn affects various intracellular signalling intermediates. **b**, nanoparticles (NP) taken up via MR, Fc γ R and CR pathways elicit an immune response but it is possible to engineer nanoparticles for uptake via non-inflammatory pathways such as the scavenger receptor (SR-) mediated pathway. IL1-R: interleukin 1 receptor; LPS: lipopolysaccharide; NE: nanoemulsion; LNC: lipid nanocapsule; IgG: immunoglobulin G.

(see Table 2 and Fig. 1b). Each pathway involves a unique set of receptors and acts on particular types of particles. This discussion is limited to phagocytosis used by macrophages to eliminate pathogens and apoptotic cells. Below we briefly discuss the

terminology and summarize some of the studies on particle uptake via phagocytic receptors.

Traditionally, the term 'phagocytosis' describes the uptake of particulate materials that are $>0.5\ \mu\text{m}$ (ref. 74). Other endocytic

Table 4 Preclinical evaluation of nanomaterials intended for use in biomedical applications. This table summarizes the key questions to be addressed during early phase preclinical characterization. CFU-GM: colony-forming unit granulocyte macrophage; TDAR: T cell-dependent antibody response; endotoxin: gram-negative bacteria cell-wall component lipopolysaccharide.

Assay category	Questions to address
<i>In vitro</i>	
Haemolysis	Do nanoparticles* change integrity of red blood cells?
Platelet aggregation	Do nanoparticles* interfere with cellular components of the blood coagulation cascade?
Coagulation time	Do nanoparticles* cause changes in coagulation factors' function?
Complement activation	Do nanoparticles* activate the complement system?
CFU-GM	Do nanoparticles cause myelosuppression (toxicity to bone marrow precursors)?
Leukocyte proliferation	Do nanoparticles* have adverse effects on leukocyte proliferative responses?
Uptake by macrophages	Are nanoparticles* internalized by specialized phagocytes?
Cytokine induction	Do nanoparticles* activate immune cells to elicit cytokine production or interfere with that caused by known immunogens?
Nitric oxide production	Do nanoparticles* induce oxidative stress? Indirect test for potential endotoxin contamination.
Cytotoxicity of natural killer cells	Do nanoparticles* interfere with the ability of natural killer cells to recognize and kill tumour target cells?
Endotoxin contamination	Pyrogen contamination test
Microbial contamination	Sterility test
Viral/mycoplasma contamination	Sterility test
<i>In vivo</i>	
Single-dose toxicity study	
Standard toxicity tests	These tests aim at answering the following questions:
Blood chemistry	Do nanoparticles* cause toxicity to immune cells and organs?
Haematology	Are there any indications for additional toxicity studies?
Histopathology	Additional toxicity studies are conducted on a case-by-case basis using weight-of-evidence approach.
Gross pathology	This test is recognized for its high predictability for human models
TDAR	
Host resistance studies	These tests are recommended for (1) testing the potential effects that particles might have on host resistance towards pathogens and tumour cells and (2) to check for contact sensitization and delayed type hypersensitivity reactions
Evaluation of cell-mediated immunity	Do nanoparticles* elicit particle specific immune response?
Repeated dose toxicity study	
Immunogenicity	

* 'Nanoparticles' refers to a given nanotechnology concept rather than the generalization for a given class of nanomaterials or to a nanoparticle in general.

pathways are associated with solutes and smaller particles⁷³. Based on the size of endocytic vesicles, some reports categorize nanoparticle internalization as receptor-mediated endocytosis⁵². Nanoparticles, which represent a very diverse class of materials, can interact with plasma proteins and be engineered to target specific delivery pathways. For example, 50-nm polystyrene particles pre-incubated in plasma are internalized by Kupffer cells (liver macrophages) via scavenger receptors and the plasma protein fetuin was shown to mediate this uptake⁷⁵. When 'function', that is the entry pathway, is used as the criterion instead of 'size', the term 'phagocytosis' is also a valid description. For the purposes of preclinical characterization, we will use the terminology 'uptake by phagocytes' because the details of nanoparticle internalization are largely unknown, likely because of the limited capabilities for visualizing nanomaterials inside cells.

