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Embracing nanomaterials' interactions with the innate immune system

Abraham J. P. Teunissen¹, Marianne E. Burnett¹, Geoffrey Prévot¹, Emma D. Klein¹, Daniel Bivona¹, Willem J. M. Mulder^{2,3}

¹Biomedical Engineering and Imaging Institute, Icahn School of Medicine at Mount Sinai, New York, New York, USA

²Department of Internal Medicine, Radboud Institute of Molecular Life Sciences (RIMLS) and Radboud Center for Infectious Diseases (RCI), Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

³Laboratory of Chemical Biology, Department of Biochemical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

Abstract

Immunotherapy has firmly established itself as a compelling avenue for treating disease. Although many clinically approved immunotherapeutics engage the adaptive immune system, therapeutically targeting the innate immune system remains much less explored. Nanomedicine offers a compelling opportunity for innate immune system engagement, as many nanomaterials inherently interact with myeloid cells (e.g., monocytes, macrophages, neutrophils, and dendritic cells) or can be functionalized to target their cell-surface receptors. Here, we provide a perspective on exploiting nanomaterials for innate immune system regulation. We focus on specific nanomaterial design parameters, including size, form, rigidity, charge, and surface decoration. Furthermore, we examine the potential of high-throughput screening and machine learning, while also providing recommendations for advancing the field.

Correspondence: Abraham J. P. Teunissen, Biomedical Engineering and Imaging Institute, Icahn School of Medicine at Mount Sinai, New York, NY. bram.teunissen@mssm.edu, Willem J. M. Mulder, Department of Internal Medicine, Radboud Institute of Molecular Life Sciences (RIMLS) and Radboud Center for Infectious Diseases (RCI), Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands. willem.mulder@radboudumc.nl.

AUTHOR CONTRIBUTIONS

Abraham Teunissen: Conceptualization; writing-original draft; writing-review & editing. **Marianne Burnett:** Writing-original draft. **Geoffrey Prévot:** Writing-original draft. **Emma Klein:** Writing-original draft. **Daniel Bivona:** Writing-original draft. **Willem Mulder:** Conceptualization; resources; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Keywords

innate immunotherapy; nanomaterials; nanomedicine; nanotherapeutics

1 | INTRODUCTION

The foundation for modern immunotherapy lies at the turn of the 19th century, when Dr. William B. Coley observed the regression of cancer following bacterial infection (Hoption Cann et al., 2003; McCarthy, 2006). Inspired by this phenomenon, he developed a treatment consisting of inoculating malignant sarcoma patients with streptococcal bacteria. A variation on this approach involving intravesical administration of *Bacillus Calmette–Guérin* (BCG) has been approved for treating non-invasive bladder cancer since 1990 (Alexandroff et al., 1999). In the past two decades, alternative approaches toward targeting the adaptive immune system have resulted in paradigm-shifting cancer immunotherapies. These include chimeric antigen receptor (CAR) T cell therapy (C. E. Brown & Mackall, 2019; Jackson et al., 2016), in which T cells are genetically engineered to target tumor cell-specific antigens, and checkpoint inhibitors, which enhance T cells' efficacy by blocking inhibitory signals produced by tumors or other immune cells (Seidel et al., 2018; Wei et al., 2018). Although these immunotherapies have proven highly effective in certain settings, their patient specificity, considerable price, and limited response rates call for the development of alternative approaches (Darvin et al., 2018; Martins et al., 2019; Verma, 2018).

The innate immune system is the first line of defense against foreign invaders and includes myeloid cells, which possess pathogen recognizing receptors (PRRs) capable of detecting distinct molecular patterns characteristic of harmful (micro)organisms (Figure 1(a); Akira et al., 2006). PRR activation initiates a nonspecific innate immune response during which phagocytes (e.g., neutrophils and macrophages), natural killer cells, and inflammatory mediators are recruited to the affected area. As the foreign pathogenic invasion continues, dendritic cells and macrophages activate T cells, thereby also prompting an adaptive immune response. Consequently, therapeutically engaging the innate immune system has the potential to treat a multitude of diseases, including cancer and a broad range of infections, in concert with facilitating a specific adaptive immune response (Binderup et al., 2019; Corrales et al., 2017; Lameijer et al., 2018; Mourits et al., 2018). The players of the innate immune system and their interconnectivity have been discussed in more detail elsewhere (Chaplin, 2010).

Nanotherapeutics have been traditionally designed to avoid myeloid cell uptake. For example, liposomal nanoparticles have been coated with polyethylene glycol (PEG) to drastically increase their blood circulation time, thereby promoting their accumulation at pathological sites, for example, a tumor (Hubbell & Chilkoti, 2012). In contrast, we advocate exploiting myeloid cell's inherent avidity for nanomaterials to therapeutically target the innate immune system. For example, nanocarriers can boost drugs' myeloid cell uptake, in particular by professional phagocytes, such as monocytes and macrophages. Alternatively, ligand-decorated nanomaterials can be employed to target specific PRRs and other cell (surface) receptors (Figure 1(b)). The strategies are especially effective in the bone

marrow, where a durable innate immune response can be generated by targeting hemopoietic stem cells (Mulder et al., 2019). Importantly, by employing either immunostimulatory or tolerogenic drugs, the type of immune response can be regulated. Furthermore, the broad scope of innate immune responses facilitates treating a wide variety of diseases and disorders. For example, we developed lipoprotein-based nanotherapeutics loaded with tolerance-inducing rapamycin and used these to effectively promote heart allograft survival (Van Leent et al., 2021). Similarly, we significantly reduced tumor growth using bone marrow-avid lipid nanocarriers decorated with immunogenic peptidoglycan derivatives, capable of activating the intracellular nucleotide-binding oligomerization domain-containing protein 2 (NOD2) PRR (Priem et al., 2020).

