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PERSPECTIVE

Mechanisms and Barriers in Nanomedicine: Progress in the Field and Future Directions

Thomas Anchordoquy, Natalie Artzi, Irina V. Balyasnikova, Yechezkel Barenholz, Ninh M. La-Beck, Jacob S. Brenner, Warren C. W. Chan, Paolo Decuzzi, Agata A. Exner, Alberto Gabizon, Biana Godin, Samuel K. Lai, Twan Lammers, Michael J. Mitchell, S. Moein Moghimi, Vladimir R. Muzykantov, Dan Peer, Juliane Nguyen, Rachela Popovtzer, Madison Ricco, Natalie J. Serkova, Ravi Singh, Avi Schroeder, Anna A. Schwendeman, Joelle P. Straehla, Tambet Teesalu, Scott Tilden, and Dmitri Simberg*

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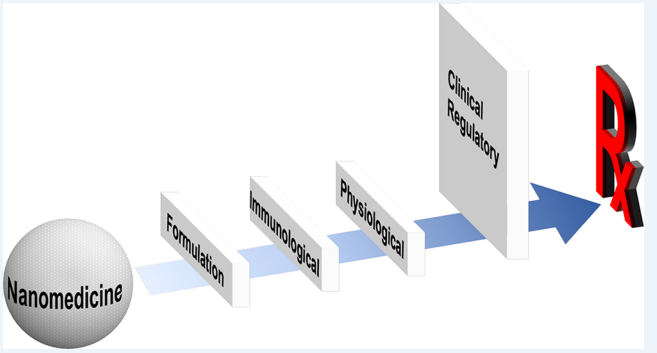
ABSTRACT: **In recent years, steady progress has been made in synthesizing and characterizing engineered nanoparticles, resulting in several approved drugs and multiple promising candidates in clinical trials. Regulatory agencies such as the Food and Drug Administration and the European Medicines Agency released important guidance documents facilitating nanoparticle-based drug product development, particularly in the context of liposomes and lipid-based carriers. Even with the progress achieved, it is clear that many barriers must still be overcome to accelerate translation into the clinic. At the recent conference workshop "Mechanisms and Barriers in Nanomedicine" in May 2023 in Colorado, U.S.A., leading experts discussed the formulation, physiological, immunological, regulatory, clinical, and educational barriers. This position paper invites open, unrestricted, nonproprietary discussion among senior faculty, young investigators, and students to trigger ideas and concepts to move the field forward.**



KEYWORDS: *nanomedicine, barriers, translation, complement, tumor, inflammation, formulation, delivery, mRNA*

1. OVERVIEW

Nanoparticles (NP) have great therapeutic potential because they can control the biodistribution of their active payload and the drug release rate, protect the drug from degradation,[1] and improve the drug delivery process.[2] Like many other nascent technologies, nanomedicine has followed a course well described by the Gartner Hype Cycle[3] (Figure 1). After an initial sharp rise of expectations, as with many emerging medical technologies, there was a trough of disillusionment when the predicted efficacy improvement from animal studies did not materialize in human trials.[4] Recently, significant advances in manufacturing, a better understanding of the delivery barriers, and a plethora of research in the areas of drug coencapsulation, imaging-based pharmacokinetics/pharmacodynamics, and interaction between the immune system and the biological milieu have reinvigorated the field of nanomedicine (the "slope of enlightenment," Figure 1). The success of mRNA-lipid nanoparticles (LNPs) for COVID-19 vaccines definitely brought attention from the wide medical community to the field of nanomedicine and spiked interest of stakeholders, and funding agencies.

Since 2016, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved several nanodrugs, and over one hundred nanodrugs, nanovaccines, and nanoimaging agents are currently in clinical trials (excellently reviewed in[5,6]). Many exciting and clinically relevant nanoplatforms are being developed,[4] although most clinically approved drugs comprise liposome- and lipid-based nanomedicines. In this context, the development of intramuscularly administered COVID-19 LNP-based vaccines

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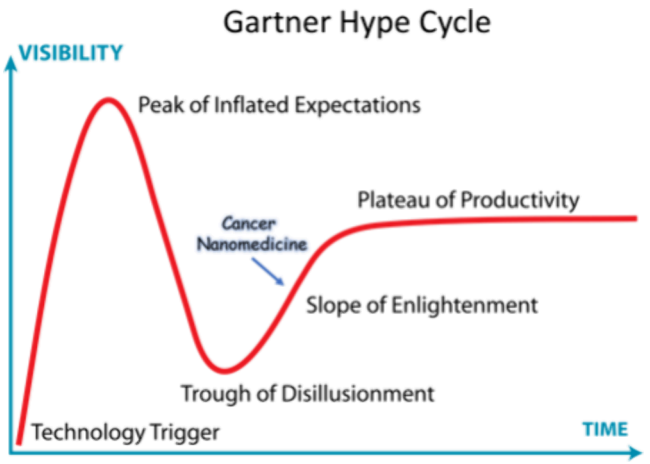