Highly sensitive techniques such as transmission electron microscopy and scanning electron microscopy detect only electron-dense materials such as metallic nanoparticles but cannot easily image polymers, dendrimers, liposomes and other 'soft' materials similar in size to cellular organelles. Because reliable visualization is difficult, most studies use indirect assays such as luminol-based detection or flow cytometry to examine phagocytosis. The former is a sensitive assay based on an increase in luminescence upon luminol oxidation in phagolysosomes, but it can be limited by particle quenching of the luminol signal when it is inside the cell. Flow cytometry assays for phagocytosis, on the other hand, cannot distinguish between particles attached to the cell membrane and internalized particles.

Conclusions based on studies that use fluorescently labelled nanoparticles are problematic because of (1) the unknown

stability of fluorophore nanoparticles under physiological conditions and (2) the lack of controls for assessing fluorophore effects on nanoparticle properties. Nevertheless, studies show that ~90-nm mannose-coated nanoemulsions, ~130-nm mannose-chitosan nanoparticles and ~200-nm gluco-mannan particles are internalized by macrophages via a mannose receptor-mediated phagocytic pathway^{66,76,77} and 20-, 50- and 100-nm lipid nanocapsules are internalized via a complement receptor-mediated pathway⁷⁸. Data also suggest that fullerene derivatives may be internalized via the Fcγ receptor route^{42,43} (Fig. 1b). Phagocytosis of microbial pathogens and nanoparticles via these pathways results in inflammatory cytokine production. Of interest are the studies with 10–20-nm superparamagnetic iron oxide nanoparticles (SPION) and ~35-nm colloidal gold nanoparticles, which demonstrate internalization via scavenger receptors^{62,79}. Scavenger receptor-mediated uptake is a 'classical' pathway macrophages use to eliminate apoptotic cells and is not accompanied by pro-inflammatory cytokine secretion⁷⁴. Indeed, neither ferumoxtran-10 (dextran-coated SPION) nor gold colloids induce inflammatory cytokines^{61,62}.

Although known to be influenced by particle size, shape, charge, and surface modification, the exact mechanism of colloidal nanoparticle uptake is still not well understood. Surface modifications with PEG, poloxamer and poloxamine polymers have been used as particle 'camouflage' to prevent phagocytosis⁸⁰. The phagocytic index, evaluated by particle number per cell, was shown to increase with lipid nanocapsule size 20 nm<50 nm<100 nm (ref. 77) and PEG5000-PHDCA particle size 80 nm<171 nm<243 nm (ref. 81). It was also shown that the uptake increases with zeta potential, that is, −3.8 mV

a

Size

- Th1/Th2 stimulation
- Adjuvant properties
- Internalization/phagocytic uptake
- Hapten properties
- Particle clearance

Charge

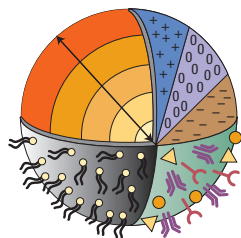
- Toxicity to immune cells
- Binding plasma proteins
- Particle clearance
- Immune cell stimulation

Hydrophobicity

- Interaction with plasma proteins
- Internalization/phagocytic uptake
- Immune cell stimulation
- Particle clearance