Here, we discuss the influence of nanomaterial design parameters on their ability to engage the innate immune system. We first detail the importance of nanomaterials' physicochemical properties, including their composition, size, and rigidity. Subsequently, we focus on nanomaterials' surface composition by discussing their surface charge and the role of ligand density and linker length. Lastly, we examine techniques that facilitate developing innate nanoimmunotherapeutics, including high-throughput screening and machine learning.

2 | NANOMATERIAL PROPERTIES INFLUENCING INNATE IMMUNE REGULATION

2.1 | Nanocarrier composition

Nanomaterials' composition determines many of their other characteristics, including their rigidity, biocompatibility, and formulation conditions (Figure 2). Designing effective nanocarriers for innate immunotherapy therefore requires matching nanomaterials' composition with their intended purposes (e.g., shielding drugs from hydrolysis, enhancing blood half-life, and targeting cell surface vs. intracellular receptors). As the number of reported nanomaterials is practically unlimited—let alone the ways in which these can be combined and organized—we will not strive to provide an all-encompassing overview of their applicability as nanocarriers for innate nanoimmunotherapy. Rather, we will demonstrate their scope and limitations using several representative examples.

2.1.1 | Polysaccharide nanomaterials—Nanoimmunotherapeutics typically consist of a nanocarrier loaded or surface-decorated with immunogenic compounds. However, some nanomaterials are inherently therapeutically active. Polysaccharides are large macromolecules capable of regulating the innate immune system by activating cell surface receptors, including tolerance inducing Siglec receptors (Crocker, 2005) and immune-activating Dectin-1 (G. D. Brown, 2005). As a result, polysaccharides can serve as the sole component of innate nanoimmunotherapeutics (Li et al., 2018; Sampaolesi et al., 2018). Their sugar monomers' alcohol moieties also facilitate derivatizing these nanomaterials with other drugs or employing them as a covalently coupled coating for other nanoparticles (Mizrahy & Peer, 2012). However, their structure also complicates site-specific modifications, poorly shields its constituents from the in vivo environment, and often displays poor water solubility (Mizrahy & Peer, 2012). Although multiple polysaccharide nanomaterials and drug conjugates have been tested in clinical trials, their

widespread clinical approval will likely require additional insight into their drug release profiles, biodistribution, and therapeutic efficacy (Miao et al., 2018).

2.1.2 | Lipid nanomaterials—Lipid-based nanomaterials are ubiquitous in nature (e.g., lipoproteins), allowing mimicking their inherent interactions with the immune system using synthetic analogs. For example, we have developed effective and highly biocompatible lipoprotein-based innate nanoimmunotherapies (Binderup et al., 2019; Kaji, 2013; Lameijer et al., 2018; Priem et al., 2020). Lipids can also be used to formulate a wide variety of other morphologies (e.g., micelles or liposomes) and these nanomaterials' sizes can be easily varied by incorporating lipophilic compounds. Furthermore, lipid-based nanomaterials' surface can be altered by including head-group functionalized lipids (Tang et al., 2016). Lipid nanocarriers are especially well-suited for delivering hydrolyzable or apolar drugs and can be formulated using mild conditions, typically by sonication or microfluidic mixing (Priem et al., 2020; Sanchez-Gaytan et al., 2017). As a result, multiple lipid nanotherapeutics have made it into the clinic, including Estrasorb (Simon et al., 2002), a soybean emulsion of estradiol, and Doxil, comprising doxorubicin-loaded liposomes (Anselmo & Mitragotri, 2016; Anselmo & Mitragotri, 2019; Barenholz, 2012). Despite their advantages, lipid-based nanomaterials are not without limitations. Most notable are their relatively disperse size and composition and the challenges associated with creating non-spherical formulations (Elizondo et al., 2012).

2.1.3 | Synthetic polymeric nanomaterials—Synthetic polymers are one of the most studied and diverse nanomaterials. Their bottom-up synthesis allows virtually unlimited variation in composition and properties. For example, polymer structure (e.g., linear, grafted, or cross-linked) can be controlled by monomer chemistry and polymerization conditions (Banik et al., 2016; Qiu & Bae, 2006). Similarly, their biodegradability and polarity can be regulated by incorporating biocleavable or charged monomers, respectively (Banik et al., 2016; Su & Kang, 2020). Nonetheless, polymeric nanomaterials tend to be relatively disperse due to challenges associated with synthesizing discrete polymers and assembling these in a controlled fashion. These nanoparticles can be comprised of single chains or clusters and exist as random coils or relatively defined structures, such as polymeric micelles (Kröger et al., 2018; Kuai et al., 2016). Polymeric nanomaterials are typically between 10 and 500 nm in size and drugs can be either covalently bound or retained through non-covalent interactions (Banik et al., 2016). Enhancing biocompatibility and biodegradability remain one of the major challenges in designing effective synthetic polymeric nanotherapeutics (Banik et al., 2016). Nonetheless, purely synthetic polymeric nanomaterials are extensively used in both preclinical studies and clinical trials (Banik et al., 2016).