Figure 1. Gartner Hype Cycle. Reprinted with permission from ref 3. Copyright 2019, Elsevier.

posed a significant milestone, leading to increased public awareness of nanomedicine and a surge in research papers, grant proposals, and clinical trials on nucleic acid–based therapeutics. Other clinical successes of locally administered nanomedicines include topical liposomes with bupivacaine for postoperative long-term pain management and inhalable liposomes loaded with amikacin for treating severe lung infections. On the other hand, intravenous delivery of nanomedicines is still plagued by nonspecific immune interactions, accelerated clearance, and failure to outperform conventional therapies. For example, systemically administered mRNA LNPs often fail to express therapeutic amounts of the translated protein, unlike mRNA vaccines that generate a protective immune response by expressing small amounts of antigen. Most FDA-approved intravenously administered nanomedicines have been approved based on improvements in safety profiles[7] and incremental improvements in surrogate efficacy end points, such as disease response rates.[8,9] AmbiSome (a liposomal amphotericin B) is an exception, being a highly successful antifungal drug product that improves therapeutic efficacy against yeast and fungal infections with a significant reduction in toxicity.[10]

For most anticancer nanomedicines, improved pharmacokinetics and safety do not always translate into increased survival. The therapeutic efficacy of PEGylated liposomal doxorubicin (Doxil), the first FDA-approved anticancer liposome, is superior in ovarian cancers and AIDS-related Kaposi's sarcoma but equivalent in multiple myeloma and metastatic breast cancer when compared with unencapsulated doxorubicin or other standard therapies.[11−13] The main benefit of Doxil is the significant (>3-fold) reduction of cardiotoxicity gained by limiting doxorubicin exposure to cardiac tissue. As evidenced by the recent withdrawal of liposomal vincristine (Marqibo) due to lack of patient enrollment[14] and by limited clinical adoption of liposomal cytarabine/daunorubicin (Vyxeos), more needs to be done to achieve clinically meaningful end points to add value over conventional formulations.

In 2016, we organized the "Mechanisms and Barriers in Nanomedicine" conference-workshop, where we defined the most relevant barriers to nanomedicine deployment in the clinic, specifically cancer-related.[15] The second edition of the conference-workshop (held on May 4−6, 2023, in Golden, Colorado, USA) aimed to assemble key opinion leaders in the field to continue an open dialogue about the formulation, toxicological, physiological, immunological, and translational barriers of nanomedicine, in cancer and beyond. While the majority of the discussed topics (understandably) focused on liposomes and lipid-based carriers, the ultimate goal of this position paper is to summarize the barriers to the development of nanomedicine in a broad context while stimulating discussion on paths to advance clinical translation.

2. FORMULATION BARRIER

There must be a clear rationale for choosing a nano delivery system over conventional delivery, and the level of encapsulation/loading of the active pharmaceutical ingredient (API) should be sufficient for delivering clinically relevant doses and ultimately treating humans. The selection of the optimal nanoformulation is dependent to a large extent on the physicochemical descriptors of the API, especially its charge and the hydrophobic/hydrophilic balance. For example, computation and machine learning could greatly facilitate the identification of APIs compatible with nanoformulations[16] and aid in the rational design of mRNA LNPs.[17] For liposomes, it was shown that only a small fraction of the APIs are compatible with a stable remote loading technology (about 70 out of ∼13,000 APIs scanned by the algorithm[18]). The same approach can be applied to other loading methods and delivery systems (Figure 2).

Significant progress has been made in the manufacturing of lipid nanoparticle formulations.[19] Major issues related to liposome and LNP technological and pharmaceutical aspects were resolved or have been circumvented to the level required by the following FDA guidance documents: 1) "Liposome drug products: chemistry, manufacturing, and controls" and 2) "Drug products, including biological products, that contain nanomaterials."[20,21] Nevertheless, producing complex nanomedicines with regulatory agencies' required quality and on a large scale remains challenging. As exemplified by mRNA vaccines, efficient manufacturing processes that consistently yield high-quality nanomaterials are essential for their widespread availability. Large-scale approaches based on rapid T-junction mixing have been introduced for these nanoparticles.

