HHS Public Access

Author manuscript

Adv Drug Deliv Rev. Author manuscript; available in PMC 2019 July 03.

Published in final edited form as:

Adv Drug Deliv Rev. 2018 May ; 130: 90–112. doi:10.1016/j.addr.2018.06.023.

Unintended effects of drug carriers: big issues of small particles

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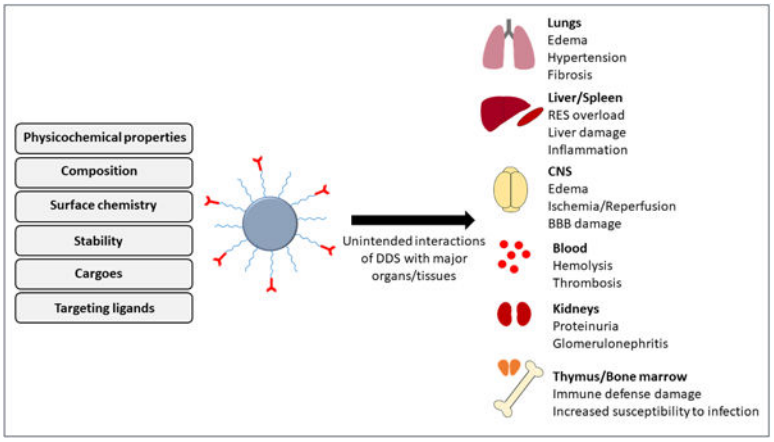
Abstract

Humoral and cellular host defense mechanisms including diverse phagocytes, leukocytes, and immune cells have evolved over millions of years to protect the body from microbes and other external and internal threats. These policing forces recognize engineered sub-micron drug delivery systems (DDS) as such a threat, and react accordingly. This leads to impediment of the therapeutic action, extensively studied and discussed in the literature. Here, we focus on side effects of DDS interactions with host defenses. We argue that for nanomedicine to reach its clinical potential, the field must redouble its efforts in understanding the interaction between drug delivery systems and the host defenses, so that we can engineer safer interventions with the greatest potential for clinical success.

Graphical abstract



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Keywords

Drug delivery system; nanoparticle; nanotoxicology; biocompatibility; immunogenicity; inflammation

1. Introduction

Drug delivery systems (DDSs) designed to improve effect of pharmacological agents inevitably elicit unintended effects in the body. Some of these effects are fairly benign or at least tolerable in the context of the medical application. Some may cause serious problems, adversities, and toxicities, which may preclude use of the DDS [1–3].

Practically every component of a DDS, including drug cargoes, may exert actions leading to undesirable effects within and outside the target. Many of these effects are distinct from those of a free drug, due to different pharmacokinetic, biodistribution, metabolism, and excretion. The carrier's interactions with body lead to additional, sometimes quite challenging safety issues [1,2,4].

Theoretically, every organ, tissue, cell, and molecule may represent a site of non-therapeutic activities of a DDS. In many cases, the intended target is the main site of side effects, which are relatively specific for each DDS. In the case of tumor eradication, such effects are beneficial, whereas in many other medical situations unintended interference with target molecule or cell has negative consequences [5,6].

There are components in the body, which are commonly involved in and/or affected by DDS-induced side effects. They include tissue components at the administration site, components of blood and vascular walls (specifically, endothelium lining the lumen), as well as main clearing organs (liver, kidneys, lymphatics, and spleen) and host defense systems (Fig. 1).

The multifaceted reactive systems of host defense are "professionally trained" to deal with natural invaders, which share many features of DDSs. Their interactions with DDS may lead to diverse potentially harmful consequences including elimination of DDS, activation of complement, white blood cells and resident macrophages. For the sake of focus and generalizability, this review will be focused on the unintended interactions of nanoscale DDS with host defense.

DDS's key physicochemical characteristics, including size, shape; surface properties such as morphology, rigidity, chemistry, and charge; materials' degradability; presence of impurities; as well as drug release kinetics can control the extent and nature of adverse effects. Several aspects of a nanoscale DDS that may contribute to its biological outcomes and unintended consequences are summarized in Fig. 2. In most cases, combination of these factors determines the fate of nanoparticle (NP) in the body. In this respect, while trying to provide the most probable NP feature connected to specific observed consequences, we understand the complex nature of such adverse effects which are generally orchestrated by multiple factors rather than a singular NP characteristic.

2. General aspects of DDS toxicology

2.1. Materials nature, excipients, and impurities

2.1.1. Biodegradable versus non-biodegradable materials—Biocompatibility of the materials is not equivalent to their biodegradation. Some non-destructible materials such as metals and ceramics are fully biocompatible in form of implants and prosthetics, while some degradable materials can exert adverse toxic effects. Biocompatibility should be considered in the context of the patient's condition, off-target interactions, route of administration, and characteristics of the materials.

Development of biodegradable and biocompatible DDSs is essential for prevention of any material-related harmful side effects. Biodegradable nanoparticles include those based on proteins, polysaccharides, and natural or synthetic polymers. Alginate, chitosan, agarose, and gelatin are examples of natural biodegradable polymers [7–10]. These natural polymers have been extensively used in development of DDSs as well as scaffolds in tissue engineering. Synthetic biodegradable polymers include poly(D,L-lactide-co-glycolide) (PLGA), polylactic acid (PLA), and poly-ε-caprolactone (PCL) [11–14]. Although biodegradable, synthetic polymeric materials have been reported to degrade into acidic byproducts (e.g. glycolic and lactic acids), which can reduce the local pH and lead to an inflammatory reaction [15,16].

Examples of non-biodegradable nanoparticles include ceramics, metal colloids, and polymers, such as (polyethylenimine) PEI and poly(dimethylaminoethyl methacrylate) (PDMAEMA) [17,18]. Non-biodegradable nanoparticles are cleared by the mononuclear phagocytic system and accumulate in the liver and spleen. This can further lead to potential irreversible toxic side effects [11]. Non-biodegradable rigid nanoparticles have been reported to induce reactive oxygen species (ROS) and autophagy. Polystyrene nanospheres, silica nanospheres, single-walled carbon nanotubes, and elongated iron oxide nanorods are among those NPs that induce ROS generation. Rigid, non-biodegradable nanoparticles, whether elongated or spherical have been found to trigger similar cellular effects, with the generation of ROS and autophagy being more severe in the elongated nanoparticles [19]. Although the precise mechanism for such enhanced ROS generation is not clear, elongated nanoparticles are believed to be able to escape endosome/lysosome [19]. In contrast to non-biodegradable NPs, reports of ROS generation as a result of biodegradable NPs has been minimal, exhibiting mainly in cases of biodegradable inorganic nanoparticles such as calcium carbonate nanocrystals and zinc oxide nanoparticles [20,21].

Polycationic polymers, such as PEI and PDMAEM are well-known for their efficiency in nucleic acid delivery [22], however they exhibit high cytotoxicity and are non-biodegradable. This can result in further complications, particularly when used as a long-term delivery system, where the non-biodegradable polymeric material continues to accumulate in the cytoplasm or the nucleus of the transfected cells [17,18]. High molecular weight (HMW) PEI (PEI 25k)-based nucleic acid delivery systems are non-biodegradable, and therefore changes have been made to these polycationic polymer to allow them to maintain high transfection efficiency, but in the same time reduce toxicity and enhance their degradability [22,23]. Some have tried to develop an efficient biodegradable PEI delivery

system by combining low molecular (LMW) PEI polymers together using degradable cross-linkers to mimic HMW constructs. These modified PEIs were reported to be biodegradable and very efficient in gene delivery. Others have developed co-polymers of PEI with biocompatible and biodegradable polymers such as poly(β-amino esters) (PBAEs) [24]. PBAEs were developed using combinatorial synthesis and screening methods, with the lead candidates demonstrating high gene delivery efficiency, excellent biodegradability, and low cytotoxicity [25,26].

The therapeutic application and target organ of the nanoparticles must be considered when fine-tuning the biodegradability of the nanoparticle. There are instances where biodegradable nanoparticles are non-biocompatible and where non-biodegradable nanoparticles are biocompatible. These can occur when the nanoparticle degradation byproducts induce inflammatory or toxic effects [1,15,27], or when a non-biodegradable nanoparticles induces anti-oxidant or cytoprotective effects [28–30].

2.1.2. Excipients and impurities—Different types of non-APIs (non-active pharmaceutical ingredients) may be used in nanomedicine formulations, similar to any other type of pharmaceutical formulation. These could encompass a variety of excipients such as solvents/vehicles, surfactants, preservatives, stabilizers, etc. Excipients in nanomedicines should be chosen carefully to avoid any side effects originated from these components of formulation. Excipient stability after nanosizing, freeze-drying, and sterilization techniques should also be clearly monitored [31]. As reviewed by Center for Drug Evaluation and Research, FDA, the required specific safety data for excipients will depend on clinical factors including route of administration, treatment duration, existing reproductive and genetic toxicity, carcinogenicity, and hypersensitivity data [32]. For example, when applied intravenously, propylene glycol can be cardiotoxic and provoke thrombophlebitis [32].

Cremophor-EL is a nanosized micellar excipient often used to solubilize hydrophobic drugs such as paclitaxel. However, it can activate the complement system and induce mononuclear cells to produce pro-inflammatory chemokine IL-8 [33,34]. In this regard, protein-bound paclitaxel, also known as nanoparticle albumin–bound paclitaxel or nab-paclitaxel (Abraxane) is a good example of an improved nanoparticle formulation design. Nab-paclitaxel does not require any toxic solvent unlike its conventional counterparts, thus avoiding solvent-related toxicity issues such as hypersensitivity and neuropathy, which was among the side effects of conventional formulations of paclitaxel [35]. A common problem with solvent-based paclitaxel is the micelle entrapment of drug resulting in non-linear pharmacokinetics (PK). This issue does not happen with nab-paclitaxel [36]. Moreover, patients taking nab-paclitaxel do not require any premedication such as antihistamines and corticosteroids to prevent hypersensitivity reactions related to the solvents [37].

Contamination with impurities such as bacterial lipopolysaccharide (LPS), or endotoxin should be also cautiously monitored in NP preparations. Due to their high surface area, small particles can easily adsorb high amount of endotoxin if available. Undetected presence of endotoxin when formulating NPs could cause misinterpretation of NP-associated toxicological and inflammatory responses [38–41]. Monocytes and dendritic cells are particularly sensitive to endotoxin and exhibit inflammatory responses to minute amounts of

LPS, usually considered as “endotoxin-free” in standard commercial products [38,42]. LPS not only interacts with well-known cell receptors such as TLR4 [43], but also stimulate intracellular LPS sensors [44,45]. It is now believed that LPS can be recognized within the cytoplasm by caspases 4 and 5 in humans and by caspase 11 in mice. Caspase-mediated LPS signal may potentially trigger inflammasome activation [46]. Therefore, it is plausible that an endotoxin contaminant transported into a cell via NP preparation could interact with caspases, inducing inflammasome stimulation in monocytes and DCs. These observations and potential unintended events make it necessary to recognize and remove even trace levels of LPS or any other similar contaminant containing pathogen-associated molecular patterns (PAMPs) when preparing NP formulations.

Several sterilization techniques such as filtration, thermal sterilization, irradiation, and other methods have been reviewed for nanoparticle formulation sterilization [47]. As far as they do not affect the stability of NP and biological activity of active ingredients, these approaches are pretty effective on microbial decontamination [40,48]. However, common sterilization procedures are usually not efficient in removing endotoxin contamination in NP samples. Techniques developed to remove endotoxin from samples are usually called ‘depyrogenation’ techniques [40,49].

Ultrafiltration was proposed as one such method. For example, endotoxin is shown to be retained by a 0.025 μm filter, as endotoxin can adsorb to the filter membrane [40,50]. A drawback of applying this procedure for depyrogenation of nanoparticle formulations is that particles may trap on membrane as well. Moreover, the contaminated particles could still escape through the filter if endotoxin is already adsorbed on the particle surface [40].

Another example is two-phase extraction technique [51], in which endotoxin can be removed with the added detergent to sample via the nonpolar interaction of lipid A. Gold nanoparticles were reported to become nonpyrogenic after treating with this two-phase method [52]. However, usually traces of detergent remain in the sample, which requires further purification steps such as adsorption or gel-filtration to remove the detergent [40].

The plasma discharge method can be given as another example of effective strategy for sterilization and depyrogenation. Active species (e.g., atomic oxygen, OH radicals) produced by the plasma discharge are very potent in killing spores and microorganisms, as well as destroying other contaminants such as pyrogens/endotoxin [53]. Although demonstrated to be effective in removing endotoxin from nanoparticle formulations, this method should be validated for each type of nanoparticle as it can significantly alter the surface characteristics of NPs [40,48].

Pyrogen inactivation techniques involving acid-base hydrolysis, oxidation, treatment with sodium hydroxide and heating, are other effective strategies to remove endotoxin [40]. These procedures have been successfully applied for microparticles such as titanium [54] or cobalt chrome particles [55]. Due to the aggressive nature of these techniques, their use for nanomaterials is usually challenging. Among these, the incineration method seems to be feasible for nanoparticles in solid form (powder) including carbon nanotubes or titanium dioxide NPs, since they can tolerate the high temperature. For example, silicon oxide,

titanium oxide, zirconium oxide, and cobalt nanoparticles were efficiently depyrogenated at 180°C for 4 h [56].