2.1.4 | Dendrimers—Dendrimers are discrete and branched structures synthesized from small (organic) molecules and typically only a few nanometers in size (Abbasi et al., 2014; Anselmo & Mitragotri, 2017; Gajbhiye et al., 2009; Okuda et al., 2006; Sharma et al., 2018; F. Zhang et al., 2015). Dendrimers' network of chains can be used to retain drugs non-covalently (Acton et al., 2013; Gajbhiye et al., 2013; Kim et al., 2018). However, therapeutically active dendrimers are typically created by functionalizing their terminal-ends

with ligands, allowing precise control over the number of ligands per nanotherapeutic. As a result of their small size and high chain density, these nanomaterials tend to display short blood half-lives due to rapid renal clearance, even compared with linear polymers with similar molecular weights (see section on “Nanomaterial form and size section”; Sadekar et al., 2011). Although dendrimer biodistribution has been studied in great detail, therapeutic studies employing these nanomaterials remain rare and typically focus on engaging the adaptive immune system (H. Chen et al., 2020; Sheng et al., 2008; J. Xu et al., 2019). Dendrimers’ efficacy as innate immunotherapeutics has been almost exclusively studied in highly controlled in vitro experiments, focused on unraveling the effects of ligand presentation (Ackun-Farmmer et al., 2020; Bezouška, 2002). Combined, dendrimers’ defined architectures make them excellent platforms for controlled in vitro studies, but their (clinical) applicability as in vivo nanocarriers remains poorly explored.

2.1.5 | Metal nanomaterials—Metal nanomaterials can be created in virtually every size, with low dispersity, and in a broad range of shapes (Abedini et al., 2016; Gugliotti et al., 2005; Mody et al., 2010). These rigid structures are typically produced using harsh processes, including vapor deposition, laser ablation, or sputtering (Jamkhande et al., 2019). As a result, therapeutic agents are usually introduced post-nanometal formation. Introducing drugs is further limited by metal nanomaterials’ solid core, allowing only surface functionalization. Consequently, the therapeutic agents in metal-based nanoimmunotherapeutics are usually in direct contact with the in vivo environment, restricting the types of drugs that can be used (e.g., hydrolyzable drugs might degrade). Together with their often poor biocompatibility, these constraints limit the use of metal nanomaterials as nanocarriers for innate immunotherapy. However, nanometals’ unique electric and optical properties make them excellent probes for studying the innate immune system (Mody et al., 2010; Weissleder et al., 2014). In such cases, these nanomaterials are often coated with organic compounds to modify their in vivo behavior. For example, iron oxide nanoparticles can be coated with lipids and antibodies to enhance their macrophage uptake, while their metal core facilitates monitoring by MRI (Briley-Saebo et al., 2011). Similarly, Feridex, consisting of dextran-coated superparamagnetic iron oxide nanoparticles, is clinically used as an magnetic resonance imaging (MRI) contrast agent (Y.-X. J. Wang, 2011).

2.1.6 | Nucleic acid-based nanomaterials—Nucleic acids can be synthesized with a precisely controlled nucleotide sequence and designed to self-assemble into pre-determined three-dimensional (3D)-structures with discrete forms and sizes (Linko et al., 2015; Martinez et al., 2017). Nucleic acid-based nanotherapeutics also allow full control over the ratio and position of conjugated drugs and probes. As a result, these nanomaterials are well-suited for the systematic investigation of nanotherapeutic design parameters (Jasinski et al., 2018). In addition, and similar to polysaccharide and lipid nanomaterials, the nanomaterials’ nucleic acid constituents can also serve as therapeutic agents (Liu et al., 2018; Wu et al., 2020). However, the complexity and poor in vivo stability of nucleic acid-based nanotherapeutics also make them challenging to formulate and employ. As nucleic acid-based nanoparticles cannot currently be economically synthesized in large quantities, their translational potential remains limited.

Combined, these examples demonstrate the large variety of nanomaterials available and the important relation between nanotherapeutics' composition and applicability. Although nearly every nanomaterial can be employed as a nanocarrier for innate immunotherapy, formulations composed of biological constituents tend to be more versatile and biocompatible.

2.2 | Nanomaterial form and size

Naturally occurring nanoparticles, including proteins (Clavant & Comper, 2003), lipoproteins (Olson, 1998; Zheng & Aikawa, 2012), and viruses (Gelderblom, 1996), have evolved distinct structural features that influence their stabilities and biodistributions. For example, albumin's tertiary structure impedes renal clearance (Clavant & Comper, 2003), and viral capsids undergo shape changes during maturation to withstand the huge pressures associated with nucleotide loading (Aznar et al., 2012; May & Brooks, 2012). Inspired by these biological examples, an abundance of synthetic nanomaterials with varying sizes and forms have been developed (Harper et al., 2008).

The majority of nanomaterials reported to-date are spherical because of thermodynamic forces, for example, due to the random coiling of polymers in polymeric nanoparticles, a minimization of surface tension when preparing metal nanoparticles by condensation, or an even distribution of both Van der Waals and hydrophobic forces in liposomes. However, in recent years nanomaterials of virtually every conceivable form have been synthesized, including cubical, cylindrical, discoidal, polyhedral, and flower-formed (Figure 3(a); Champion et al., 2007; Venkataraman et al., 2011; X. Zhu et al., 2019).

In vitro experiments reveal how nanomaterials' form affects their cellular internalization and ability to activate cell surface receptors (Toy et al., 2013). These dependencies predominantly stem from the contact angle between a nanomaterial and the cell membrane (Figure 3(b); Champion & Mitragotri, 2006). Typically, a small contact angle hampers formation of the actin networks facilitating endocytosis (Champion & Mitragotri, 2006). In contrast, relatively large contact angles (e.g., cells binding spherical particles or the sides of discoidal ones) promote cellular uptake. However, this behavior is not universal. In contrast to mononuclear phagocytes, neutrophils internalize rod-shaped particles faster compared with spherical ones, presumably due to their higher flexibility and surface motility (Safari et al., 2020). The contact angle between a nanomaterial and cell membrane also influences cell-surface receptor activation (see section on "The role of ligand density and linker length"; Decuzzi & Ferrari, 2006; Kolhar et al., 2013; Muro et al., 2008).