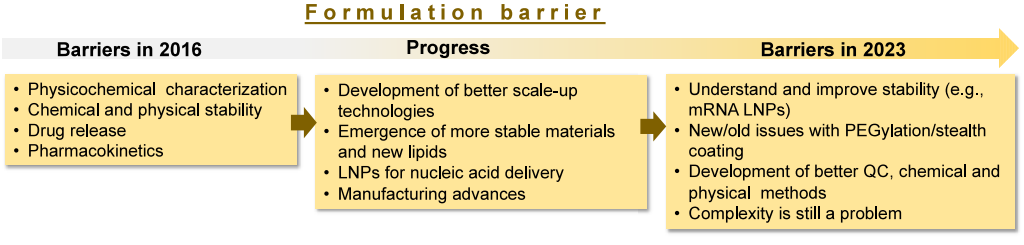

Figure 2. Our view of the progress in overcoming the formulation barrier.

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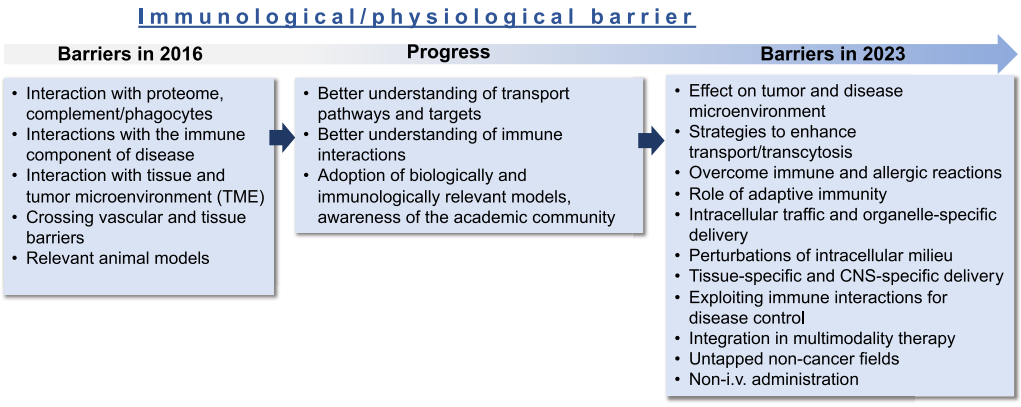

Figure 3. Our view of the progress in overcoming the immunological/physiological barrier

At the same time, microfluidics is still challenging to scale up[22] and, anecdotally, can result in different efficiencies depending on the mixing method.[23] Hopefully, microfluidic-based approaches can be adapted in the near future for many nanomedicine classes, including liposomes. However, even with efficient manufacturing, the high expense of developing and producing nanomedicines limits their accessibility and adoption. Finding ways to reduce costs while maintaining tight quality controls is pivotal for their widespread use.

The complexity and unstable character of multicomponent drug delivery nanosystems is rooted in a plethora of factors that must be tightly controlled, including geometry and morphology (size, shape, polydispersity), surface charge, surface ligand density, and characteristics, clustering of the surface moieties, stealth coating, etc. Insufficient command of structural features can result in chemical instability, aggregation, and dissociation. For example, a lack of control over biophysical parameters and interactions between LNP components and impurities, such as aldehydes, can contribute to both mRNA and LNP instability.[24,25]

Formulating API in a nanoparticulate form affects the interactions with the body milieu, driven by physiological and pathological factors of biological fluids, biomechanical stresses, cells, and tissue components. In this context, there is still a limited understanding of the changes a nanoparticle undergoes *in vivo*. However, it is known that extensive remodeling, exchange, and degradation of LNPs (e.g., Onpattro, lipoprotein nanoparticles, liposomes, and lyotropic nonlamellar liquid crystalline nanodispersions[26]) occur upon exposure to plasma. Many lipids used in liposomes and LNPs undergo breakdown upon exposure to animal and human sera.[27] Furthermore, the incorporated cargo molecules significantly affect not only the formulation processes but also the biophysical properties of the final nanoproduct and the mode of its interactions with the body's internal milieu. The cargoes—nucleic acids, proteins, small molecule drugs/prodrugs, enzymes, gene editing machinery, *etc.* are sometimes so complex, diverse, and susceptible to degradation that identical nanocarriers loaded with two different cargoes may emerge with varying physicochemical and functional characteristics, thus affecting the nanoformulation toxicity and biodistribution. The predominant strategy for reducing undesirable nanobio interactions and stabilizing particles utilizes a "stealth" polyethylene glycol (PEG) coating.[28] High PEGylation density (typically 5 mol % of total lipid, e.g., Doxil) is required for the long-circulating properties of liposomes. On the other hand, some current products, including mRNA vaccines, include lower mol % of PEG[29,30] or no PEGylation at all, which is dictated by the formulation requirements and the desired pharmacokinetics and pharmacodynamics. Furthermore, PEGylation may affect the immunological properties of the nanoformulation (see below).