Other than strategies mentioned above, there are less-investigated methods that have been suggested for endotoxin separation. These include ion-exchange chromatography, affinity adsorption chromatography, and gel filtration chromatography [40]. Overall, it is obvious that endotoxin removal is not an easy task and its applicability is highly dependent on the nature of NP formulation that needs to be decontaminated. Therefore, preventing contamination by using endotoxin-free reagents and glassware during the NP synthesis seems to be the best way for obtaining endotoxin-free particles [40,57].

2.2. Size

Unlike large drug delivery systems that are usually restricted to limited body areas, nanoparticles typically travel throughout the body quickly and face several cellular and non-cellular systemic components. Therefore, sub-micron DDS adversities may include mechanisms that are not ignited by large delivery systems of the same material. Similarly, nanoparticle is also different from bulk materials. A common diameter size range for NPs injected systemically is 50–300 nm [58]. Enhanced surface/mass (S/M) ratio in NPs compared to larger counterparts exerts significant impact on their circulation, degradation, and intracellular delivery (Table 1). For instance, highly increased S/M may enable degradation via burst release instead of slower, safer surface erosion. S/M ratio also enhances the ability of NP to absorb, activate, and deplete biomolecules [59,60]. Extravasation, tissue penetration, and localization may also differ from larger delivery systems.

Due to the small size and high surface area in nanoparticles, chemically reactive groups are highly exposed and could play a role in the adverse biological effects. For instance, the enhanced surface reactivity may lead to protein unfolding, membrane impairment, DNA damage, immune reactivity, and inflammatory responses [1]. It has been demonstrated that titanium dioxide (TiO_2) NP size modulate the affinity of plasma proteins such as fibrinogen towards the TiO_2 surfaces [61,62]. NP aggregates with extensive surface area and correspondingly increased number of contact points with fibrinogen [61] triggered elevated inflammatory responses [62].

Smaller particles bind to cells and may internalize via vacuolar uptake mechanisms unavailable to larger objects. Particles in the size range of 10–500 nm and even up to micron size were reported to get internalized into cells via vesicular pathways [63–65]. The large particles can be engulfed via macropinocytosis [64], whereas particles with sizes ranging 10–300 nm were reported to enter through clathrin-mediated mechanism [63,66]. Caveolae-mediated pathway may facilitate the entry of nanoparticles of 60–80 nm [64,67] and in some cases up to 100 nm in diameter [68]. It should be noted that modulation of the uptake and trafficking by size of particles is also cell-type specific. For example, professional phagocytes consume relatively larger particles in the micron range faster, whereas endothelial cells prefer an order of magnitude smaller particles [69].

Some adverse effects of internalizable DDS may relate to their interactions with sub-cellular organelles. Endoplasmic reticulum (ER) is a vital sub-cellular compartment mediating protein synthesis, protein folding, Ca^{2+} storage, and lipid biosynthesis [70]. The effect of NP exposure on ER stress has been reviewed elsewhere [1,71,72]. As depicted by Hussain et al. [72], NPs are capable of inducing ROS production via their inherent effect on catalysis of oxidation reduction reactions through their surfaces. They may also interact with cellular components and/or normal ROS production mechanisms such as mitochondria and NADPH oxidase system. Moreover, NPs can decrease the cellular anti-oxidant defense mechanisms through savaging/inactivation or decreased production of anti-oxidants [72]. A hierarchical oxidative stress model was proposed by Nel et al. [1] as 1) inducing anti-oxidant enzymes at low levels, 2) activating pro-inflammatory responses at intermediate levels and 3) triggering cell death at very high oxidative stress levels. For instance, ER stress may evolve into mitochondrial-dependent apoptosis due to the surge in cytosolic Ca^{2+} and ROS levels [73,74]. ER stress may also trigger autophagy to enable the clearance of accumulated proteins [70]. Another subcellular compartment, the lysosome, functions as a cellular digestive organelle. If the function of a lysosome is impaired by the accrual of nanomaterials, autophagy flux can be blocked by subsequent autophagosome accumulation. Furthermore, the release of cathepsins and other associated lysosomal hydrolases into the cytoplasm may trigger inflammation responses and mitochondrial depolarization [70,73,75].

NP size may also affect changes in cell phenotype. Particle size along with other characteristics play a role in inducing M1 polarization in macrophages [2]. Ma et al. [76] reported a size-dependent interaction between graphene oxide (GO) nanoparticles and plasma membrane. They demonstrated that larger (750–1300 nm) GO could drastically stimulate TLRs (e.g., TLR4) leading to macrophage M1 polarization through canonical NF-κB signaling, which could promote pro-inflammatory responses. The authors correlated this effect to a robust adsorption of larger GOs onto the plasma membrane rather than massive phagocytosis that mainly occurs with smaller (50–350 nm) GO sheets. A slightly reversed phenomenon was reported by Yen et al. [77] for gold nanoparticles (AuNPs), in which the macrophages treated with smaller AuNPs showed giant spread morphology representative of macrophage activation. Moreover, AuNPs at 1 ppm led to up-regulation of pro-inflammatory cytokines such as IL-1, IL-6, and TNFα in macrophages shortly (3 hrs) after the treatment. Here, the inflammatory response was described as being activated by NP uptake rather than spread over macrophages. Serum adsorption onto negatively-charged AuNPs could possibly result in internalization via complicated endocytic pathways that led to the pro-inflammatory reaction. Size, therefore, can be used as a property of NP to target specific cells. For example, particle size range of 20–40 nm has been suggested to be ideal for NPs uptake into dendritic cells (DCs) [78,79].

Nanoscaled DDS usually consists of different entities including carrier components, excipients, and therapeutic content; each may hold toxicity induction capabilities. Hence, any study evaluating the adverse behavior of DDS demands careful investigation of each entity individually, and in combination with other parts as a whole nanoparticulate system. Nanomaterials may induce complex cellular signaling mechanisms or modify existing signaling pathways resulting in adverse/unexpected consequences [72]. Mechanistic studies specifically focused on the fate of nanoparticles *in vivo*, such as the effect of each individual

NP building block/feature on interaction with plasma components as well as cellular entities and the subsequent potential side effects will allow us to design safer DDS moving forward.

2.3. Shape

Shape of NP can be considered as even more of a determining factor in cellular uptake than size. The rate by which phagocytic cells engulf nanoDDS changes with the geometrical properties. Champion et al. [80] demonstrated that the local shape of polystyrene beads at the point of initial contact with macrophages, and not their size, determine whether cells will proceed with phagocytosis or simply spread over the particle. For instance, elongated prolate ellipsoidal shaped polystyrene particles attach to phagocytic cells better than spheres, but are phagocytosed less efficiently [81]. Therefore, it was suggested that elongated particles deliver drugs to various types of cells more efficiently by eluding internalization into phagocytic cells [80,82,83]. Similarly, disk-shaped polystyrene beads presented longer half-lives in circulation and greater targeting specificity in mice compared to analogous spherical particles [84]. In rodents, long worm-shaped PEG-polyethylethylene filomicelles, block copolymer micelles that form into filaments, exhibited prolonged circulation time, escaped macrophage internalization, and accumulated highly in tumors [85,86].

Although non-spherical particles seem to evade the clearance mechanism to some extent, one can imagine that the higher contact area of elongated shaped NPs with the cells may lead to more damage to cell membrane [87]. In this context, rod-shaped silver NPs were found to be toxic whereas spherical NPs with the same mass concentration were shown to be safe on the human lung epithelial cell (A549 cells). The direct contact and accumulation of long wires onto the cells was proposed to cause toxicity by inducing small pores in the cell membrane. A similar trend was observed by comparing the toxic behavior of rod-shaped zinc oxide (ZnO) NPs versus spherical counterparts on A549 [88], although it should be noted that A549 cells are not highly phagocytic in nature. Using different types of cells, similar conclusions were made in the work of Wang et al. [89], in which long anodic alumina nanotubes (AANTs-L) triggered significant alternations in cell morphology, TNF-α release, lysosomal membrane permeabilization (LMP), and ER stress leading to cell death and inflammation to a greater extent than shorter nanotubes. This study was performed on both RAW 264.7 mouse macrophage cells and MDA-MB 231-TXSA human breast cancer cells. A combination of NP properties including size, shape, and surface chemistry may direct the particles into sub-cellular compartments including lysosomes and orchestrate their subsequent mechanical and chemical vacuole rupture. The resulting release of danger signals into the cytoplasm will potentially induce toxicity, although this effect has been exploited to enhance cancer vaccine efficiency [90,91].

Particle shape has also been linked to the genotoxicity observed by some NPs [92]. For example, tubular-shaped particles such as carbon nanotubes (CNTs) induced greater DNA damage than ZnO particles. It should be noted that since ZnO particles create a higher level of oxidative stress, the superior DNA damage detected by CNTs likely comes from mechanical injury and not oxidative effect. The fact that there is evidence on CNTs crossing the nuclear membrane and reaching the nucleus justifies this physical contact-based theory [93,94].

The impact of particle shape on the biological outcome of NPs was also reported in large animals. Wibroe et al. [95] showed that spherical particles were more rapidly cleared from the blood as compared to non-spherical particles (rods and disks) when injected directly in pigs (Fig. 3a–d). Although initially, spheres, rods, and disks did not stimulate the complement system five minutes post I.V. injection, delayed complement activation was induced more robustly by rods and disks, rather than spheres (Fig. 3e and f). In the same study, rods and disks did not induce any notable cardiopulmonary distress when compared to spheres (Fig. 3g). It was implied that immediate and robust particle phagocytosis by resident pulmonary intravascular macrophages (PIMs) may lead to cardiopulmonary responses [95]. In a different context, wire-shaped aluminum oxide nanoparticles exhibited pro-inflammatory effects such as NLRP3 inflammasome activation and enhanced TGF-β level on primary splenocytes more significantly than spherical counterparts. Nanowires led to more profound inflammation and enhanced degree of lung metastasis in a syngeneic mouse tumor model relative to spheres [4].

2.4. Elasticity

Careful control of the mechanical properties of nanomaterials has emerged as a design consideration in nanomedicine [58,96]. The mechanical flexibility of a nanoparticle can tune biomedical outcomes including pharmacokinetics and *in vivo* targeting, which in turn modulate clearance mechanisms and off-target behaviors.

Most notably, flexible nanoparticles have delayed clearance relative to stiff counterparts. Red blood cells represent a natural blueprint for engineering of materials that are benign during prolonged circulation in the blood. Among their salient characteristics, red cells are capable of avoiding splenic filtration and undergoing repeated extrusion through capillaries of ~1/10 their diameter [97]. Highly flexible hydrogel nanoparticles designed to mimic the shape and size of red cells have circulation times prolonged relative to similar particles with higher crosslinker density [98,99]. However, even without mimicry of the size and shape of red cells, more flexible nanoparticles have reduced RES interactions. PEG diacrylate nanogels with elastic moduli better resembling those of cells (~10 kPa) circulated longer and more effectively targeted the lungs as compared to harder (~3000 kPa) PEG diacrylate particles [100]. Filomicelles manifest a combination of high aspect ratio elongated shape and low rigidity [85,101,102]. Their properties uniquely allow alignment with blood flow and, all told, the filament particles circulate ~10 times longer than similar spherical particles [85,101]

Beyond behavior in flowing blood, minimized RES clearance of flexible filomicelles may also be explained by the minimal interactions of the particles with cells *in vitro* in the absence of antibody-directed adhesion to cell surfaces [101]. Indeed, the mechanical properties of nanoparticles may generally represent a variable affecting nanoparticle interaction with and uptake by a variety of cell types. Noting that mechanical flexibility of the microenvironment or substrate hosting a cell can dramatically affect the behaviors of the cell [103], it is perhaps unsurprising that the mechanical flexibility of nanoparticles interacting with cells can impact the tendencies of the cell-particles interaction. It was confirmed *in vitro* that rigid disks were consumed up to 3-fold more efficiently by mouse

bone marrow-derived macrophages and macrophage-like cell lines in comparison to softer counterpart particles [104,105]. During the phagocytic process, soft particles can be easily deformed, which makes their internalization less energetically favorable than rigid particles [106]. Polyallylamine particles escaped phagocytosis when they were made softer by reduction of crosslinking density, with soft polyacrylamide particles avoiding Fc-mediated uptake *in vitro* [107,108]. Similarly, the PEG diacrylate particles described above more effectively avoided non-specific uptake in tumor cells, endothelial cells, and macrophages when they were manufactured with ~10 kPa modulus, as opposed to ~3000 kPa [100].

While there is evidence that nanoparticle flexibility may reduce non-specific uptake in immune cells and avoid RES clearance, lower elastic moduli may conversely enhance nanoparticle targeting in certain cases. Soft platelet-mimetic polyallylamine hydrochloride-BSA particles exceeded counterpart rigid particles with identical size, shape, and surface chemistry in targeting fibro-collagenous surfaces [109]. Increased membrane fluidity of antibody-functionalized lipid particles has been shown to enhance selectivity of targeting to endothelial markers [110]. Beyond direct effects on nanoparticle affinity interactions, the synergy of affinity targeting and prolonged circulation due to mechanical flexibility has yielded enhanced targeting to intercellular adhesion molecule (ICAM) in the case of PEG diacrylate nanogels [100]. There is also evidence of a role for nanoparticle mechanical flexibility in non-affinity targeting. Mammary epithelial cells and breast cancer cells more avidly took up lipid-alginate particles with lower Young's moduli (as controlled by alginate crosslinking density). *In vivo*, the highly flexible lipid-alginate particles successfully targeted tumors in an orthotopic model, while concurrently manifesting reduced liver uptake, relative to rigid counterparts [111].