Investigating nanoparticles under microfluidic flow revealed that spherical nanoparticles display relatively straight trajectories, while elongated ones have higher tumbling rates and oscillatory trajectories; presumed to promote their in vivo margination and adherence to vessel walls (Figure 3(c); Blanco et al., 2015; Toy et al., 2011, 2013). In agreement, elongated nanoparticles display longer blood half-lives than spherical ones (Blanco et al., 2015; Toy et al., 2011, 2013; Y. Zhao et al., 2017). Nonetheless, thorough preclinical studies are required to gain comprehensive insight in the relation between nanomaterial form and in vivo behavior (in circulation).

Nanomaterials' size also impacts their behavior and can be accurately controlled for most nanomaterial types (Hickey et al., 2015; Richard et al., 2017; Suchomel et al., 2018; Vreeland et al., 2015). How a nanomaterial's size affects its myeloid cell uptake is poorly understood, but computational and in vitro studies suggest internalization proceeds most rapidly for particles approximately 25–60 nm in size (Chithrani et al., 2006; Jiang et al., 2008; Jin et al., 2009; Lu et al., 2009; S. Zhang et al., 2009). The influence of nanomaterials' size on their organ-level biodistribution is much better understood. Nanomaterials smaller than approximately 6 nm are efficiently removed by the kidneys, while larger ones increasingly accumulate in the liver and spleen (Hoshyar et al., 2016; Perrault et al., 2009; Popovi et al., 2010). Consequently, (innate) nanoimmunotherapies are best designed to be approximately 10–150 nm, although optima will depend on the nanomaterials used, their application, and the type of cells involved.

In part, the lack of detailed in vivo investigations into nanomaterial size and form result from the challenges associated with varying these parameters independently (i.e., varying a nanomaterials' form often also changes their volume, surface area, and rigidity). Furthermore, the in vivo adhesion of proteins and other biomolecules affects nanomaterials' dimensions and cannot be accurately mimicked in vitro (see section on “Nanomaterial surface charge”; Amici et al., 2017; Bertrand et al., 2017; García-Álvarez et al., 2018). Lastly, any effects observed might result from rare nanomaterial populations with properties distinctly different from the bulk. Studies investigating discrete (i.e., monodisperse) nanomaterials with identical forms but varying sizes—and vice versa—are therefore especially valuable. For instance, Jasinski et al. created RNA-based nanosquares of different sizes and observed large differences in their biodistributions in tumor-bearing mice (Figure 3(d); Jasinski et al., 2018).

Combined, and in general, in vitro cultured myeloid cells most efficiently internalize roughly spherical nanoparticles approximately 20–60 nm in size. Nanomaterials in this size range are also relatively slowly cleared by the kidney, spleen, and liver, facilitating their uptake by (bone marrow) innate immune cells. However, insight into how nanomaterials' size and form affect their in vivo engagement of the innate immune system remains scarce. Advancing the field will require improving the syntheses of discrete nanotherapeutics as well as techniques to study their behavior on a microscopic level in vivo.

2.3 | Nanomaterial rigidity

Nanomaterials' rigidity also affects their in vivo behavior (Box 1). Naturally, nanomaterials' bulk composition is a good indicator of their rigidity (e.g., metal nanomaterials are more rigid than lipid-based ones, Figure 4(a)). However, nanomaterial rigidity can in some cases be finely tuned, for instance, by varying the cross-link density of polymeric nanoparticles (Key et al., 2015; Merkel et al., 2011).

In vitro experiments have revealed that flexible nanoparticles' cellular uptake typically proceeds slower than that of rigid ones (Hui et al., 2020; Key et al., 2015; Sun et al., 2015). This difference in internalization kinetics results from flexible nanoparticles' deformation during endocytosis, leading to shape changes that impede it (Figure 4(b)). In agreement, the interactions between cells and flexible nanoparticles result in less actin filament formation

compared with more rigid analogs (Anselmo & Mitragotri, 2017; Beningo & Wang, 2002). However, an opposite trend (i.e., slower internalization of rigid nanoparticles) has been observed for nanoparticles in which the primary mode of cellular uptake involves their fusion with the cell membrane (Figure 4(c); Guo et al., 2018). In such cases, a high nanomaterial rigidity impedes membrane fusion and this effect can outcompete the increase in endocytosis brought about by the nanoparticles' enhanced rigidity.

Nanomaterial flexibility also influences in vivo behavior. Comparing nanomaterials with similar composition, size, and surface charge, but varying rigidity, reveals that flexible variants display longer blood half-lives. This is attributed to the relative ease with which flexible nanomaterials can pass physical barriers, thereby reducing their removal by clearance organs (Figure 4(d); Anselmo et al., 2015; Yu et al., 2018; L. Zhang et al., 2012).

The effect of nanomaterial flexibility on pharmacokinetics and biodistribution was investigated by Anselmo et al. (2015). A range of nanoparticles with uniform size (~200 nm), but varying elastic moduli (0.255–3000 kPa) were created by developing polyethylene glycol-based hydrogels cross-linkable by UV-light. Intravenous injection of radiolabeled hydrogels in mice revealed that rigid variants display faster blood clearance and higher macrophage accumulation 30 min post-injection.

In a similar study, cylindrical polymer brushes were synthesized in two different lengths and with either a rigid or flexible core (Müllner et al., 2015). Fluorophore-labeled brushes were incubated for 24 h with RAW264.7 macrophages and their cell-association quantified by fluorescence microscopy. Results showed that polymer length, but not rigidity, influences the polymers' in vitro cell adhesion (Figure 4(e)). Interestingly, administering radiolabeled polymer brushes to rats did reveal an influence of the nanoparticles' rigidity, with both rigid variants displaying shorter plasma half-lives compared with their flexible analogs. These results highlight the importance of in vivo experiments and suggest that nanomaterials' overall flexibility, rather than just that of their surface, dictates their in vivo behavior (Figure 4(f)).