In summary, researchers must meticulously analyze the physicochemical properties of the resulting formulations because they can affect the nanoformulation's stability, toxicity, biodistribution, and efficacy. High throughput approaches, machine learning, and modeling should be explored to understand the parameters and develop materials to improve stability and efficacy.

3. IMMUNOLOGICAL/PHYSIOLOGICAL BARRIER

3.1. Immunological Barrier. The immune system is a significant determinant of disease status and nanoparticle pharmacology; therefore, investigators must pay careful attention to the immune interactions (Figure 3). Nanocarriers interact with serum immune proteins, such as immunoglobulins and complement proteins, leading to uptake by immune cells and the release of anaphylatoxins.[31−35] In the context of underlying inflammatory diseases such as acute respiratory distress syndrome, complement activation could exacerbate the disease. Complement is implicated as the potential cause of infusion reactions in patients,[36] but its precise role needs to be better understood. Infusion reactions also occur with nanoparticles that are weak complement activators, thus demonstrating the involvement of coordinated and multifaceted mechanisms at the cellular level.[37] Nanoparticle design, including surface ligand density, nanoparticle size, the mode of surface pattern presentation (particularly at angstrom-scale periodicity[38]), and the use of inhibitors, can mitigate some of the complement responses in a preclinical setting.[39−43] For example, targeted complement inhibitors with good safety profiles could be a powerful strategy to improve hemocompatibility and immune uptake of nanoparticles[42] that can be deployed in the clinic (Figure 4). In addition to the complement, other serum proteins and biomolecules, collectively called the protein corona,[44,45] could modulate nanomedicine stability, pharmacokinetics, and toxicities. Still, the knowledge of the other "players" is limited, and further investigations are encouraged.

It was reported over two decades ago that repeated injections of empty PEGylated liposomes exhibited accelerated blood clearance in animal models[46] More recent work has

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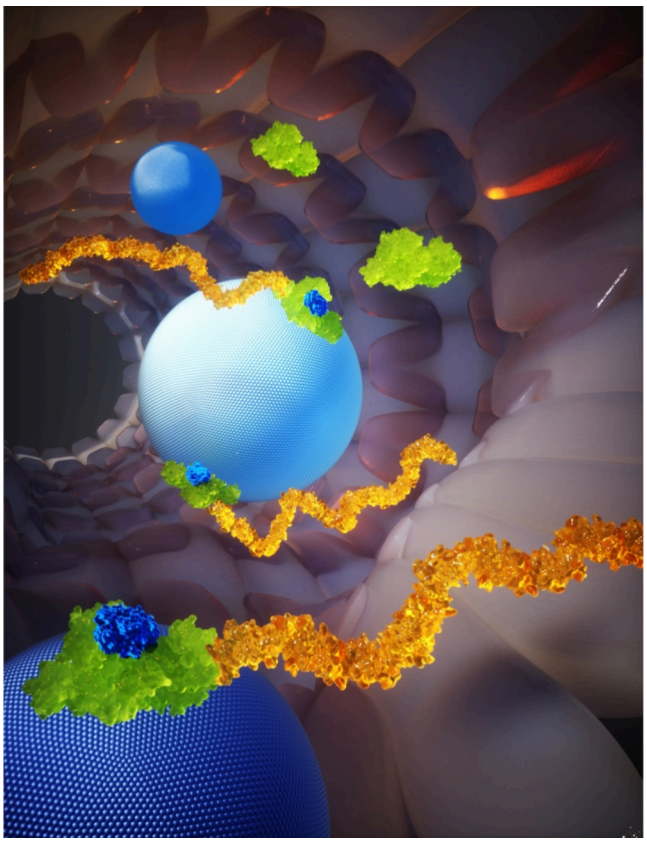

Figure 4. Targeted complement regulators (orange) are natural inhibitors that bind to initial complement depositions (green) on nanoparticle surfaces (blue) in blood and protect them from full-scale complement attack.[42] Artistic illustration by Ella Mary Studio.