Of note, precise tuning and characterization of the mechanical flexibility of biocompatible nanomaterials is an area of ongoing research [85,112,113]. Further consideration given to the role of physical structural properties for injectable nanomaterials may identify additional effects and side effects of mechanical properties. For instance, recent studies have explored nanomaterials that either mimic or interact with blood components. A critical role for materials flexibility has been identified for nanoparticles incorporating in the coagulation process, where nanogels targeted to fibrin have been shown to affect clot structure during the process of clot contraction. Inflexible particles disrupt the process of clot contraction and solidification, while flexible counterparts participate in and enhance that process [114].

Conversely, modifying the mechanical properties of nanomaterials may come with new unintended side effects. Liposomes, hydrogels, and polymer-lipid composites can be modified via chemical structure of molecular components or features of the supramolecular assembly [96,110,111,115]. A standard and direct approach to modification of the flexibility of polymeric nanomaterials is the inclusion of crosslinkers [107,115]. However, inclusion of crosslinking (either intrinsic to the polymer or via introduction of exogenous bifunctional linkers) entails variation of polymerization conditions and possible modifications to the size distribution and surface chemistry of the resultant nanomaterials. Therefore, interpretation of flexibility effects on biomedical behavior of nanomaterials may be subject to assessment of the interplay of mechanical properties with chemistry, size, and shape. Likewise, outcomes

regarding side effects and pharmacokinetics, including those enumerated here, may require further investigation exploring a more thorough space of physical design parameters [96].

2.5. Surface chemistry and charge

Multiple surface properties such as charge, hydrophobicity, and specific functional groups can contribute significantly to nanoparticle-associated toxicity. Even slight changes in surface chemistry may modulate extent of NPs interaction with cells. As an example, galactose- and mannose-modified silver nanoparticles showed considerably less toxicity than glucose- and citrate-modified NPs on both a neuronal-like cell line (Neuro-2A) and a hepatocyte cell line (HepG2) [116]. Pre-coating of particles with serum proteins also appears to reduce NPs harmful impact [117]. Serum protein coat on TiO2 particles prevented photo-generated radical production, suggestive of the barrier role of serum proteins [118].

The net charge on the particle surface is a major factor controlling not only the interaction with plasma components or cells, but also with subsequent cargo release. Nucleic acid carriers such as polyethylenimine (PEI), poly(propylene imine) (PPIs) dendrimers, polyamidoamine (PAMAM) dendrimers, etc. take advantage of their highly positive charged surface to interact with cell membranes effectively [22]. Polycationic vehicles have been shown to destabilize endosomal membranes, buffer the acidic endosomal pH, and mediate lysosomal rupture, leading to high cargo release into cytoplasm and enhancing transfection efficiency [119]. The same effective interaction with several layers of membranes may also lead to membrane perturbation and toxicity, especially when used at high doses.

Both linear and branched PEIs induced rapid plasma membrane disturbance in three clinically relevant human cell lines (Jurkat T cells, umbilical vein endothelial cells, and THLE3 hepatocyte-like cells) within 30 min of exposure. These early necrotic-like changes include substantial lactate dehydrogenase release and phosphatidylserine translocation from the inner plasma membrane to the outer cell surface. Later toxic events (24 hrs post-treatment) are characterized by activation of a mitochondrially-driven apoptotic program [120]. In a similar way, highly positively charged amidine functionalized polystyrene (PS) particles impaired lysosome function. Possibly PS particles displayed their disruptive effect on lysosomes through a similar lysosomal rupture mechanism as explained for PEI [121]. Compared to linear PEI, branched PEI architecture led to an enhanced intracellular ROS levels. The branched PEI structure also induced a concentration-dependent collapse in glycolytic flux reducing glucose flux through the pentose phosphate pathway (PPP). As mentioned above, leakiness to key player enzymes of glycolysis such as LDH could probably disturb the rate of glycolytic flux [122].

The presence of dense clusters of surface cationic charge in polycations and polyplexes enhances complement activation [123]. Cardiopulmonary distress observed upon PEI injection in the porcine model [124] can be explained in part by complement activation. Moreover, a strong polycation and polyplex clearance by intravascular pulmonary macrophages in pigs independently of complement stimulation leads to a surged release of thromboxane A2, prostaglandin, and prostacyclin molecules which could subsequently mediate periods of peak vasoconstriction, bronchoconstriction, and pulmonary hypertension [125].

Although not extensively discussed, anionic NPs can also initiate adverse effects. Positively charged AuNPs were reported to adsorb proteins with a pI <5.5 such as albumin, whereas negatively charged AuNPs were covered with a protein corona mainly composed of proteins with a pI >5.5 such as apolipoprotein [126]. In another report, negatively charged poly(acrylic acid) (PAA)-conjugated AuNPs were shown to adsorb fibrinogen on their surface. Upon attachment to the surface of NPs, fibrinogen becomes unfolded and induces inflammatory cytokine release via the integrin receptor, Mac-1, leading to subsequent NF-kB signaling [62]. Negatively-charged NPs have been also found to induce the classical complement cascade [127].

It is generally believed that scavenger receptors, a family of cell surface glycoproteins, recognize some anionic surfaces [128]. Different types of scavenger receptors on macrophages are reported to remove a variety of negatively charged particles such as liposomes containing negatively charged phospholipids [129], and negatively charged polystyrene nanospheres [130]. Nagayama et al. [130] demonstrated that a serum protein called fetuin, associated on the surface of negatively charged polystyrene nanospheres, directed its uptake by Kupffer cells via scavenger receptors. It was reported that scavenger receptor SR-B1, which is involved in the uptake of AgNPs, could activate immune system by inducing pro-inflammatory cytokines and up-regulation of co-stimulatory molecules [131,132]. Furthermore, macrophages from SR-B1 deficient mice internalized lower number of AgNPs and showed a reduced inflammatory response as measured by neutrophilic influx and IL-6 mRNA expression [131,132].

2.6. *In vitro-in vivo* correlation in nanotoxicology

Numerous types of *in vitro* toxicity assays (summarized in Table 2) exist to evaluate the toxic behavior of NPs, however, any extrapolation from *in vitro* data to *in vivo* behavior should be made with caution. Additionally, caution should also be taken when making comparison among different *in vitro* studies. For example, the type of cells used for an experiment impacts the observed toxicity pattern, explained by differences in phagocytic ability, cell proliferation capability, and functional status of cell in body [133,134]. The RAW 264.7 cell line is an extensively studied macrophage cell line for nanotoxicology, largely due to its defense roles in the immune system. Cancer cell lines have also been widely used for toxicity evaluations; however, their common apoptosis resistance characteristics may counteract the accuracy of the toxicity analysis. Hence, it is suggested to complement *in vitro* toxicity studies in cancer cell lines with primary cells to help finding adverse trends more accurately [135].

Particle size combined with particle number and surface area will ultimately determine the actual dose of exposure to nanoparticles. NP properties such as size and shape may change markedly after injection due to agglomeration and adsorption of biomolecules [58]. Notably, *in vivo* parameters such as diet, body temperature, health status, dynamic and fluidic variations could possibly challenge all assumptions based on *in vitro* assays. Therefore, it would be ideal to study both therapeutic and side effects of NPs in a relevant environment. A list of *in vivo* assays evaluating toxicity behavior of NPs is provided in Table 3.

3. Unintended interactions of DDSs with host defenses

Formation of superstructures from primary NPs can occur during formulation, storage, and/or administration. Size, surface chemistry, and charge can control the agglomeration/aggregation propensity of particles. Agglomerate/aggregates may have significantly different biodistribution and organ accumulation relative to their primary building blocks. Unstable nanoparticles may form large micron-sized aggregates, which can be trapped in the capillary bed of the lungs and pose danger to patients [74]. Below, we will discuss the interactions with host defense largely occurring at nanoparticle level rather than micron-sized aggregates.

3.1. Protein corona formation and subsequent events

Immediately upon NP exposure to biofluids, principally plasma, protein components start coating NP surface forming a protein corona (PC). PC can be categorized as “hard corona” and “soft corona”. Hard corona is usually composed of proteins with higher affinity to the NP surface that may permanently bind to NPs. Soft corona consists of lower affinity proteins, which are reversibly bound to NPs; the content of soft corona can be changed over time due to the loose interactions. Nguyen and Lee [188] reviewed the parameters affecting protein corona formation on nanoparticles such as media composition, protein concentrations, exposure time, temperature, and pH, and the effect of different nanoparticle characteristics on the protein corona composition. Evidently, PC content and conformation depends not only on size, but also on curvature, flexibility, surface chemistry, charge, functional groups, and hydrophobicity [117].

It is now believed that protein composition within the PC strongly impacts the NPs fate. If the PC is rich in dysopsonins, namely albumin or apolipoproteins (Apos), PC-coated NPs have longer circulation times. In contrast, if complement factors, fibrinogen, or IgG are abundant in protein corona, these may enhance rapid clearance of NPs by superior uptake into macrophages [189–192]. Close proximity of PC proteins to NPs may disturb their biological functions. For example, fibrinogen has been reported to bind several types of nanomaterials in plasma, and plays a critical role in leukocyte activation [193] and blood coagulation [194]. NPs interference with coagulation factors via binding to plasma fibrinogen may potentially dismantle clotting events. For instance, when cationic 7th generation PAMAM interacted with blood, fibrinogen aggregation was induced in a thrombin-independent manner [195]. Binding of coagulation proteins onto NP surface could also inactivate those proteins or others of the coagulation cascade, thus leading to deficiency in coagulation reactions [194]. Furthermore, unfolded proteins may lead to enhanced immune responses. It has been shown that negatively charged poly(acrylic acid) (PAA)-conjugated AuNPs induced unfolding of fibrinogen and triggered release of inflammatory cytokines via the integrin receptor, Mac-1, and NF-kB signaling [62]. Similarly, unmodified silica NPs interact with intrinsic coagulation factors such as factor XII leading to their damage on coagulation pathway [196]. However, the amine-modified silica NPs prevented abnormal activation of the coagulation cascade after systemic administration in mice, probably due to lower affinity to factor XII [197].

Multiple IV injections of NPs with selective binding to particular proteins may deplete those proteins, preventing their effective role in several biological events. Moreover, recruitment of

immune cells such as macrophages for NP clearance may hinder them from their foremost function of combating disease or infection [198,199]. This may make the individual more susceptible to real threats such as infectious particles. Additionally, engagement of both innate and adaptive immunity may induce harmful effects on immune cells, as well as all other tissues and cells in the body affected by the aggravated immune system.

3.2. Innate immunity and nanoparticles

3.2.1. Complement activation—The complement system, acting through a network of over thirty proteins both in circulation and membrane bound [200], plays a key role innate immunity, acting to identify and eliminate particulate matter and pathogens, driving interactions with these nanoscale foreign objects [201]. Fig. 4 illustrates the interdependent nature of NP interactions within the complement system, and its impact on therapeutic efficacy and viability. The complement cascade is proteolysis driven, acting through three main pathways, which converge as the third complement protein (C3) is cleaved generating C3 [2]. Classical pathway activation involves antibodies binding nanoparticles, activation of the C1 complex, C2, C4 and C3 leading to the C3 and C5 convertases.

Generally speaking, activation of the alternative pathway of the complement system by nanoparticles is initiated through the spontaneous hydrolysis of the thioester in C3 to form C3(H2O), which subsequently bind complement factor B to form C3(H2O)B. Furthermore, factor B is cleaved and activated by complement factor D which forms C3(H2O)Bb, the fluid phase convertase. This converts C3 into the anaphylatoxin C3a and the opsonic molecule C3b, which binds covalently to hydroxyl and amino groups presented on surfaces of nanoparticles [204]. Once bound on the surface, C3b is converted to C3bBb through binding of factor B and subsequent cleavage by factor D, whose complex product amplifies the alternative pathway, and through interactions with protein factors cleaves C3 into C3a and C3b, thereby feeding a positive feedback loop generating more C3b deposition on the nanoparticle surface [205].

The earliest mention of a nanoparticle interaction with complement proteins is of liposomes, the earliest synthesized nanoparticles. In 1969, only a few years after liposomes were described by Bangham and Horne [206], Haxby et al. [207] described the utility of bilayer ‘model’ membrane interactions with complement proteins and antibodies as a tool to study ‘the complement lytic mechanism’. Haptenized liposomes were used as a tool to study interactions between complement proteins and biological membranes, leading to the understanding that all charged phospholipid/cholesterol bilayers intrinsically activate complement proteins [208], although the consequences of the phenomena vary by the species, the individual, and the vesicle properties. Further studies revealed the impact of these interactions results in the increased clearance of the opsonized particles and the release of complement proteins C3a and C5a [208].