Combined, optimal myeloid cell uptake can be achieved using nanomaterials flexible enough to avoid rapid clearance, while sufficiently rigid to aid cellular internalization. However, no single optimal nanomaterial rigidity exists. Rather, this varies between nanoparticle types and intended application (e.g., their shape and hydrophobicity, their desired biodistribution, and if receptors on cells' surface or interior are targeted; L. Chen et al., 2018; Huang et al., 2013; Shen et al., 2019; S. Wang, Guo, et al., 2019).

2.4 | Nanomaterial surface charge

Nanomaterial surface charge is typically studied using the experimentally easier to determine zeta-potential (Blanco et al., 2015; Lundqvist et al., 2008). It can be controlled by decorating nanomaterials' exterior with ionizable moieties, such as carboxylic acids and amines (Figure 5(a); Blanco et al., 2015; Mout et al., 2012). For instance, most metal nanoparticles inherently possess a positive surface charge in aqueous solution, which can be modulated by surface-functionalizing them with negatively charged compounds (Schaeublin et al., 2011).

In vitro studies have demonstrated the impact of nanomaterials' surface charge on their colloidal stability and interaction with cells. Nanotherapeutics are typically designed to have a pronounced surface charge, as the lack of electrostatic repulsion between neutral nanomaterials facilitates their aggregation by Van der Waals forces, complicating their storage and employment (Figure 5(b)) Jin et al., 2009. In contrast, nanomaterials with a net surface charge adsorb biomolecules, which typically promote their cellular uptake (Figure 5(c); Deng et al., 2012; Gunawan et al., 2014; Quach & Kah, 2017; Tenzer et al., 2013). Importantly, nanomaterials' properties influence the type and quantity of biomolecules adsorbed. Parameters affecting this process include the sign, density, and magnitude of nanomaterials' surface charge, but also their size and surface chemistry (Chandran et al., 2017; Gessner et al., 2002; Gunawan et al., 2014; Piella et al., 2017; Walkey & Chan, 2012). The composition of the resulting "biocorona" strongly affects nanoparticles' behavior by influencing their biocompatibility, stability, size, rigidity, and form (Bertrand et al., 2017; Mohammad-Beigi et al., 2020; Ritz et al., 2015). It has been suggested that the negative membrane potential of most cells might further enhance the uptake of positively charged nanomaterials (Arvizo et al., 2010; Shin et al., 2013).

The interconnection between nanomaterials' surface charge, protein adsorption, and myeloid cell uptake was demonstrated by Xiao et al. (2011). The authors formulated neutral micelles consisting of PEG-oligocholeic block-copolymers as well as positive or negatively charged analogs in which the PEG chains' distal ends were terminated with lysine or aspartic acid residues, respectively. Incubating fluorophore-loaded micelles with murine-derived macrophages revealed that the positively charged variants were internalized faster than their negative or neutral counterparts. However, preincubating negatively charged micelles with mouse serum significantly enhanced their macrophage uptake, likely because of protein adsorption. Interestingly, the uptake of positively charged particles was slightly reduced after serum incubation, presumably due to differences in the types of proteins adhered.

Although a net surface charge generally promotes nanomaterials' in vitro uptake by innate immune cells, there are some limitations. For example, excessive charge-induced protein adsorption can disrupt nanomaterials' integrity (Abstiens, Maslanka Figueroa, et al., 2019; H. Chen et al., 2008), and the uptake of charged nanomaterials can alter cells' membrane potential by blocking their ion channels (Nakamura et al., 2019; Shin et al., 2013; Warren & Payne, 2015). Lastly, zwitterionic nanoparticles—containing both positive and negative charges—poorly retain biomolecules (Ladd et al., 2008; Murthy et al., 2013; Schlenoff, 2014; W. Yang et al., 2009).

In vivo studies on nanomaterial surface charge are rare, but highlight the differences between in vitro incubation and in vivo circulation. Typically, such studies involve recovering nanomaterials from the bloodstream by a combination of centrifugation and filtration steps (Amici et al., 2017; Bertrand et al., 2017; García-Álvarez et al., 2018). Amici et al. compared employing liposomal amphotericin B (AmBisome) in vitro or in vivo by studying the resulting protein corona (Amici et al., 2017). Incubating the liposomes for 10 min in blood from FVB/N mice increased their hydrodynamic diameter (from ~190 to 330 nm) and zeta-potential (from approximately -60 to -25 mV), indicating the adsorption of biomolecules (Figure 5(d)). Similar trends were observed after circulating the liposomes

for 10 min in mice, although there were notable differences with the in vitro incubated sample (size ~260 nm and zeta-potential -20 mV). Tandem mass spectrometry of the in vitro incubated and in vitro circulated liposomes revealed substantial differences in the total number of protein species adhered as well as their molecular weights and functions (Figure 5(e)).

Efficient and durable innate nanoimmunotherapy requires engaging myeloid progenitor cells in the bone marrow and therefore limiting clearance by other organs. However, both processes are typically promoted by an enhanced nanomaterial surface charge (Deng et al., 2012; Gunawan et al., 2014; He et al., 2010; Quach & Kah, 2017; Tenzer et al., 2013; Xiao et al., 2011). Nanomaterials that change surface charge in vivo might provide a solution to this problem. The pH dependence of hydrolyzable or ionizable surface modifications has been used to influence nanotherapeutics' tumor uptake and intracellular drug release (H. Y. Yang et al., 2020). Similar strategies in which a nanomaterial's net charge increases upon reaching the hemopoietic organs will likely boost its ability to engage the innate immune system. Potential environmental cues could include bone marrow's relatively low pH and high K^+/Na^+ -ratio compared with blood (Miettinen et al., 2012; Radovic-Moreno et al., 2012).