Targeting

Immunogenicity



b

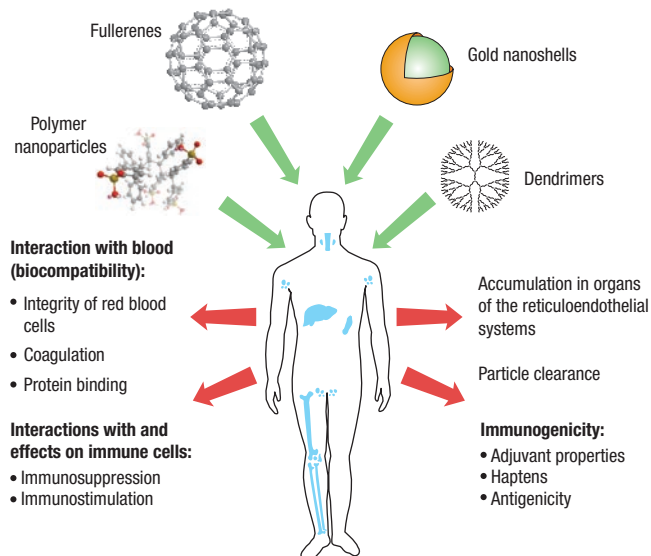


Figure 2 Nanoparticle properties determine their interaction with the immune system. **a**, The effect of nanoparticle size, charge, hydrophobicity and targeting on immunotoxicity. **b**, Some nanoparticles (shown schematically) can trigger certain immune responses as listed here. A characterization scheme for nanoparticles intended for biomedical applications must include testing for these responses. Such tests may exclude a potentially harmful drug candidate from the development pipeline and inform future studies relevant to the immunomodulatory properties of nanoparticles.

(size 169 nm) < -10.6 mV (171 nm) < -14.8 mV (172 nm) (ref. 81) and -0.1 mV (200 nm) < +40 mV (200 nm) (ref. 82). Of great interest is a recent study using large particles (>500 nm), which shows that shape decisively determines phagocytosis initiation, whereas particle size primarily impacts phagocytosis completion⁸³. More studies are required to conclude whether this holds for nanoparticles as well. In general, cationic or anionic and large particles are internalized more rapidly than smaller and neutral particles. Furthermore, the pathway for nanoparticle uptake is

defined by surface groups or by opsonins, which are proteins that bind to the particles in the blood stream.

Phagosome-mediated processing and presentation of nanoparticles may differ from that of 'canonical' antigens. Certain biodegradable nanoparticles can be taken up through conventional pathogen-specific routes and can stimulate inflammatory reactions just like pathogens^{66,76–78}. More mechanistic studies are required to understand how the immune system manages non-biodegradable components of nanoparticles (for example, metallic cores). Many questions remain regarding processing of multi-component and multi-functional nanoparticles. Are the individual components (the coating, core, and so on) stable inside the phagosome or do they separate? Are the biodegradable and non-biodegradable components processed together or individually? It is also interesting to know if nanoparticles will change the hydrolytic environment of maturing phagosomes — an important prerequisite for efficient antigen processing and presentation of MHC (major histocompatibility complex) class II proteins. The fundamental question, however, is whether manipulation of nanoparticle surfaces can direct particle-bound antigens to a particular pathway for more efficient processing, presentation and clearance of the antigens. This is also a central question for nanovaccine development. Challenges of vaccine delivery by nanotechnology-derived carriers are reviewed elsewhere⁵².

IMMUNOSUPPRESSIVE PROPERTIES

Immunosuppression is a down-regulation of the immune response. Undesirable immunosuppression may result in an impaired ability to cope with cancer and infections, whereas pharmaceutical down-regulation of the immune response may be beneficial in the treatment of inflammatory disorders. Some examples of nanoparticle-related immunosuppression are highlighted below.

Generation-3.5 PAMAM dendrimers conjugated to glucosamine strongly inhibited induction of inflammatory cytokines and chemokines in human macrophages and DCs exposed to bacterial endotoxin⁸⁴. Haematology studies of these conjugates did not reveal any overt toxicity, suggesting that the immunosuppressive effects of dendrimer-glucosamine conjugates can potentially treat and prevent scar tissue formation⁸⁴. Amino-terminated generation-5 PAMAM dendrimers modified with 2-hydroxyhexyl groups suppressed inflammatory cytokine secretion *in vitro* and *in vivo*, and were protective against endotoxin-induced sepsis — the fatal overwhelming immune response towards bacterial lipopolysaccharide⁸⁵.