Particle physicochemical characteristics obviously matter here. Particles larger than a few microns present markedly reduced complement activation [209]. As discussed earlier, spheres are less prone to activate complement than rod- and disk-shaped NPs [95]. Deposition of complement protein fragments on the surface of NPs such as liposome significantly alters blood level kinetics and particle integrity [200]. Studies have shown

across a diversity of particles that complement activation is sensitive to surface coatings varying by charge, thickness, surface density, and accessibility to reactive groups. In terms of surface charge, negatively-charged NPs mainly activate the classical complement cascade, while their positively-charged counterparts induce the alternative pathway [127,204]. Modulation of complement activation through modification of surface chemistry, coatings, and charge has been studied in a wide array of nanoparticle species [202,205,210–215]. As an example, the addition of PEG or poloxamine 908 surface coating reduced nanoparticle activation of complement, and dextran coatings increase it. In another example, complement activation was prevented with >90% efficiency by coating NPs with negative regulatory complement factor H [216]. Similarly, the lower level of complement activation observed by galactose polymer modified NPs compared with the glucose modified NPs, was attributed to adsorbing the complement H protein on their surface [217].

After more than forty years of data since the first clinical availability of the PEGylated liposomal doxorubicin drug (Doxil™), some patients have demonstrated a complement activation-related hypersensitivity syndrome called C activation-related pseudoallergy (CARPA). This has highlighted the need, as Doxil's patent expired and alternatives emerge on the market, that generic formulations be carefully evaluated for bioequivalence since modifications of formulation and processing may produce variable immunity and toxicity profiles [203]. The drawback from the standpoint of formulating and producing potentially useful drug delivery vehicles is striking; the particle may not only trigger damaging biological reactions, it also may not be retained long enough to produce any therapeutic benefit, or slight variability in production may elicit unforeseen consequences. Existing literature on these important issues is extensive [2,202,208,210,212–214,218–222] and distinctions of nanoparticle species, e.g. inorganic engineered nanoparticles (gold, silica, superparamagnetic iron oxide) versus liposomes, solid lipid nanoparticles, polymer particles (either solid or vesicular) or protein-based nanoparticles dictate the different interactions that particulate matter experiences via phase transformations, particle aggregation, surface reconstruction and dissolution. These processes then influence the nanoparticles functional interactions, reactivity, bioavailability, pharmacokinetics, and potential for immunotoxicity [223]. Our necessarily limited discussions herein relate mainly to medical and diagnostic nanoparticles that contact the bloodstream.

3.2.2. Resident intravascular leukocytes patrol the blood, apprehending NPs and activating inflammation—The blood is full of vigilant patrolmen, leukocytes, which surveil for pathogens and particulate matter, subsequently engulfing them and setting off a cascade of immunological responses. Many of these leukocytes are freely moving within the blood. However, many leukocytes sit in the vasculature of one organ, which can give them an outsize role in NP effects and distribution, as described below. Therefore, here we define an oft-overlooked grouping of these patrolling leukocytes which we give the term Solid-organ-Associated Intravascular Leukocytes (SAILs). We define SAILs as leukocytes that reside permanently or for prolonged periods in one organ, but reside *inside the blood vessel lumen* of that organ's vasculature, not in the tissue parenchyma. This latter feature distinguishes SAILs from the common term of "tissue resident leukocytes". For example, the Kupffer cells of the liver can be considered SAILs, as they sit in the intravascular space,

where they grab pathogens and nanoparticles (NPs) from circulation. Because of their unique position, SAILs have been shown to play important roles in NP biodistribution, effects, and toxicities.

The term SAILs may at first seem redundant with the more common terms of the reticulo-endothelial system (RES) or monocyte-macrophage system (MPS). The capture of NPs by intravascular leukocytes is widely recognized by NP engineers in the form of the RES macrophages (Kupffer cells) that take up large fractions of NPs into the liver [224,225]. The RES is thus portrayed as a single cell type in one primary organ (liver), removing NPs from the circulation without consequence beyond the loss of NPs for the target organ. However, as we outline below, this simplified model leaves out numerous other key features of SAILs' interactions with NPs: the liver is *not* the only organ with patrolling SAILs; these SAILs are *not* just macrophages or monocytes; SAILs often appear only in a diseased organ; and the uptake of NPs into these SAILs is not just a loss of NPs (as often portrayed by nanomedicine engineers), but rather can have major impacts on the immune system and even survival. In this section, we explore the diversity of SAILs that can take up intravascular NPs and the consequences of such interactions. Fig. 5 shows the different classes of leukocytes able to interact with nanoparticles (NPs), many of which at times act as SAILs.

Before exploring the diversity of SAILs, we will first focus on the most well-known SAILs and RES organ in nanomedicine: the Kuppfer cells of the liver. In most studies of intravascular NPs, the liver does indeed have the highest NP uptake, usually attributed to its RES function, via intravascular macrophages. Indeed, a recent meta-analysis showed that < 0.7% of anti-cancer NPs localize to solid tumors, with the vast majority going to the liver [226]. Most of that liver uptake is in Kupffer cells, the resident tissue macrophages that sit in the liver sinusoids, where they screen the passing blood for pathogens and then engulf the pathogens [227]. The role of these Kupffer cells in liver uptake was recently quantified by killing the Kupffer cells using clodronate liposomes (CL), followed by measuring the distribution of a variety of NPs. For example, 50 nm gold NPs had ~80% of the injected dose (%ID) go to the liver at 24 hours post-injection in naive mice, while CL treated mice had ~20%ID in the liver [228]. Thus, intravascular macrophages in the liver account for a very large proportion of total NP uptake.

Beyond the liver, there are multiple other organs that have classically RES-type functions. The most well known of these is the spleen, where red pulp macrophages contribute to splenic uptake in the 5–10%ID range in the studies cited above for Kupffer quantification [228]. Additionally, the bone marrow has resident macrophages exposed to the blood and thus likely acts in an RES fashion for NPs, but this has been explored very little. Thus, these cells would be considered SAILs.

The most overlooked RES organ is the lung, where at least two major SAILs have been defined. Nanomedicine may have overlooked the lung as an RES organ largely due to high species variability and a minimal role in healthy mice. The large inter-species variability is attributed to the presence of pulmonary intravascular macrophages (PIMs). PIMs are resident macrophages which are adhered to the capillary endothelium of the alveoli (air sacs) of the lungs (Fig. 6), thus exposed to the bloodstream in the same orientation as Kupffer

cells [229]. Of many animals tested, a great number, including pigs, sheep, and cats, have large numbers of PIMs constitutively present even in healthy animals. However, a few species, notably including rodents and humans, do not have such “constitutive” PIMs, but instead only manifest PIMs in pathological states (“induced PIMs”). These interspecies differences in PIMs cause enormous differences in the localization of nanoparticles in the lungs versus liver. For example, IV-injected 20 nm gold NPs injected into sheep (which have very high PIM numbers [230]) had 60%ID (injected dose) in the lungs at 30 min, compared to 0% for mice [231]. Notably, eliminating PIMs with CL greatly reduces lung uptake of NPs in pigs [95]. Since mice and humans do not have constitutive PIMs, it might be assumed that PIMs are not important to NP engineering. However, in numerous important pathological states, induced PIMs develop in great numbers in the lungs of rodents and humans [229]. For example, rat models of sepsis and cirrhosis both develop PIMs, and these rat PIMs efficiently take up adenovirus, which is the same size as many NPs [232,233]. Thus, PIMs may become major patrolmen for the RES in major diseases, though significantly more research is needed to determine the extent and resulting effects. The study of PIMs can thus serve as a prototype for a type of SAIL involved in nanomedicine other than the oft-studied Kupffer cells.

With respect to the relatively hidden revelation that there are key RES organs besides the liver, there are also SAILs besides macrophages within the RES. There are multiple types of leukocytes in the blood, including in descending order of numbers neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Most of these have not been examined in detail or at all for their interactions with NPs, especially in the intravascular space [2]. However, their free circulation in the blood gives them direct access to systemically delivered NPs, and thus a very real chance of having a role in the disposition and side effects of NPs.

A glimpse into the possibility of other ILs regulating NP disposition was garnered by the recent finding that ~100 nm NPs made of denatured albumin are taken up by intravascular neutrophils in mice [238,239]. The neutrophils recognized and phagocytosed the NPs via the Fc-gamma receptor, and then were able to cross the endothelial barrier of inflamed tissues carrying therapeutic drugs. Neutrophils are not only the most abundant ILs (60% of ILs), they also have a unique physical position within the vasculature to allow them to capture NPs. A large fraction of neutrophils are “marginated”, meaning that they adhere to the capillary lumen with much slower transit through the capillaries than red blood (Fig. 6) [234,235]. The marginated pool is largest in the lungs; e.g., 70% of neutrophils in rabbits are in the pulmonary marginated pool [236]. This marginated pool of pulmonary intravascular neutrophils has been shown to form a defensive barrier, capturing pathogens such as bacteria very rapidly after their introduction into the blood [237]. Future studies are needed to determine whether the marginated pool of neutrophils also takes up NPs, how pathology plays a role, and whether such NP uptake leads to either positive or negative consequences.

The consequences of NP uptake into SAILs are important to understand in the development of nanomedicines. SAILs evolved in part to detect intravascular pathogens, and such detection usually leads to inflammation, and adverse health effects. Numerous *in vitro* studies have shown that phagocytes such as macrophages and monocytes can release pro-inflammatory cytokines when exposed to various NPs [240–242]. For example, one such

inflammatory pathway is the inflammasome NLRP3, which responds to a wide array of NPs by upregulating IL-1β and IL-18 [243,244]. However, far fewer studies have demonstrated the *in vivo* consequences of NP-IL interactions. One recent exception found that in pigs, PIMs rapidly take up polystyrene NPs, causing a sudden spike in pulmonary artery pressures [95]. Because of the paucity of *in vivo* studies, for new NPs moving towards clinical translation, it will be crucial to understand which ILs take up the NPs in animal models of pathology, and determine the consequences, by measuring IL responses, cytokine levels, and physiological endpoints with clinical relevance.

In summary, the term SAILs is a convenient descriptor for the numerous types of leukocytes that reside within the blood vessels of multiple organs and greatly affect NP biodistribution, efficacy, and toxicity. It is now important to more thoroughly investigate other SAIL types (e.g., neutrophils) in organs beyond just the liver.

3.3. Adaptive immunity and nanoparticles

Upon administration, nanoparticles encounter not only a range of plasma proteins and first cellular line of defense (leukocytes), but several classes of immune cells that influence their immunogenicity. Following uptake by antigen-presenting cells, the fate of nanoparticles depends on their composition and physicochemical properties. This can consist of simple clearance of the particles or multilevel activation of the immune system involving innate and/or adaptive immune responses [245]. Of interest to the drug delivery field is how to design nanomedicine that evade the immune system or are recognized as self.

3.3.1. Mechanism of adaptive immune response—Adaptive immunity (also called acquired immunity) consists of T and B cells that are activated in response to specific antigens. There are two types of adaptive immune responses: cellular immunity involving activation of cytotoxic T cells and humoral immunity response generated by activated B cells producing antigen-specific antibodies. Helper T cells are essential cells in the adaptive immune system that control the direction of the immune response. Upon uptake of antigens by APCs, the antigens are processed and presented to naive T helper cells that can differentiate into either a Th1 or a Th2 effector cell. Th1 cells secrete cytokines such as TNFα and IFNγ that lead to activation of antigen-specific cytotoxic T cells. Th2 cells secrete cytokines such as IL-4, −5, −6, and −10, which can stimulate B cells to produce antigen-specific antibodies [246,247].

Antigen presenting cells play a major role in priming the adaptive immune system. A three-signal model has been proposed to describe T cell activation (Fig. 7). Signal 1 is the engagement of peptide/MHC (pMHC) on APCs with T cell receptor (TCR). Insufficient to activate the T cells alone, signal 1 has been reported to induce T cell anergy and silencing. Signal 2 activates T cells through co-stimulatory receptors expressed by APCs. On the other hand, stimulation by co-inhibitory receptors on APCs can also lead to T cell anergy or formation of regulatory T cells (Tregs). Signals 1 and 2 may be sufficient to induce T cell activation, however a third signal is generally required for T cell activation. Signal 3 is the secretion of cytokines by APCs which activate the T cells and polarize by differentiation into various types of effector T cells [247–249]

3.3.2. Interaction of nanoparticles and adaptive immunity components—

Nanoparticles have been reported to activate the adaptive immune response. Several factors can influence the immunogenicity of nanoparticles such as size, charge, hydrophobicity, surface characteristics, solubility, and its composition [245]. Some nanoparticles can serve as adjuvants and enhance the immunogenicity of weakly antigenic cargoes as in the case of both lecithin- and polymethylmethacrylate-based nanoparticles [250,251]. Lipid-coated polysaccharide particles have been used as adjuvants for rabies vaccine. The mixed nanoparticles/rabies antigens generated a much higher antibody response than immunization with Alum adjuvants [252]. Fifth generation PAMAM dendrimers were also found to have immunopotentiating effect when used as adjuvants, generating both Th1 and Th2 type immunity [253]. Correlation of nanoparticle size with the resulting immune response has been rather controversial, with varied responses being reported. Large nanoparticles (>1um) have been found to be generally associated with inducing a Th1 response, compared to smaller nanoparticles (<500nm) that induced more Th2 responses. However, there have been exceptions, with smaller nanoparticles such as PLGA, dendrosome, nanoemulsions, and PEG-PHDA nanoparticles inducing Th1 responses. Polystyrene nanoparticles (<100nm) were found to induce both higher cellular and humoral response than larger polystyrene particles (>500nm) [254,255]. Nanoparticle surface charge has been reported to play a significant role in its immunogenicity. Charged nanoparticles (cationic or anionic) are phagocytosed at a higher rate than neutral nanoparticles. Cationic nanoparticles have been found to show greater immunogenicity with increased production of pro-inflammatory cytokines, whereas anionic nanoparticles had lower immunogenicity [256–258].