Another still largely unexplored avenue of research is the effect of nanomaterials' surface charge anisotropy on myeloid cell regulation. Janus nanoparticles (e.g., nanomaterials with different surface chemistry on each hemisphere) have interesting biological properties (Gao & Yu, 2015; Han et al., 2013). For instance, nanoparticles in which only one hemisphere is PEGylated show a similar in vitro reduction in macrophage uptake as fully PEGylated ones and their surface anisotropy allows determining their orientation during phagocytosis (Sanchez et al., 2017). Similar control over nanomaterials' charge distribution will likely yield unique and unforeseen in vivo properties.

Combined, nanomaterials' surface charge, as well as its anisotropy and responsiveness to the in vivo environment, play a prominent role in determining their biodistribution and ability to modulate the innate immune system.

2.5 | The role of ligand density and linker length

Myeloid cells have evolved cell surface receptors that "taste" their surroundings, allowing these cells to effectively detect dangerous pathogens. For example, Toll-like receptors (TLRs) induce inflammation upon recognizing pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPS, respectively; Crocker, 2005; Li et al., 2018; Lübbers et al., 2018). Importantly, co-activation and clustering multiple receptors typically results in a disproportionally strong immune response, allowing cells to modify their response to infections' severity.

Cell surface receptors can also be activated using nanomaterials that are surface-functionalized with ligands capable of activating these receptors. For example, the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptor can be engaged using nanomaterials functionalized with peptidoglycan derivatives (Priem et al., 2020). However, employing nanomaterials to effectively activate and cluster cell surface

receptors requires precise control over their ligands' density and orientation (Figure 6; Duan et al., 2018; Poupot et al., 2018; Weissleder et al., 2005). Increasing nanomaterials' ligand density can greatly enhance receptor activation and it is not uncommon for multivalent constructs' *avidity* (i.e., the combined binding strength of multiple receptor–ligand interactions) to be several orders of magnitude greater than the sum of the individual receptor–ligand binding sites' *affinities* (Figure 6(a); Bandyopadhyay et al., 2011; Hong et al., 2007; Silpe et al., 2013). This phenomenon mainly stems from the enhanced local ligand concentration (i.e., effective molarity) brought about by connecting multiple ligands (Karush, 1976; Kiessling et al., 2000; Kitov & Bundle, 2003; McKenzie et al., 2018; Teunissen et al., 2016; Vauquelin & Charlton, 2013). Nonetheless, very high ligand densities can negatively affect a nanomaterial's biodistribution or impede receptor activation as a result of steric hindrance (Alkilany et al., 2019; Elias et al., 2013).

The linkers connecting a nanocarrier to its ligands also play important roles in activating cell surface receptors (Figure 6(b); Abstiens, Gregoritz, & Goepferich, 2019; B. Wang et al., 2014). Generally, optimal efficacies are achieved using linkers just long and flexible enough to allow ligand–receptor binding, as this maximizes the local ligand concentration in the receptors' proximity (Kapadia et al., 2019; Stefanick et al., 2013; Teunissen et al., 2016). Nanomaterials' interaction with cell-surface receptors is also influenced by their shape. Elongated nanomaterials are typically more effective due to their ability to bind more receptors simultaneously (Figure 6(c)). For example, in vitro stimulation of macrophages with rod-shaped nanomaterials elicits a stronger TNF response compared with spherical particles with similar ligand densities (Garapaty & Champion, 2019). In addition to clustering identical receptors, nanomaterials can also cluster different types of receptors (Figure 6(d); Gref et al., 2003; Peiris et al., 2018; S. Xu et al., 2013). Such receptor heteromultimerization is impossible using unconnected ligands and plays an important role in many biological pathways, including those related to the innate immune system (Barnes, 2006; Singh & Jois, 2018; S. Xu et al., 2013). However, modulating the innate immune system using nanomaterials functionalized with multiple types of ligands remains poorly explored, likely due to the complex multivalent and non-linear effects involved.

The importance of nanomaterial ligand design for cell surface receptor engagement was demonstrated by Abstiens et al. (Figure 6(e)). A library of PEG-decorated polymeric nanoparticles was created by varying both PEG linker length and the fraction of PEG chains terminated with cyclic arginylglycylaspartic acid (cRGD). A small fraction of fluorophore functionalized polymer was also incorporated. The nanoparticles were incubated in vitro with U87MG glioblastoma cells, which overexpress the cRGD receptor $\alpha_v\beta_3$ integrin and analyzed by flow cytometry. Although cRGD surface functionalization boosts the uptake of all nanoparticles, this cRGD-dependency was much stronger for nanoparticles decorated with short (2 kDa) PEG chains (Figure 6(f)). Analyses by dynamic light scattering revealed that these nanoparticles assemble into larger aggregates (Figure 6(g)), which greatly enhanced the density of cRGD ligands presented (Figure 6(h)). Unfortunately, similar studies focused on ligand-functionalized nanomaterials' engagement of the innate immune system are rare and nearly exclusively performed in vitro.

Combined, the activation of myeloid cell surface receptors can be best achieved using elongated nanomaterials with a relatively high ligand density and with linkers just long enough to allow receptor activation. However, detailed (in vivo) studies on how these design parameters influence the activation of innate immune cells' surface receptors remain largely lacking.

3 | FUTURE DEVELOPMENTS

Nanomaterial research has produced virtually unlimited nanoparticle variations and future technologies will presumably generate even more exotic morphologies and material properties (Champion et al., 2007; Venkataraman et al., 2011; X. Zhu et al., 2019). The employment of biological entities as nanotherapeutics also holds great potential (Brenner et al., 2018; Burnouf et al., 2018; Y. Wang, Zhang, et al., 2019; Wayteck et al., 2016). Nonetheless, predicting nanomaterials' in vivo behavior based on their composition and material properties remains a major challenge.