documented the presence of anti-PEG antibodies that can potentially compromise the safety and efficacy of PEGylated formulations[46−49] and promote drug release from liposomes.[50] Unlike other antidrug antibodies, anti-PEG antibodies appear to be preexisting,[51] potentially due to exposure to everyday commercial products (e.g., food, cosmetics).[48,52] PEGylated nanovaccines can also trigger the formation of antibodies, but the levels of anti-PEG antibodies induced by Moderna and BioNTech COVID-19 mRNA vaccines, both of which contain PEGylated lipids in their composition, vary significantly between the two vaccines and among vaccine recipients.[53,54] The administration of free PEG prolongs the circulation of PEGylated proteins and PEG-liposomes, but this strategy has yet to be tested in clinical trials.[55,56] The relevance of anti-PEG antibodies to infusion reactions, drug release, and premature clearance is unclear and could depend on the nanoparticle/drug and its PEGylation method.[57] For example, despite the high prevalence of anti-PEG antibodies, adverse reactions to mRNA vaccines are extremely low.[58] Some mRNA LNP formulations use PEGylated lipids with shorter hydrocarbon chains that promote faster desorption.[25] These alterations, along with the surface presentation of PEG chains, may explain why the avidity of the mRNA-LNP to anti-PEG antibodies is low.[59]

While many hydrophilic polymer coatings are being explored as an alternative, studies have cautioned that the immune system can evolve to recognize repeating motifs inherent in polymers.[60] However, PEGylation is a scalable and well-understood technology, which makes it familiar to regulatory bodies and relatively straightforward to apply in the laboratory. Moreover, for many biologics (e.g., monoclonal antibodies, proteins), antidrug antibodies are routinely induced but often do not result in loss of efficacy.[61] Considering the continued widespread use of this polymer in nanomedicine, there is a need for clinical studies to understand the clinical correlates of the antibody titers with safety and efficacy, which will be essential to elucidate and overcome the potential PEG immunogenicity of nanoformulations.

The broad impact of the uptake of nanocarriers by immune cells on systems pathophysiology remains to be fully elucidated. For example, macrophages metabolize and transport endogenous cholesterol particles (e.g., low-density lipoproteins).[62,63] Cholesterol metabolism generates oxysterols that are potent regulators of cellular signaling pathways implicated in the pathogenesis of many diseases, including cancer, Alzheimer's disease, and atherosclerosis.[64−66] Considering the predominant use of cholesterol in lipid-based nanomedicines, it is imperative to address the gap in the current understanding of nanoparticle-associated cholesterol and lipids' *in vivo* metabolic fate. Another example is the effect of anticancer nanomedicines on immune cells.[67,68] In this context, it is known that systemically administered nanomedicines are internalized by tumor-associated macrophages (TAMs), dendritic cells, and myeloid-derived suppressor cells (MDSCs), and these cells orchestrate resistance to immunotherapies through various mechanisms.[69−75]

Given that TAMs prime and activate T-cells against tumor antigens, remodel the tumor immunologic milieu, and promote vascular permeability,[76] ablation of macrophages may impact therapeutic responses. Also, TAMs have been shown to serve as drug depots, releasing the payload to surrounding tumor cells,[77,78] which can be crucial in hypo-perfused tumors. A promising strategy is delivering amino-bisphosphonates encapsulated in stealth liposomes (PEGylated liposomal alendronate, PLA) to enhance internalization in TAMs and MDSCs.[74] Studies have demonstrated that PLA polarizes TAMs toward an M1 antitumor phenotype, increases tumor infiltration of tumor antigen-specific cytotoxic T cells, and increases antitumor cytokine responses in T cells without significant depletion of macrophages in the spleen, liver, or bone marrow.[74] These findings suggest that liposomal uptake can be exploited for immunotherapy of diseases where macrophage dysfunction perpetuates pathogenesis, such as in cancer and atherosclerosis.

Uptake by macrophages in the liver and spleen plays a predominant role in the clearance of systemically administered nanoparticles. Reducing uptake by these organs should serve the goal of improving delivery to diseased tissues. Preadministration of nanosized agents can temporarily and reversibly block the phagocytic activity of organ-specific macrophages, reduce the entrapment in the clearance organs and possibly in TAMs, and enhance the preferential accumulation of nanomedicines in the cancer cells.[79] A concern is that complete blockade of Kupffer cells decreases their bacterial clearance activity and may pose a significant risk in controlling infectious diseases.[80,81] Interestingly, splenic immune cells play an essential role in systemic antitumor response after intravenous injection of nanoformulated immunoadjuvants,[82] suggesting that uptake of nanoparticles to spleen-resident macrophages can achieve multiple effects. Immune cells in the spleen (and perhaps other secondary lymphoid organs) are an essential