The association of cargoes with the nanoparticles can result in their conformational changes leading to their immunogenicity. C60 fullerene nanoparticles have been reported to have anti-inflammatory and anti-oxidative properties and induce immunosuppressive response, however conjugation of C60 fullerene derivative to bovine serum albumin, resulted in immunostimulation and generation of antibodies toward the nanoparticle [259]. Conversion to an immunostimulatory response was also observed with conjugation of polyaminoamine dendrimers to BSA, resulting in the generation of dendrimer-specific antibodies. Polyamidoamine (PAMAM) dendrimer conjugated to cytokine human interleukin-3 (hIL-3) induced dendrimer-specific antibody response, whereas the unmodified PAMAM dendrimer did not [260]. Conjugation of targeting ligands such as antibodies to nanoparticles has been one of the main approaches in development of targeted drug delivery system. However, the addition of antibodies to nanoparticles may also increase their immunogenicity resulting in poor pharmacokinetics, as has been shown with immunoliposome studies in mice [261,262].

It is very important to maintain a controlled nanoparticle manufacturing process. The immunogenicity of nanoparticles could vary from batch to batch as result of small differences in composition or conformation changes that can be introduced during the manufacturing process. For example, the liposome manufacturing process could affect its immunogenicity. Liposomes produced by high shear extrusion technique can lead to denaturation of encapsulated proteins. Another source for induction of immunogenicity of nanoparticles is contamination by endotoxins which can be introduced during the manufacturing process and has been discussed earlier in this review [245,256].

4. Approaches to alleviate the NP-associated adverse effects

4.1. Stealthiness, the easiest strategy to avoid unintended uptake

As we have described, the physicochemical properties of nanoparticles can influence how they are recognized by the immune system. Many different hydrophilic agents including polyethylene glycol (PEG), chitosan, hyaluronic acid, poloxamer, polyvinylpyrrolidone (PVP), and dextran have been used to introduce stealthiness on the nanomaterials preventing recognition by the immune system [188,263–266]. Among all polymers mentioned, PEG is the most commonly used for NP surface modification. In liposomes, the addition of as much as 10% surface coating of PEG resulted in ‘stealth’ liposomes and increasing the blood circulation half-life of the vesicles by orders of magnitude [210]. The addition of PEG in polymer and lipid nanoparticles has been shown to reduce complement activation [211]. To improve the “stealth effect” observed by PEG, many studies focused on increasing the PEG density on the surface of NP by using branched PEGs [267], using a hydrophobic layer as spacer [268], or by incubating NPs in an excess amount of PEG [269].

Although very promising at first glance and extensively used, PEG is a non-biodegradable polyether and its accumulation in the body may cause adverse effects especially if PEG-coated NPs are going to be administered over a long period. Indeed, there have been reports that PEG-specific antibodies are generated following administration of PEG-coated liposomes, which then resulted an accelerated blood clearance (ABC) phenomenon of PEG-coated liposomes (Fig. 8). Interestingly, the anti-PEG antibody response was not found to be induced by the commonly known T helper-based activation of the humoral immune response, but rather by direct B cell activation via a T independent antigen 2 (TI-2) based activation of B cells inducing a strong anti-PEG IgM antibody response. It was suggested that the PEGylated liposomes activated the B cells in splenic marginal zone, which is the splenic compartment central in clearance of blood-borne pathogens [270–273]. Notably, a significant number of the normal population have pre-existing anti-PEG antibodies in their blood without any treatment with PEGylated therapeutics [274]. As discussed earlier, PEG has been also found to activate complement cascade inducing anaphylactic reactions in sensitive individuals [275].

Degradable polymers including hydroxyethyl starch (HES), polysialic acid, dextrin, and poly(phosphoester)s (PPEs) have been proposed as PEG alternatives [276,277]. As a biological alternative to PEG, NP surfaces have been coated with cell membrane components [278,279]. Hu et al. [280] reported platelet membrane-cloaked NPs (PNPs) prepared by fusing human platelet membrane with 100-nm PLGA nanoparticles. PNPs internalized into human THP-1 macrophage-like cells less than uncoated counterparts and did not induce complement activation in autologous human plasma. Moreover, Docetaxel- and Vancomycin-loaded PNPs had elevated therapeutic efficacy in a rat model of coronary restenosis and a mouse model of systemic bacterial infection, respectively. Parodi et al. [278] reported a similar technology with a different membrane source and different particle core. They coated the surface of nanoporous silicon particles (NPS) with cellular membranes isolated from freshly harvested leukocytes, and named them LeukoLike Vectors (LLVs). The membrane coating protected particles from protein opsonization and markedly decreased

cellular uptake. Furthermore, unlike NPS, LLV did not induce any significant change in the cell membranes or any vesicle formation when internalized. The same group showed that the membrane coat improved particle interaction with tumor blood vessels, providing enhanced targeting and strong adhesion at the tumor site [279]. Besides plasma membrane fragments, there have been some attempts on coating the NPs using individual membrane proteins such as CD47 to reduce their phagocytic uptake. Although all these strategies proved to extend the circulating time of NPs, there is still not enough quantitative data on the biodistribution of such engineered particles in major organs such as liver, spleen, and lung.

4.2. Replacement of antibodies by safer fragments that do not elicit Fc-mediated side effects

Myriad targeting molecules have been developed for use in targeted drug delivery. These include monoclonal antibodies, single-chain variable fragments (scFvs), antigen-binding fragments (Fabs), single domain antibodies (sdAb), DARPins, peptides, aptamers, and small molecules (Fig. 9) [282–284]. Monoclonal antibodies consist of two immunoglobulin (Ig) heavy chains and two Ig light chains, each with constant and variable domain regions. Fragments of the parental antibody (scFv and Fab) have been developed in order to increase tissue penetration, reduce immunogenicity, and by lacking the Fc region, prevent interaction with Fc receptors. ScFvs have been developed by fusion of immunoglobulin variable heavy (VH) and light chains (VL) regions. Fabs consist of a single constant and variable domain form each heavy and light chains. Compared to conventional IgG antibodies (~150kDa), single-chain variable fragment (scFv) antibodies (~27kDa) and antigen-binding (Fab) fragments (~57kDa) are smaller in molecular size and weight [282,285,286].

Single domain antibodies (sdAbs), which may be man-made or natural, have attracted a lot of attention from the pharmaceutical industry due to their small size, accessibility to cryptic structures, high affinity, and deep penetration into tissue. Man-made sdAbs have been developed using phage display technology. Phage display immune libraries have been used to display antibody fragments on bacteriophages to generate highly diverse populations. A biopanning technique is then used to select the candidates with the highest affinity and specificity. Non-conventional immunoglobulins have been discovered in nature, and used to develop nanobodies. Camelids (camels and llamas) and cartilaginous fish (wobbegong and nurse sharks) have single variable domains with a constant domain framework. The single domain regions of camelids (VhH) and shark antibodies (V-NAR) have long surface loops that are able to reach protein cavities better than conventional antibodies. These nanobodies are around 12–15 kDa and have very small dimensions of 2.5nm by 4nm [285,287]. DARPins (designed ankyrin repeat proteins) are another interesting non-immunoglobulin targeting molecules derived from natural ankyrin repeat proteins, that can vary in molecular weight from 14 to 21 kDa. DARPins are around one-tenth the size of conventional IgG molecules and have high thermal stability. Another type of single domain targeting molecules are affibodies [282,288]. Affibodies are very small (−6.5kDa) targeting moieties developed by combinatorial protein engineering of the staphylococcal protein A-derived Z-domain scaffold. As compared to the above mentioned targeting moieties, the affibodies can be produced by peptide synthesis because of their small size and rapid folding [289,290].

Other molecules that have also been used as targeting ligands include peptides, aptamers, and small molecules [282,291].

Smaller antibody fragments or moieties have several advantages over larger intact antibodies for nanoparticle-mediated targeting such as, lack of binding to Fc receptors or complement activation, lower chance of immunogenicity due to lack of Fc region, and increased ligand multivalency because of their smaller size. The constant fragment (Fc) of the antibodies binds the Fc receptors on macrophages and other antigen-presenting cells. Conjugation of intact antibodies to nanoparticles could increase their immunogenicity by increasing their uptake and presentation by the reticuloendothelial system (RES), whereas smaller fragments could potentially avoid direct interaction with the RES [292]. In one study, the immunogenicity of doxorubicin-loaded nanoparticles was diminished potentially by the destruction of the antigen-presenting cells [292,293]. Others have attempted to utilize ‘don’t eat me’ signals such as CD47 to evade uptake by the RES [294–296]. There are currently no FDA-approved antibody targeted-nanoparticle therapeutics on the market, however with the advances in antibody engineering and development of more novel biocompatible antibody fragments and single domain antibodies, and with possible incorporation of immunoevasion strategies, targeted nanomedicine have a very promising future [297,298].

4.3. Innovative approaches for immune tolerance

Tolerance-inducing approaches function by either suppressing antigen-specific effector T cells or inducing generation of regulatory T cells. These include interventions at the Signal 1 and Signal 2 such as through modulation of co-stimulatory and co-inhibitory molecules to induce T cell anergy or death. Other strategies have included the use of T cell depleting antibodies, immunotoxins, and modulation of cytokines. One approach that has shown a lot of promise for induction of antigen-specific immune tolerance has been through the use of tolerogenic nanoparticles.

There have been three primary strategies using tolerogenic nanoparticles for induction of tolerance to foreign material, which include exploitation of the natural tolerogenic environments or processes, targeting of tolerance-inducing receptors, or use of nanoparticles carrying tolerance-inducing payload. These innovative approaches could potentially be used to induce tolerance to nanomedicine. The first strategy is utilizing the natural tolerogenic processes such as targeting the liver to take advantage of the hepatic tolerogenic environment, targeting the GI tract for induction of oral tolerance, or apoptotic cell mimicry [256]. In addition, it is possible to use the natural tolerogenic processes such as apoptotic cell death, which largely induce a tolerogenic response. One example is targeting of aged RBCs that clear in a tolerogenic fashion. Antigen-specific tolerance has also been reported by attachment of immunogenic proteins on surface of RBCs. Lorentz K.M. et al. [299] attached *e. coli* L-asparaginase enzyme on surface of RBCs which led to antigen-specific reduction in antibody titer by more than 1000-fold. This was achieved by chemical conjugation of glycophorin-A binding peptides (ERY1) on asparaginase enzyme. This approach was used to take advantage of the natural apoptotic signaling mechanism of aged RBCs to elicit humoral immune tolerance. Second approach for tolerance induction is the development of tolerogenic nanoparticles targeting tolerogenic receptors such as CD22

inhibitory receptor, FAS receptor, and aryl hydrocarbon receptor [256]. One example is induction of antigen-specific immune tolerance by sialic acid–binding Ig-like lectin (SIGLEC)-engaging tolerance-inducing antigenic liposomes (STALs). STALs display on their surface, the antigens and the B cell co-inhibitory receptor CD22 ligands, resulting in selective binding and suppression of antigen-specific B cells [300]. Third is development of tolerogenic nanoparticles loaded with tolerance inducing payload (such as NF-κB inhibitors or mTOR inhibitors) to induce formation of tolerogenic antigen presenting cells [256].

Targeted modulation of APCs has been one of the key strategies used to induce antigen-specific immune tolerance. Nanoparticles in particular have made excellent vehicles for APC modulation. Tolerogenic nanoparticles can be in the form of nanoparticle encapsulated with antigens along with tolerogenic pharmacologic agents, or nanoparticles encapsulating only the tolerogenic pharmacologic agents that is admixed with the free antigen. Selecta Biosciences has been one of the main leaders in developing a platform to prevent the formation of anti-drug antibodies (ADAs) against biologic therapeutics. The tolerance induction platform consists of biodegradable polymeric nanoparticle encapsulated with immunomodulator agents such as the mTOR inhibitor Rapamycin. SEL-212 is an example of tolerance-inducing nanoparticle technology currently in Phase II clinical trials for treatment of gout. It is a combination therapy that consists of co-administration of PEGylated uricase (pegsiticase) with rapamycin-containing tolerogenic nanoparticles. Pegsiticase has been reported to induce immunogenicity in 90% of patients. In Phase I clinical trial, SEL-212 prevents ADA formation and allowed sustained control of serum uric acid levels for 30 days following a single dose administration. This type of tolerance induction approach holds great promise for a range of biologic therapeutics, including nanomedicine. Nanoparticle therapeutics could be admixed with tolerance-inducing nanoparticles to prevent ADA formation and aid in their immune evasion. Another example is the SEL-403, an admixture of anti-mesothelin antibody fragment/pseudomonas exotoxin A recombinant fusion immunotoxin with SVP-Rapamycin as combination therapy for mesothelioma and pancreatic cancer patients. This has been designed to prevent ADA formation against the fusion protein, and in particular the immunotoxin which is derived from a bacterial host and is highly immunogenic. This platform is also being investigated for viral gene therapy treatments (SEL-302 and SEL-313) to induce tolerance to the viral vector and the transgene. Work is also being carried out along with Spark Therapeutics for developing a non-immunogenic hemophilia gene therapy medicine [301].