Fullerene derivatives can quench the production of nitric oxide by macrophages because they scavenge free radicals like superoxide dismutase — the enzyme that protects against superoxide-induced damage to cells⁸⁶. Polymerized lipid nanoparticles that bind preferentially to selectins (cell adhesion molecules) on inflammation-activated endothelial cells attenuated both peribronchial inflammation and airway hyper-reactivity induced by an allergen. It is thought that the nanoparticles prevent eosinophils (a type of lymphocyte) from binding to the targeted selectins, which in turn decreases local inflammation and prevents tissue damage⁸⁷. Similarly, cholesteryl-butylate-conjugated solid lipid nanoparticles reduced neutrophil adhesion to inflammation-activated endothelial cells and are currently considered for treating colon ulcers⁸⁸. PLGA nanoparticles (100–200 nm in diameter) functionalized with betamethasone reduced inflammation in experimentally induced arthritis in rats⁸⁹.

All these studies demonstrate that nanoparticles may be engineered to be immunosuppressive for use as anti-inflammatory therapeutics. Concerns may arise, however, about undesirable immunosuppression. Will administration of nanoparticles designed for cancer therapy or imaging interfere with the body's immune

protection against cancerous cells and bacterial and viral pathogens? Tests for undesirable immunosuppression are an important part of preclinical nanoparticle evaluation. The great potential of biomedical engineering is that particles may be manipulated to reduce/eliminate immunosuppression and yet retain their therapeutic potential⁹⁰.

CHALLENGES IN IMMUNOTOXICITY TESTS

Immunotoxicological analysis of new molecular entities is not a straightforward process, and there is no universal guide for immunotoxicity. For example, whereas European authorities mandate the application of immune function tests as an initial screen for immunotoxicity in sub-chronic rodent toxicity studies, the US Food and Drug Administration recommends such tests only when previous evidence indicates a need. Japanese guidelines also follows the as-needed approach^{91–94}. According to an inter-laboratory survey of both European- and US-based pharmaceutical companies, an overwhelming amount of immunotoxicity studies (91%) were based on case-by-case reviews. In 55% of cases, decisions were made on the basis of a change in one or more parameter in routine toxicity tests such as lymphoid organ weight, cellular composition or histopathology⁹⁵.

As nanotechnology-based products are relatively new drug candidates¹, there are currently no agreed-upon guideline for assessing their immunotoxicity. However, data strongly suggest that nanoparticles do interact with the immune system (Fig. 2). In an attempt to provide a framework of clinical concerns for various aspects of immunotoxicity, we have suggested in Table 4 the important parameters to be addressed during an initial evaluation of nanotechnology-derived pharmaceuticals (composition and intended application of a product will define other considerations).

The main challenge in immunological studies of nanomaterials is choosing an experimental approach that is free of false-positive or false-negative readouts. The majority of the standard immunotoxicological methods are applicable to nanomaterials. However, as nanoparticles represent physically and chemically diverse materials, the classical methods cannot always be applied without modification, and novel approaches may be required. For example, many nanoparticles absorb in the UV–Vis range and some particles may catalyse enzyme reactions or quench fluorescent dyes commonly used as detection reagents in various end-point or kinetic assays. These and other methodological challenges in preclinical evaluation of nanoparticles are reviewed in detail elsewhere⁹⁶.

Both ‘classical’ and novel immunotoxicological assessments of nanomaterials clearly need a scrupulous stepwise validation, standardization, and demonstration of their physiological relevance. Industry, academics, and federal agencies are now collaborating to identify critical parameters in nanoparticles characterization and to establish acceptance criteria for nanomaterial-specific assays^{97–100}.

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Competing financial interests

Authors declare no competing financial interest.

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