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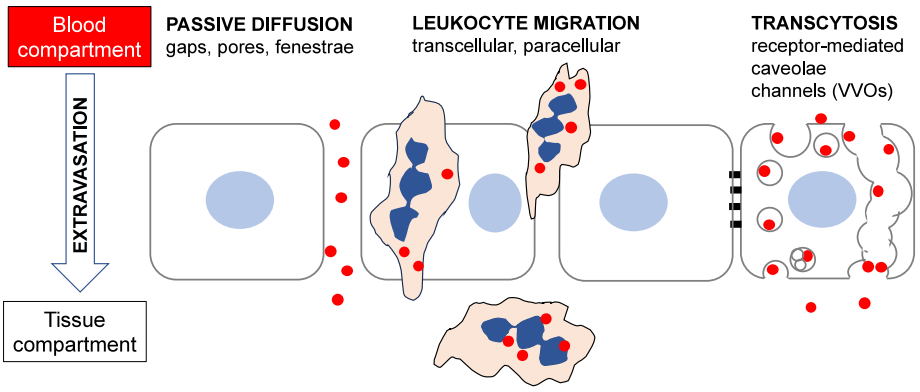

Figure 5. Nanoparticle movement (extravasation) from blood to tissue compartments may include a) passive diffusion, b) immune cell uptake and hitchhiking, c) transcytosis, for example, via vesiculo-vacuolar organelles (VVOs) or endocytosis. Nanoparticles can extravasate intact or as individual components after degradation/disintegration in blood and endothelial/immune cells.

target for generating long-lasting antitumor memory. As an interesting example of nanoimmune system interactions, STING-agonist-loaded mRNA LNPs have been shown to initiate the production of type I IFN by liver macrophages, leading to the systemic activation of NK cells and synergism with immune checkpoint blockade.[83]

Studies reveal the exploitation of nanoparticle-induced immune responses to reduce off-target uptake and increase bioavailability and tumor accumulation in mice. Specifically, lipoplex administration promotes the release of IFN-λ, which reduces normal tissue permeability and off-target deposition of subsequently administered nanoparticles in organs and tissues, leading to increased uptake by tumors.[84] This study suggests that a better understanding of the interplay between nanoparticles and immune systems can greatly benefit nanodrug delivery systems' distribution, biocompatibility, safety, and efficacy.

Lastly, preclinical research demonstrated differences in immunological interactions, pharmacokinetics, and pharmacodynamics of nanomaterials between young and old mice and male and female mice.[85,86] Funding agencies now require researchers to consider the sex of animals as a research variable. Intersubject differences leading to immune responses and related toxicities can be dramatic[87] but are often not readily revealed in animal models,[40,88] necessitating combined studies of genetics and immunology in preclinical models and human cohorts.

3.2. Physiological Barrier. A complex relationship exists between the net accumulation of a nanoparticulate drug in the target site and its efficacy. Differential uptake of particles by cell populations in the target tissue and intracellular delivery to specific compartments are critical aspects to consider. Potential transfer mechanisms between cells (e.g., cancer cells and immune cells, Kupffer cells, and hepatocytes) should be considered, especially for the design and synthesis of stable formulations, which can move from one cell type to another with its cargo remaining intact.[82]

Mechanisms of nanoparticle accumulation have been studied extensively in tumors,[89] and multiple processes can explain the extravasation of nanoparticles (Figure 5). Diffusion through leaky neovasculature appears to be one of several mechanisms of passive nanoparticle accumulation in tumors. Another transport pathway across blood vessels that nanoparticles can utilize is transcytosis, and recent work has suggested that this can play a significant role in uptake from the circulation and delivery to the tumor.[90−92] This mechanism may depend on the nanoparticle's chemical composition (e.g., solid metal particles versus lipid nanoparticles), size, shape, charge, hydrophobicity, surface properties, and the presentation of specific receptors.[89,93−95] Physicochemical targeting of nanoparticles is currently achieved by empirically screening many nanoparticle formulations, and the selectivity mechanisms have yet to be fully understood.[96]

Transport processes within nontumor tissues have yet to be extensively studied, but they appear to be at least partially similar to those in tumors. In the case of inflammation and bacterial infections, there is an enhanced endothelial permeability leading to passive targeting followed by inflammatory cell sequestration.[97,98] Nanoparticles may utilize normal or/pathological cellular components of blood, lymph, interstitial, and edematous fluids as natural vehicles. For example, about 30% of neutrophils are normally transiently positioned in the lumen of the pulmonary microvasculature, and these cells have been shown to collect nanocarriers depending on the surface architecture.[99] Circulating neutrophils take up complement- and immunoglobulin-opsonized liposomes and shuttle them to inflamed joints, as was shown in rheumatoid arthritis[100] and lung inflammation[99] models. Distortion of tissue by physical forces (*e.g.* pressing) can influence nanoparticle extravasation. Notably, a recent study showed that liposomes and nanoparticles accumulate in pressured skin via a passive extravasation mechanism,[101] which may be responsible for hand-foot syndrome, one of the significant side effects of Doxil.