Development of a non-immunogenic targeted drug delivery system can be challenging. Factors that can influence immunogenicity of targeted nanomedicine include their physicochemical properties, modifications made to attach targeting ligands or immunoevasive coatings, cargoes or ligands that can be structurally perturbed during the manufacturing process, aggregation, and so on. Targeted nanomedicine face many obstacles in achieving complete immune evasion, nevertheless, there are many tolerance-induction strategies available that offer solutions which could be applied to nanomedicine.

5. Biomedical and translational aspects

5.1. Role of health (disease) status of the recipient

The health and physiological condition of the individual receiving a nanoparticle therapeutic may impact the extent of DDS's toxicity. Pre-existing pulmonary, allergic, inflammatory, thrombotic, or metabolic conditions, malignancies, infection, smoking, and pregnancy may further complicate the nanoparticle administration consequences. The term "personalized protein corona (PPC)" was introduced by Hajipour et al. [302] to emphasize that small alterations among patients/individuals (protein source) could affect the composition of the hard corona at the nano-biointerface. In fact, their data demonstrate that the composition of the hard corona differs among healthy individuals, as well as among patients with diverse diseases/medical conditions. Even temperature change in body could possibly facilitate the NP interaction with proteins in blood leading to changes in biodistribution and bioavailability [188,303]. One can imagine that such variations may result in diverse scenarios in terms of biological and toxicological fate of nanoparticles *in vivo*.

To evaluate how sensitized and non-sensitized mice may react differently to nanoparticles exposure, Gustafsson et al. [304] compared the responses in sensitized mice with an established allergic airway disease to healthy mice after exposure to iron oxide (hematite) nanoparticles intratracheally. Exposure to hematite NPs induced a significant increase in number of inflammatory cells such as neutrophils and eosinophils in the airways and lymphocytes in the draining lymph nodes of healthy mice. In contrast, same particles led to unspecific cell reduction in the alveolar space and the lung-draining lymph nodes in mice with established eosinophilic inflammation. The authors suggested that ionic irons released from hematite NPs in the acidic eosinophilic environment may potentially amplify generation of ROS and cell toxicity. Surprisingly, in another study, competing pro-inflammatory response elicited by titanium dioxide particles could potentially be a key factor in their significant downregulation impact on Th2 type inflammation in allergic mice [305].

Tracking specific nanoparticles interactions *in vivo* will help pinpoint potential harmful effects in susceptible subjects. As mentioned earlier, negatively charged nanoparticles can unfold fibrinogen and promote activation of the Mac-1 receptor pathway [62]. Considering the role of the fibrinogen-Mac-1 pathway in inflammatory responses observed in the pathogenesis of disorders such as Alzheimer's [306] and arthritis [193], the risk of exacerbating these conditions by nanoparticles known to interfere with that specific pathway should be carefully examined [62]. In another example, aberrant NLRP3 activation was shown to contribute to the pathology of various infectious and sterile inflammatory diseases, such as gout, obesity, atherosclerosis, alzheimer's disease, and type 2 diabetes mellitus [307–309]. Several types of nanoparticles including silica, asbestos and alum, TiO_2, SiO_2, and carbon nanotubes have been proved to activate NLRP3 inflammasome, leading to inflammation through IL-1β release [310–312]. Whether administration of such active particles in susceptible subjects can potentiate the existing inflammatory conditions remains an open question.

While NPs with immune stimulation properties may develop into promising immune adjuvants, their application in patients with elevated immune activity/inflammatory responses should be cautiously investigated. Manshian et al. [4] demonstrated that administration of aluminum oxide NPs amplified inflammatory responses and enhanced lung metastasis in syngeneic tumor mouse model. To demonstrate the response *in vitro*, they showed that spherical and wire-shaped aluminum oxide nanoparticles could trigger a clear activation of NLRP3 inflammasome and TGF-β secretion in primary splenocytes. Cancer cells exposed to these cytokines exhibited an augmented level of epithelial-to-mesenchymal transition, indicating cancer metastasis. Exposure to the nanoparticles themselves did not manifest such characteristics.

Pregnant women are a highly vulnerable population, since NPs can transport to developing embryos/fetuses through the placenta [313–315]. Any perturbation to placenta's regular performance will have possibly permanent and even life-threatening prenatal and postnatal effects on fetus. Polystyrene particles (50–500 nm) were taken up by the human placenta and crossed the placental barrier in an *ex vivo* study [316]. Similarly, quantum dots passed the placental barrier in mice [314]. Upon systemic administration in mice, silica and titanium dioxide NPs were found in the placenta, fetal liver, and fetal brain [317]. A few studies also investigated the effect of NPs on fetus-baring animal rather than the fetus itself. For example, silica nanoparticles with diameters of 70 nm could induce pregnancy complications when injected intravenously into pregnant mice, whereas larger (300 and 1,000 nm) silica particles did not cause such complications.

5.2. Auxiliary drugs to manage inflammation and other side effects

Given the outsize role macrophages play in the early side effects of NPs, the future of nanomedicine may depend on strategies to reduce macrophage-related toxicities. We have considered in previous sections ways to reduce macrophage toxicities by modifying the properties of the NPs themselves. However, an additional approach is to deliver an *auxiliary* agent concurrent with the NP delivery that would modulate the macrophages' response. Such auxiliary agents have been proposed in two main classes: 1) agents that decrease the macrophage's uptake of NPs; 2) agents that decrease macrophage inflammatory responses.

Auxiliary agents that decrease macrophage uptake of NPs include both small molecule drug therapy and NPs themselves. The best example of the former is chloroquine. Pre-treating mice with cholorquine (a small molecule drug that is FDA-approved for malaria) for 2 days decreased Kupffer cell phagocytosis of IV-injected NPs, thereby slightly improving their delivery to tumors [318]. NPs themselves can decrease the uptake of other NPs, possibly by saturating the RES. For example, injection of very high doses liposomes increased the circulation time of inorganic NPs, thus very slightly improving uptake in tumors of the inorganic NPs [319]. These methods are thus proof-of-principle, but need significant optimization to create an effect of significant magnitude.

Auxiliary agents that decrease macrophage inflammatory responses also include small molecule drugs and NPs themselves. Small molecule drugs used to limit NP-induced inflammation have seen few if any studies for non-biological NPs, but have been well studied for one of nature's NPs, viruses. In the field of viral gene therapy, it was found that

intravascular leukocyte-mediated inflammation can be reduced by pre-treating with corticosteroids (CS). This prophylaxis of NP-induced inflammation can be modified by packaging the prophylactic drugs into NPs themselves. An example of modulating macrophage inflammatory responses with NPs comes from the study of a mouse model of multiple sclerosis [320]. In that study, it was shown that while free CS strongly affected T cells, liposomal CS instead accumulated most in macrophages, altering the macrophages from a proinflammatory state to an anti-inflammatory state similar to the classic M2 phenotype. The ability of CS-loaded liposomes to modulate macrophage inflammatory status has even been demonstrated in isolated human macrophages, though the picture was more complex than that found in mice, with CS-liposomes downregulating most inflammatory markers but upregulating some [321]. Thus, while inflammation-modifying drugs should in theory be able to modulate the negative consequences of engineered NPs, studies are needed to prove this is true and devise clinically practical strategies to do so.

5.3. Species-specific aspects of DDS toxicology

Despite all attempts on moving novel NP formulations to clinic, the field is still suffering from poor clinical translation. One reason could be due to significant differences between animal models and humans in biodistribution, pharmacokinetics, and even adverse reactions towards NP administration. For example, protein corona composition is different among species and even among individuals at different health conditions. Using lipid-based NP formulations, Pozzi et al. [322] demonstrated that the protein corona in human plasma was much less dense in proteins compared with that formed in mouse plasma. In a similar study, incubation of PEGylated lipid NPs with mouse and human plasma resulted in a significantly different composition of formed protein corona. Notably, only half of the 25 most abundant corona proteins identified on these NPs, were in common between mouse and human coronas. Compared to human plasma-based corona, mouse plasma-based corona was more enriched in apolipoproteins and less enriched in opsonins [323]. These different coronas may modulate a different fate for the NPs governed by specific body environment in each species.

Structure and function of the immune system varies among different species. For example, the human pulmonary alveolar macrophages (PAMs) are larger and have superior phagocytic capability as compared to rodents, dogs, or non-human primate species. Although pigs and sheep are often used as predictive models of nanomedicine-mediated reactions in humans, significant differences have been observed between these species and human. For instance, pulmonary intravascular macrophages (PIMs) are more abundant in pig and sheep lungs than in humans. PIMs are giant (20 to 80 um) phagocytic cells which are capable of clearing NPs a few minutes after the injection [324,325]. This extensive phagocytosis of NPs may also trigger the release of thromboxane A2, prostaglandin, and prostacyclin molecules leading to vasoconstriction, bronchoconstriction, and pulmonary hypertension [324–326]. As mentioned above, the human lung lacks PIMs [229,324,325], instead hepatic Kupffer cells are the predominant phagocytic system in direct contact with the blood and therefore, responsible for principle clearance of particles from the blood.

Conclusion

Host defenses can attack and react to nano-scaled DDS the same way they face real enemies such as viruses and bacteria. This reaction could possibly trigger a variety of toxic/inflammatory events. While these challenges are numerous and powerful, engineered DDSs can certainly overcome them. The clinical success of engineered nanoparticles such as Doxil and Abraxane shows that therapeutic benefit can be realized even in the face of host-defense-mediated toxicities. The key lies in studying the host defense interactions with DDSs, so that the DDSs can be modified, co-administered with auxillary therapies, or in some cases, given up for more promising options. If nanomedicine continues to focus disproportionately on therapeutic effects and not on off-target side effects, clinical success will remain largely out of reach. Since host defenses are one of the main causes of nanomedicine side effects, future research in nanomedicine must pay more attention to this area to ensure that nanomedicine can reach its full potential to help patients.

Acknowledgments

This work is supported by National Heart, Lung and Blood Institute (NHLBI) grants HL125462, HL128398 and HL126874 to VRM, and T32 HL007915 to HP.

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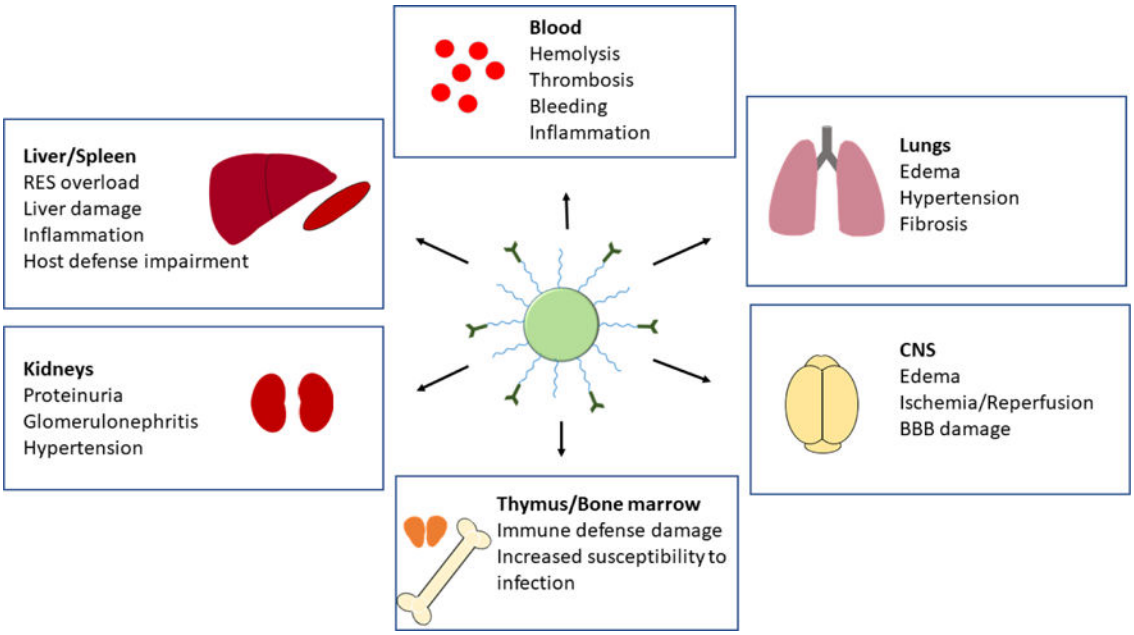

Fig.1.

Examples of unintended interactions of drug delivery systems with major organs/tissues and their potential subsequent side effects. RES: reticuloendothelial system, BBB: the blood-brain barrier.