Nanomaterial heterogeneity—both on an intra-particle and inter-particle level—plays an important role in their interaction with biological entities (Rabanel et al., 2019). Especially in highly disperse nanotherapeutic formulations, it is likely that nanotherapeutics' efficacy stems from small fractions of extremely efficacious variants. The development of innate nanoimmunotherapeutics will, therefore, benefit from techniques that either produce formulations with low dispersity or study nanoparticles on a single particle level (Rabanel et al., 2019). For example, Cleland et al. developed a microfluidic-based analyzer capable of measuring the individual size of 500,000 nanoparticles per second (Fraikin et al., 2011). Similar technologies will allow investigating, and potentially isolating, rare nanomaterial subsets challenging to study using bulk techniques.

A better understanding of the interplay between nanomaterial design parameters is also required. The design aspects discussed in this review represent important tools to control nanomaterials' interaction with the innate immune system. However, many of these parameters are interdependent. For example, changing a nanomaterial's form also affects its rigidity and ligand presentation, while changes in surface charge affect nanoparticles' effective size by influencing protein adsorption (Garapaty & Champion, 2019). As a result, nanomaterials' in vivo behavior cannot be predicted by a straightforward evaluation of their material properties. Developing effective nanotherapies therefore still requires (in vivo) screening of multiple promising formulations, demanding extensive resources and time.

High-throughput formulation and screening technologies will accelerate the development of innate nanoimmunotherapeutics. In recent years, the “barcoding” of nanoparticles with nucleic acids has been demonstrated (Dahlman et al., 2017; Lokugamage et al., 2018; Yamankurt et al., 2019). In one example, high-throughput microfluidic mixing was used to produce more than 300 different liposomal formulations labeled with unique DNA sequences (Lokugamage et al., 2018). Using a combination of fluorescence-assisted cell sorting (FACS) and next-generation sequencing, the authors were able to simultaneously assess the biodistribution of over 150 nanoparticles ex vivo. Given the fast developments in this field, and the practically infinite nucleotide sequences available, it is likely that the

formulation, characterization, and ex vivo screening of thousands of formulations will soon become a reality. As such experiments will inevitably produce large amounts of data, we expect machine learning to play a vital role in interpreting these results (Ban et al., 2020; Ho et al., 2019; Yamankurt et al., 2019).

Furthermore, and as made clear by the examples discussed in this review, nanomaterials' in vivo behavior cannot be fully mimicked in vitro. Although ex vivo analyses provide a useful alternative, they typically offer only snapshot information. Developing effective innate nanoimmunotherapies will require comprehensive and non-invasive insight into nanomaterials' in vivo behavior, both on a macroscopic and cellular level. For example, little is known about how nanomaterial design parameters influence their in vivo propensity for uptake through specific endocytotic pathways (J. Zhao & Stenzel, 2018). A focused effort toward developing innovative nanomaterial imaging techniques is therefore essential (Pérez-Medina et al., 2020; Schoormans et al., 2019; Senders et al., 2020; Teunissen et al., 2018; X. Zhu et al., 2015).

Lastly, engaging distinct myeloid cell subsets (e.g., macrophages vs. dendritic cells) will likely influence the resulting immune response, thereby providing additional avenues for therapeutic intervention. Unfortunately, insight into how nanomaterial design parameters affect their interaction with specific myeloid cell-types is virtually absent. Although techniques to study cell-specific nanomaterial uptake are widely available (e.g., flow cytometry and CyTOF), the effect of distinct design parameters cannot be easily untangled due to the relatively high dispersity of most formulations. Techniques to create highly defined nanomaterials would therefore benefit this area of research.

4 | CONCLUSION

Immunotherapeutics targeting the adaptive immune system have revolutionized the treatment of disease. Therapeutically engaging the innate immune system remains largely unexplored, but is rapidly gaining ground. Myeloid cells' inherent phagocytic features make nanomaterials compelling platforms for innate immune regulation. We have examined the relationship between nanomaterials' design parameters and their efficacy as nanocarriers for innate nanoimmunotherapy. Our evaluation revealed general characteristics of effective innate nanoimmunotherapeutics, including a net surface charge, moderate rigidity, and 20–150 nm size. However, we also found that most reports on nanomaterial behavior involve in vitro studies, and caution against straightforwardly extrapolating their results to in vivo behavior. Instead, we advocate a concentrated effort toward developing high-throughput in vivo screening methodologies that integrate non-invasive imaging and machine learning, thereby avoiding the costly and time-consuming successive screening of promising nanotherapeutic formulations.

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FURTHER READING

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BOX 1**Mechanical properties of materials**

Nanomaterials are exposed to various stresses in vivo, which have the potential to alter their size, form, and integrity. These include shear stresses in the bloodstream, abrasion against vessel walls, deformation upon passing physical barriers, and complex distortions during cellular internalization (Hui et al., 2019; Korin et al., 2012; Yu et al., 2018). Nanomaterials' behavior under force can be described using a variety of terms with related, but nonidentical, meanings (see below; Bhandari, 2013).

In this review, we predominantly focus on nanomaterials' rigidity and flexibility as their resistance to deformation purportedly underlies the reported differences in blood half-lives and cellular internalization rates. However, nanomaterials' ability to retain their initial size and form after deformation (related to their elasticity and resilience) and resistance to abrasion (hardness) likely also affect their in vivo behavior.

Rigidity (i.e., stiffness)—Flexibility: A materials' ability to resist deformation.

Elasticity: A materials' ability to absorb energy without permanently deforming.

Resilience: A materials' ability to store energy upon deformation and release this when unloaded.

Plasticity: A materials' ability to absorb energy while permanently deforming.

Strength: A materials' ability to resist deformation without permanent deformation or fracturing.