Active targeting using ligands that bind to receptors on target cells has a long history in the delivery field. Whereas antibody-drug conjugates have had commercial success, ligand-mediated nanoparticle delivery has yet to result in an approved product. A significant challenge is that translating the target selectivity of targeting ligands (antibodies, aptamers, homing peptides) to modulate the tropism of synthetic nanoparticles requires extensive optimization of the binding affinity, linkers, and ligand density.[102−104] Unfortunately, significant increases in accumulation are often not observed even after optimization of these factors. This limitation arises partly from nonspecific off-target interactions, limited binding sites, and hindered access to the target cells. For example, a recent study showed that much of the enhanced tumor uptake of antibody-labeled nanoparticles is due to nonspecific binding of the Fc-binding domain of the antibody to receptors on the immune cells

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associated with tumors and is not dependent on tumor expression of the receptor to which the antibody is targeted.[105] In contrast, the presence of a "binding site barrier" frequently prevents targeted nanoparticles from getting deeper into the diseased tissue. Developing tumor-penetrating peptides (and potentially other tissue-penetrating ligands) holds promise to overcome the above limitations. iRGD, a prototypic tumor penetrating peptide, allows the extravasation of conjugated and coadministered molecular and nanoparticle drugs and their penetration deep into the tumor parenchyma.[106,107] In particular, the coadministration mode of iRGD holds promise as it can be used to increase the efficacy of unmodified clinically approved nanoparticles such as Abraxane without the risk of saturating the receptors and limiting the capacity of targeted delivery. The iRGD peptide (CEND-1) is currently undergoing clinical testing to help target chemotherapeutics for pancreatic cancer and other solid tumors.[108]

Another approach to address these limitations is to engineer each dose of nanoparticles, cells, or other therapeutics as a capturing surface for the next dose of therapeutics (pretargeting). In that way, the targeting surface can be continuously amplified to increase drug accumulation at the disease site. An interesting example was presented: boosting the targetable surface area through heterodimerizing leucine zippers,[109] which led to a substantial increase in accumulation and enhancement in prolonged retention. This pretargeting approach could be applied to different heterodimerizing ligands and carrier systems (i.e., micro- and nanoparticles) for enhanced accumulation and retention. The downsides are the need for multivalent interactions to occur in situ at the target and the complexity of this approach, which requires separate optimization of both the pretargeting and the therapeutic components.

Instead of targeting the extravascular compartment, the intravascular targeting of endothelium or blood cells appears more straightforward but still challenging. Nanoparticles tend to distribute into the center of vessels containing red blood cells (RBCs), which makes them less likely to localize near, interact with, or extravasate through the walls of blood vessels.[110,111] Conversely, RBCs may also play an essential role in nanoparticle-endothelium interactions via "massaging" as they squeeze through the capillary beds. Nanoparticles passively or actively adsorbed onto RBCs are transferred to the capillary walls during this process, facilitating delivery to the vascular endothelium.[112] This cell-mediated "hitchhiking" approach can be enhanced by decorating nanoparticles with ligands to target receptors on specific cell types. Using this strategy, it is conceivable that nanoparticle delivery could be enhanced in inflamed tissues or tumors with irregular vascular networks, but questions remain as to whether pathology will affect the vasculature associated with disease sites and how that might alter the effectiveness of this approach.

3.3. Central Nervous System (CNS) Barrier. The blood-brain barrier (BBB) represents a significant obstacle to drug delivery. Drugs must not only gain access to the brain but also to the correct region and, ultimately, the target cells. In this regard, drug delivery to the brain should be described in terms of 'CNS exposure,' which includes crossing the BBB, diffusing within the brain parenchyma, and reaching the biological target, as opposed to the ubiquitous but poorly defined term "brain penetrant" drug delivery.[113] Different pathologies (e.g., cancer versus neurodegenerative disease) possess distinct properties in uptake and distribution. For instance, primary brain tumors and brain metastases heterogeneously modulate the integrity of the blood-brain tumor barrier. Although primary brain tumors undergo surgical resection whenever possible, the high probability of residual cancer cells requires further treatment with radiotherapy and local drug delivery. The use of multistage systems is an exciting strategy to facilitate transport where an implant is inserted into the brain. During tumor resection surgery, the implant releases nanoparticles that would release even smaller nanoparticles capable of penetrating deeper into the brain tissue.[114]