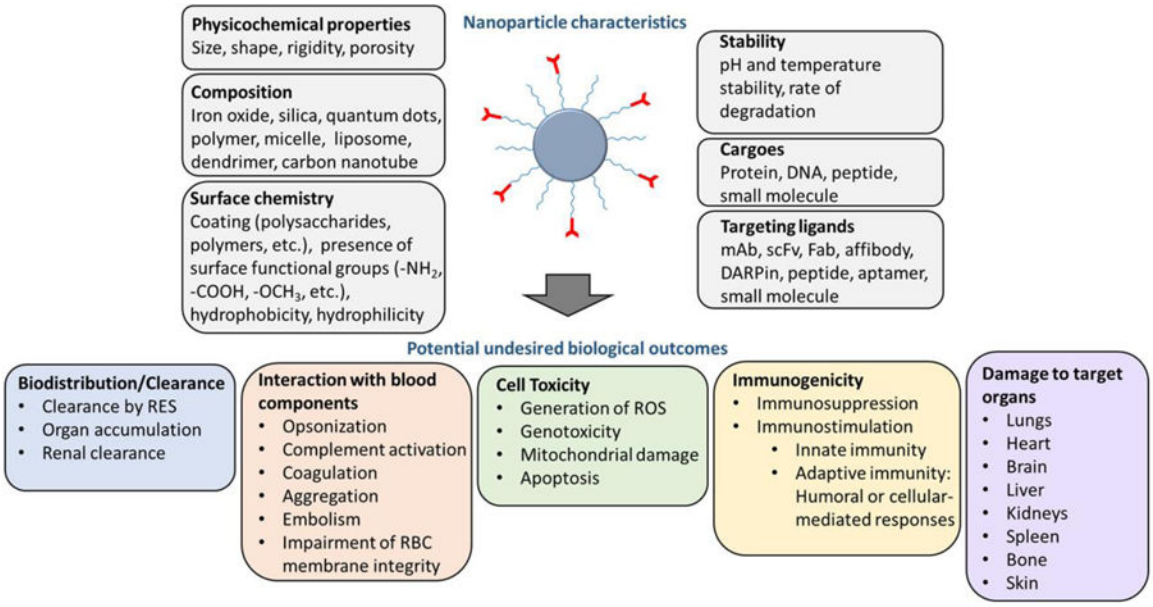

Fig. 2.

Nanoparticle characteristics and their influence on biological outcomes. Several features of drug delivery systems can impact their outcome *in vivo*. These include nanoparticle physicochemical properties, its composition, surface coating, type of cargo, and the particular targeting ligand. All of which determine the biocompatibility and the efficacy of the targeted nanoparticles *in vivo*.

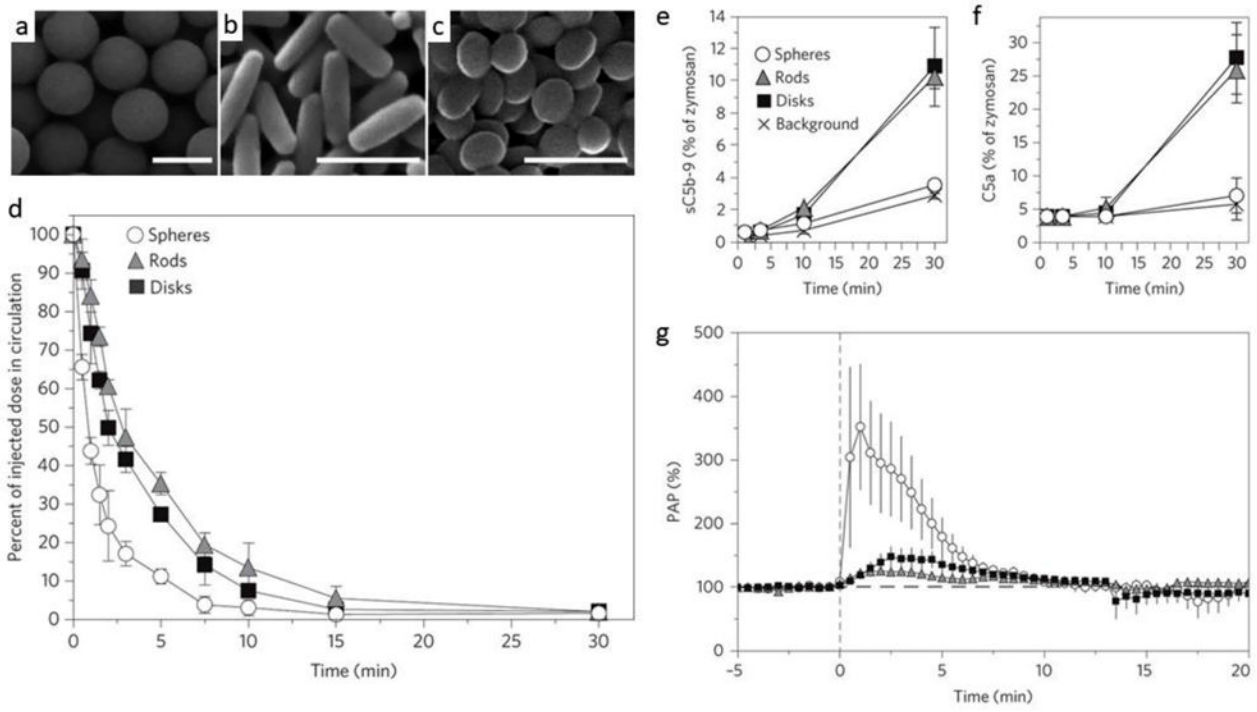

Fig. 3.

The effect of nanoparticles' shape on biological outcome in pigs. (a) Scanning electron microscopy (SEM) of spheroids, rods (prolate spheroids), and disks (oblate spheroids). (b) Circulation profile of particles in pigs following intravenous injection at 1.5×10^{11} particles per 20 kg body weight. Complement activation in blood (c), and pulmonary arterial pressure (PAP) (d) after injection of spheres (circles), rods (triangles) and disks (squares) in pigs was compared with background level. Reprinted with permission from ref [95].

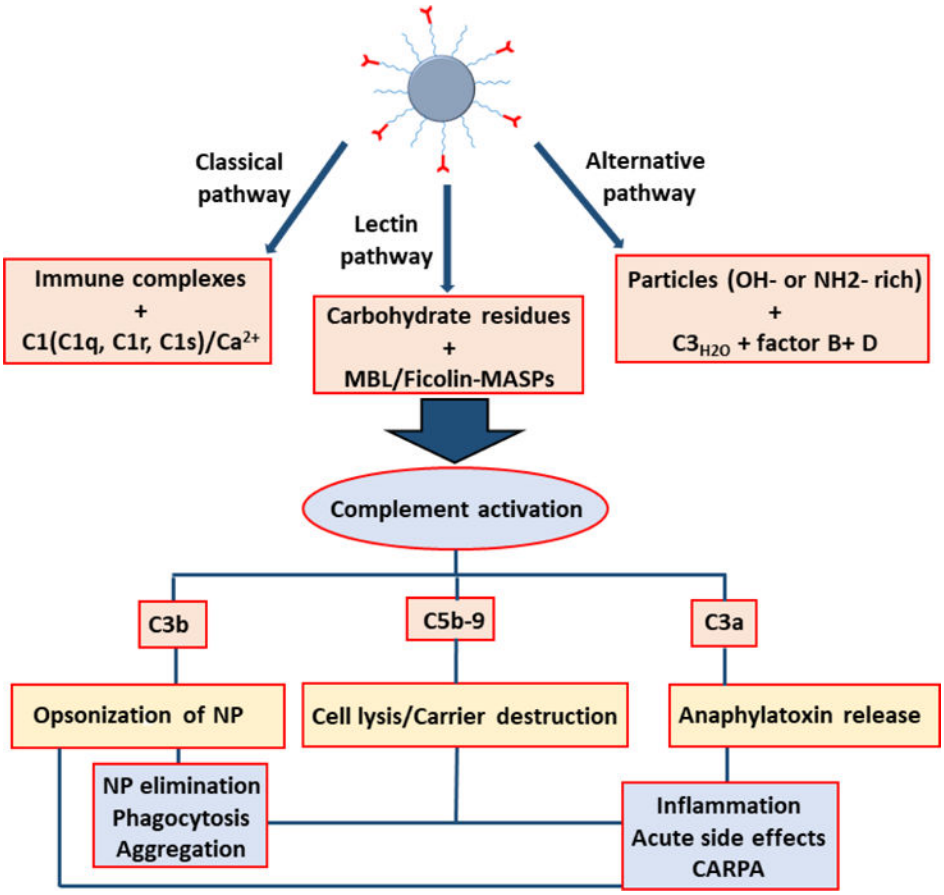

Fig.4.

Interdependence of effects of complement activation on nanotherapuetics. Partially adapted from [200,202,203]. CARPA: C activation-related pseudoallergy, MBL: mannose-binding lectin, MASP: mannose-associated serine protease.

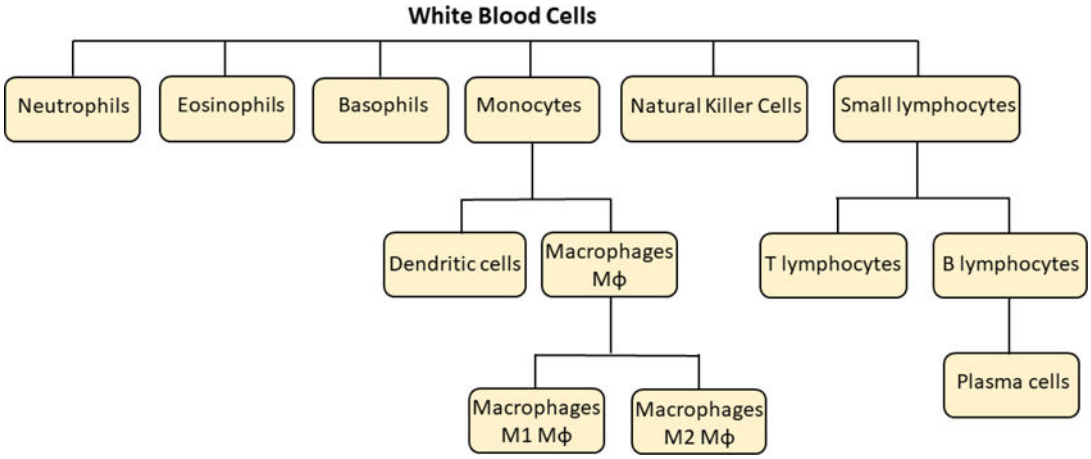

Fig.5.

Different classes of leukocytes that participate in recognition of, interaction with and destruction/elimination of nanoparticles.

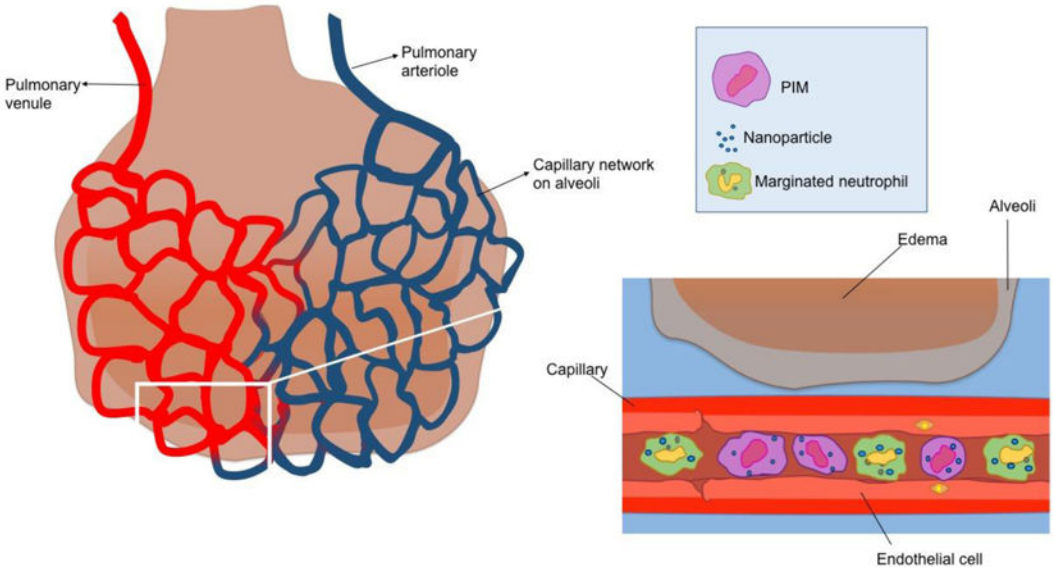

Fig. 6.

Illustration of pulmonary intravascular macrophages (PIMs) and marginated neutrophils that can phagocytose a significant amount of IV-injected nanoparticles are presented in capillaries adjacent to alveoli [229–237].