Toughness: A materials' ability to absorb energy without breaking.

Hardness: A materials' ability to resist localized permanent deformation (e.g., abrasion).

FIGURE 1.

(a) Schematic overview of cell types associated with the innate (top) and adaptive (bottom) branches of the immune system. The depicted residence organs serve as representative examples. (b) Nanocarriers can be loaded with drugs to enhance these compounds' intracellular delivery or surface-functionalized with specific ligands to target cell (surface) receptors

FIGURE 2.

Schematic representation of nanomaterials commonly employed as nanocarriers or nanotherapeutics for (innate) nanoimmunotherapy (Almeida et al., 2014; H. Chen et al., 2020; Fadel & Fahmy, 2014; Hawkins et al., 2008; Herrera Estrada & Champion, 2015; Hess et al., 2017; Kean & Thanou, 2010; Lee et al., 2019; Mehnert & Mäder, 2012; Okholm & Kjems, 2017; Raut et al., 2018; Savage et al., 2013)

FIGURE 3.

The effect of nanomaterial size and form on in vivo behavior. (a) Transmission electron microscopy (TEM) micrographs of nanomaterials with various forms, scale bars represent 100 nm. (b) A small contact angle (θ) between nanomaterials and cells' surface impedes endocytosis. (c) The increased blood half-life typically observed for elongated nanoparticles is presumed to originate from their tumbling in the bloodstream, promoting vessel wall adherence. (d) Schematic depiction of highly defined RNA nanosquares of different sizes and ex vivo fluorescence imaging of organs obtained from tumor-bearing mice (subcutaneous xenografts established using KB cells) intravenously injected with these RNA nanosquares. All nanosquares were labeled with the fluorophore Alexa Fluor 647, Li = liver, Sp = spleen, Ki = kidney, Tu = tumor. Panel a was adapted with permission from Castagnola et al. (2017), panels b and c are based on Toy et al. (2013), panel d is adapted with permission from Jasinski et al. (2018)

FIGURE 4.

The effect of nanomaterials' rigidity on their in vivo behavior. (a) Nanoparticles' rigidity largely depends on their bulk composition, but in some cases can be precisely controlled. (b) Flexible nanomaterials deform upon endocytosis, hindering the formation of actin filaments and impeding endocytosis (Anselmo & Mitragotri, 2017; Beningo & Wang, 2002). (c) In some instances, enhanced nanoparticle rigidity can inhibit fusion with the cell membrane and thereby reduce their uptake. Panel adapted with permission from Guo et al. (2018). (d) Flexible nanoparticles pass through membranes easier than rigid variants, reducing their clearance from the bloodstream. (e,f) Polymer brushes were synthesized in two different lengths and with either flexible or rigid cores (I = flexible 220 ± 70 nm, II = rigid 260 ± 50 nm, III = flexible 1150 ± 300 nm, and IV = rigid 1200 ± 200 nm). (e) Fluorophore-labeled polymer brushes were incubated with RAW264.7 macrophages for 24 h and analyzed by fluorescence microscopy. Significantly more cell association was observed for both longer brushes, but the brushes' rigidity had no clear influence. (f) Radiolabeled polymer brushes were i.v. administrated to rats and their plasma concentration monitored. Results revealed that rigid variants clear notably faster than their flexible analogs. Panels e and f were adapted with permission from Müllner et al. (2015)

FIGURE 5.

The effect of nanomaterial surface charge on in vitro and in vivo behavior. (a) Introducing surface modifications can alter nanomaterial surface charge. (b) Surface charge influences nanomaterial (spheres) colloidal stability and protein (blobs) adsorption. (c) Protein adsorption alters nanomaterials properties and thereby their cellular uptake. (d,e) Amici et al. incubated liposomes for 10 min at 37°C in blood-derived from FVB/N mice before recovering the nanoparticles (in vitro sample). Alternatively, the liposomes were intravenously administered to FVB/N mice, allowed to circulate for 10 min, and subsequently recovered from the bloodstream (in vivo sample). (d) Dynamic light scattering revealed that protein adsorption increases the effective size and zeta-potential of both in vitro incubated and in vivo circulated liposomes, as compared with their unused counterparts.

However, differences in size and zeta-potential were observed between the in vitro and in vivo used nanomaterials. (e) The number, molecular weight (Mw), and function of the types of proteins adsorbed after in vitro incubating or in vivo circulating the liposomes; as determined by tandem mass spectroscopy. Panels d and e were adapted with permission from Amici et al. (2017)

FIGURE 6.

Factors influencing nanomaterial-mediated activation of cell surface receptors. (a) The avidity between multivalent receptor–ligand scaffolds is often several orders of magnitude greater than the sum of the individual receptor–ligand affinities. (b) The linkers connecting a nanomaterial and its ligands should ideally be just long enough to activate and cluster multiple receptors. Further increasing linker lengths reduces the ligands' local concentration and thereby the nanoparticle's receptor avidity. (c) Elongated nanomaterials generally bind cell surface receptors more efficiently than spherical ones with identical ligand density. (d) Functionalizing nanomaterials with multiple types of ligands allows clustering different kinds of receptors. (e) Schematic depiction of a nanoparticle library comprised of PEG (blue), poly-lactic acid (PLA, yellow), and cRGD (green). (f) In vitro uptake of the various polymeric nanoparticles in U87MG glioblastoma cells after 45 min incubation at 37°C, as determined by flow cytometry. (g) The hydrodynamic diameter of nanoparticles

containing 2 or 5 kDa PEG chains as determined by dynamic light scattering. (h) Schematic depiction of the effect of PEG linker length on the nanomaterials' colloidal stability and receptor activation. Panels d–h were adapted with permission from Abstiens, Gregoritz, and Goeperich (2019)