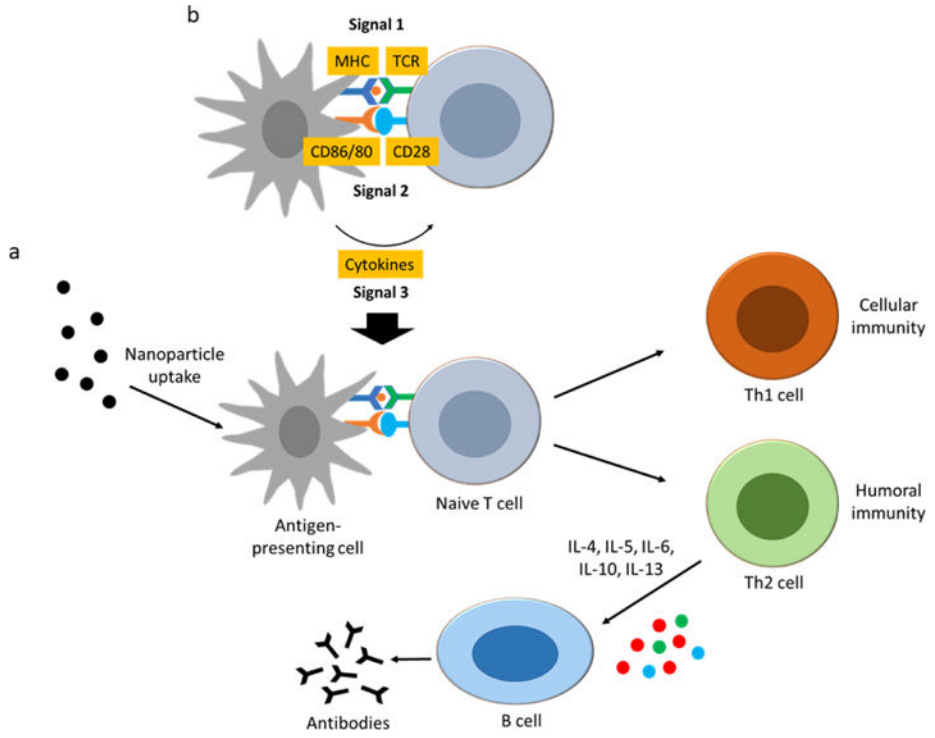

Fig. 7.

Illustration of adaptive immune system interactions with nanoparticles. (a) Uptake of nanoparticles by antigen-presenting cells leads to processing and presentation of the antigens to naive T cells that can differentiate into T helper cells Th1 or Th2 effector cells, leading to activation of cytotoxic T cells or activation of B cells to produce antigen-specific antibodies. (b) Three-signal model proposed for activation of naive T cells. Signal 1 is the engagement of peptide/MHC (pMHC) on APCs with T cell receptor (TCR). Signal 2 is activation by co-stimulatory receptors expressed by APCs. Signal 3 is the secretion of cytokines by APCs to stimulate T cells. MHC: major histocompatibility complex.

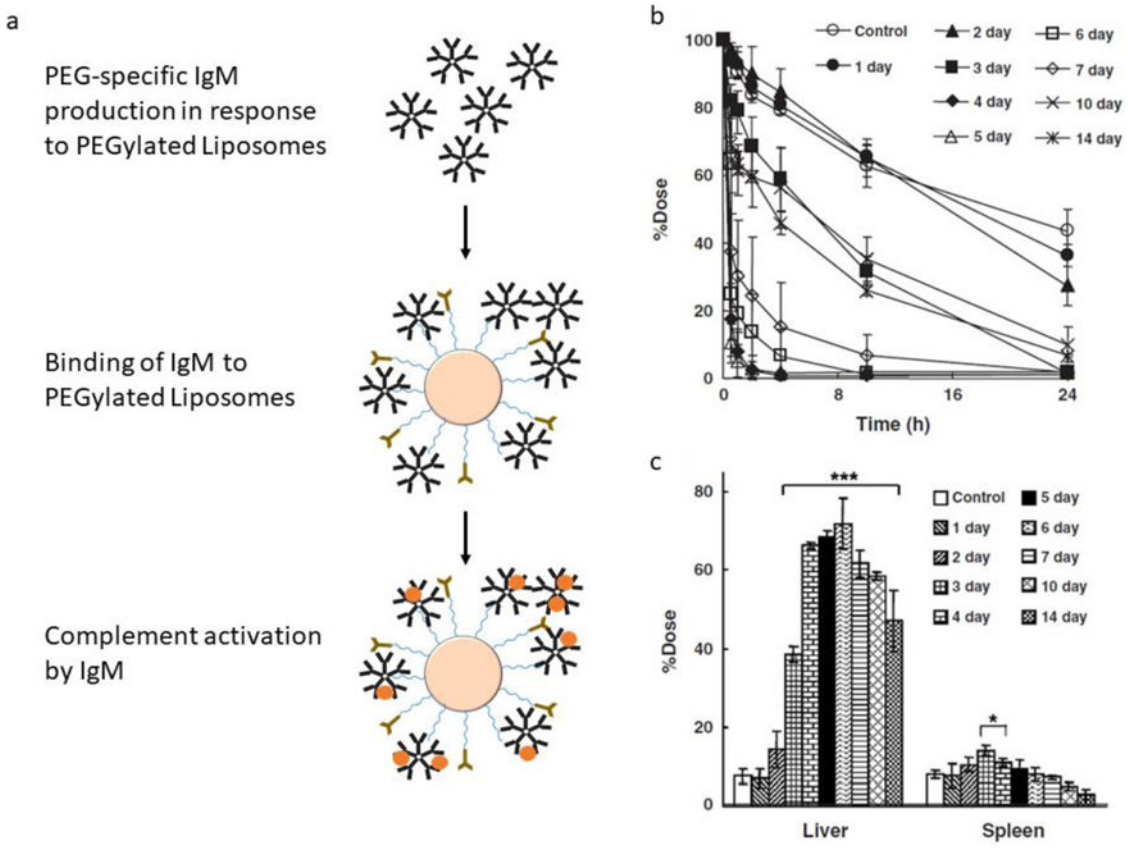

Fig. 8.

Accelerated blood clearance (ABC) phenomenon of PEGylated liposomes. (a) Illustration of anti-PEG IgM in response to PEGylated liposomes. (b) Blood clearance levels of PEGylated liposomes following injection at different days after post-first injection. (c) Accumulation of PEGylated liposomes in liver and spleen 24 hrs after injection. Reprinted with permission from ref. [281].

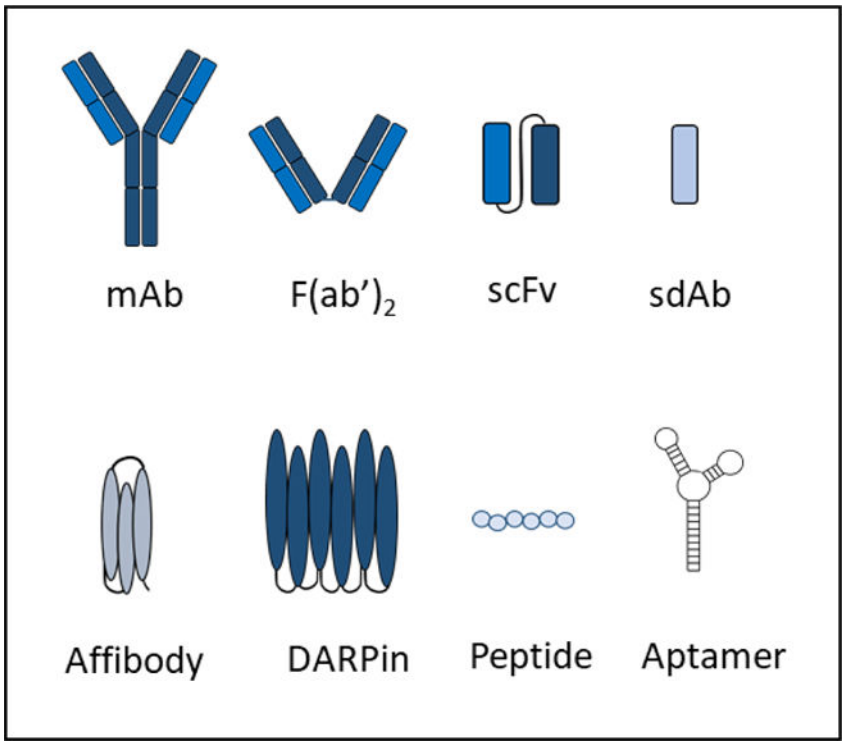

Fig. 9.

Different types of targeting molecules that have been used in targeted drug delivery systems. mAb: monoclonal antibody, scFv: single-chain variable fragment, F(ab')2: antigen-binding fragment, sdAb: single domain antibody, DARPin: designed ankyrin repeat protein.

Table 1.

Distinct features of small vs. large DDSs of the same materials [58–60].

Features	Large vs. small DDS	
Size	Large (millimeters)	Small (sub-micron)
Surface/mass ratio	Low	High
Release kinetics	Surface erosion	Burst release
Blood circulation	No	Yes
Cellular uptake	No	Yes
Capacity of interactions with biomolecules	Modest	Extensive

Table 2.

In vitro assays to evaluate toxicity of NP preparations.

Assay	Mechanism	Advantages/use	Limitations	References
Chromium-51 release assay	Detection of irreversible damage to cell plasma membrane.	High sensitivity, accuracy, and simplicity.	Requires handling of hazardous radioisotopes, and needs pre-labeling of cells.	[136–138]
LDH release assay	Detection of damage to cell membrane by measuring the release of intracellular LDH enzyme.	No need in cell labeling and use of isotopes.	Less sensitive, takes more time and less high-throughput than above.	[139,140]
Metabolic activity assay (MTT, MTS, etc.)	Colorimetric assay measuring cellular metabolic activity, such as ability to reduce tetrazolium dye.	Simple, Cost-effective, Indicates total metabolic status.	Numerous intracellular factors can influence reduction of dye leading to inaccurate results.	[141–144]
Protease activity assays (CytoTox-Glo)	Luminescent assay measuring activity of intracellular protease released from cells, which have lost their membrane integrity.	Different protease activities can be measured.	Costly, Signal interference from fluorescent nanoparticles.	[145–147]
Calcein AM assay	Calcein AM is a permeable molecule that enters cells and is hydrolyzed by intracellular estrases to Calcein which is fluorescent and is retained inside cells.	Simple, Indicative of membrane damage.	Signal interference from fluorescent nanoparticles.	[148–150]
Oxidative stress assays (DCFH assay)	Measures the release of reactive oxygen species by detecting conversion of substrates into fluorescent or colorimetric outputs.	Simple, Direct measure of cellular redox state.	Not a direct measure of H_2O_2.Several other oxidative species can oxidize DCFH. Released cytochrome C can oxidize DCFH.	[151–153]
Apoptosis assays (Annexin V staining, caspase activity assays)	Detects presence of phosphatidyl serine in apoptosing cells by annexin V staining; or measurement of caspases such as caspase 3 activated during apoptosis.	Simple, Distinguishes apoptosis from necrosis.	Annexin V staining requires live cells, and for adherent cells must use detachment agents that do not damage the cell membrane.	[154–156]
Genotoxicity assays (comet assay)	Single cell gel electrophoresis assay that measure DNA strand breaks.	Rapid, Indicates DNA damage.	Requires an internal reference to avoid variations. Inability to detect specific mutations generated.	[157–160]
Hemocompati bility assays (Hemolysis assay)	Spectrophotometric measurement of the amount of hemoglobin released.	Simple, Indicates RBC damage.	Nanoparticle binding to complement proteins can alter the hemolytic activity.	[161–164]
Macrophage cytokine profiling	Measures cytokines released from macrophages, using systems such as ELISA or Luminex bead array assays.	Simple, extensively used, High throughput assay for several cytokine measurement in one assay.	Endotoxin contamination can produce false positive results.	[165–167]
Leukocyte proliferation assay	Leukocytes are incubated with different concentration of nanoparticles, followed by measurement of effect on leukocyte proliferation, such as by 3H-thymidine incorporation.	Quantitative analysis of lymphocyte activation and proliferation.	Some assays require radioisotopes, which are biological hazards.	[168–170]

Table 3.

In vivo assays for evaluation of NP preparations.

Assay	Mechanism	Advantages/use	Limitations	References
Acute, subacute, subchronic, and chronic toxicity assays (Clinical chemistry tests, Hematological tests, Coagulation tests, Weight change assessment, Carcinogenicity tests, and Histopathology)	To evaluate systemic response by testing different routes of administration and durations of exposure.	Thorough assessment of biological changes as a result of substance administration.	Time-consuming, and expensive.	[171–175]
Reproductive toxicity tests (Mammalian germ cell cytogenetic assay, Heritable translocation assay, Mouse spot test, Micronucleus test, Chromosomal analysis)	To evaluate effects on fertility of the host anddevelopment of offspring by looking for changes at the genomic or embryonic level.	Accurate detection of mutations, deletions, and chromosomal aberrations.	Skill-demanding, time-consuming, and expensive.	[176–179]
Ocular- and skin-irritation tests (Eye irritation draize test, and Trans-epithelial water loss (TEWL) test)	Measurement of skin and eye irritancy by dripping the test substance on eye or skin of the host and looking for irritations such as inflammation, bleeding, ulceration, swelling, or permanent damage.	Accurate assessment of harmfulness of substances to the eye or skin.	Ethical concern over animal welfare.	[180–184]
Hypersensitivity tests (Skin prick test, Intradermal test, Patch test)	Treatment of skin with various concentration of test substance and observing for any immediate skin reactions, itching edema, erythema, urticaria, angioedema, signs of anaphylaxis; as well as testing for release of histamine, IgE, IgG, and Histopathological analysis.	Accurate detection of immediate contact reactions.	Difficult to attain sensitivity and specificity values, variations in symptoms.	[185–187]