Strategies for enhancing the transport across the BBB for nanoparticle delivery to the CNS, such as trans-nasal penetration of various therapeutics, have been investigated.[115] In this case, the epithelial barrier and fast clearance from the nasal cavity are likely to require disruption of the epithelial barrier to enhance the penetration of nanoparticles in the brain. However, with more advanced technologies, the convection-enhanced delivery of nanoparticles is a viable option.[116,117] Several exciting strategies to boost systemic brain delivery have been developed, including conjugating natural transporting ligands that enable transcytosis. For example, insulin conjugation can be used as an efficient "BBB shuttle" to facilitate the brain uptake of gold nanoparticles and further target the particles to specific brain regions involved in neurodegenerative and neuropsychiatric disorders.[118] In neurodegenerative conditions like Parkinson's, the BBB is compromised, allowing enhanced accumulation of 100 nm PEGylated liposomes into the brain and its cells. In addition, conjugating transferrin to the surface of these liposomes further improved their ability to cross the BBB and neuronal uptake.[119] Other examples include phage display to discover targeting peptides and engineered conjugates that self-assemble into nanoparticles (NanoLigands). This approach shows promising penetration of intact BBB while targeting neurons and microglia.[120] Alternative strategies involving nano/microbubbles and focused ultrasound can allow transient permeabilization and are being tested clinically.[121,122] Importantly, BBB modulation brings safety concerns[123] that require rigorous preclinical testing in nonhuman primates. While there is an overwhelming need for better approaches to treat cancer and neurodegenerative diseases, it is vital to consider the potential for negative consequences of chronic repetitive use of BBB disruptors or permeation enhancers.

As opposed to enhancing the BBB permeability, there is a potential to target nanoparticles to the BBB endothelial cells to reduce vascular permeability. Targeting liposomes or LNPs loaded with cargo that promotes endothelial barrier resistance to the pathological endothelium has proven effective in ameliorating stroke.[124]

4. CLINICAL/REGULATORY BARRIER

In vitro-in vivo correlation (IVIVC) plays a key role in the pharmaceutical development of dosage forms, primarily oral dosage forms. IVIVC is a tool used in quality control for scale-up and postapproval changes, to improve formulations or to change production processes, with the goal of reducing the number of animal and human studies during the development of pharmaceuticals.[125] The establishment of effective IVIVC requires understanding the physicochemical and biopharmaceutical properties of the drug, including solubility, drug absorption, drug pK_a, logD at various relevant pHs, LogP, hydrophilic surface area, as well as the physiological environment and microenvironment relevant to drug pharmacoki-

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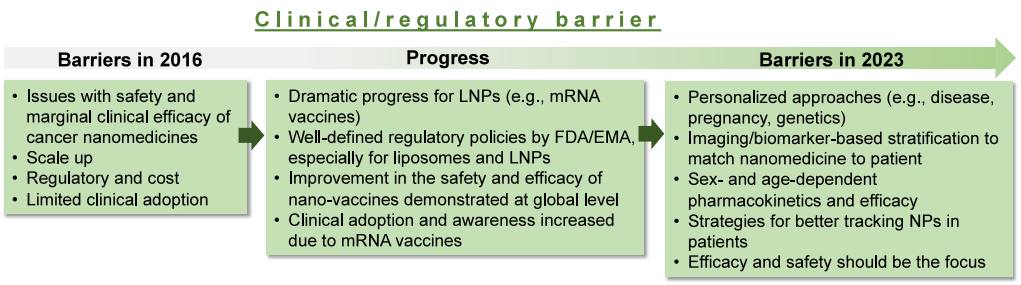

Figure 6. Our view of the progress in overcoming the clinical/regulatory barriers.

netics, biodistribution and mechanism of action. Only limited work related to IVIVC has been done for nonoral nanoparticle forms, including liposomal formulations.[126,127] Among the challenges in creating IVIVC for nonoral dosage forms, including liposome-based formulations administered locally or systemically, are the lack of *in vitro* dissolution media and conditions relevant to the *in vivo* situation and burst release.[126,127] Overcoming these challenges would enormously facilitate the development of generic and new nanomedicines (Figure 6).

Physiologically relevant *in vitro* models that mimic cellular interactions of nanoparticles and other transport barriers would benefit the field. Emerging technologies include patient-derived organoids and microfluidic organ-on-a-chip or tumor-on-a-chip. There is a concern that *in vitro* systems and animal models can be unreliable in predicting nanoparticle behavior *in vivo*. The absorption, distribution, metabolism, and excretion may be unique to the administration route or disease stage, presenting nanobio interactions not accounted for by the *in vitro* systems. For example, certain tumor types are prone to accumulating more nanoparticles because they are not cellularly dense, are not well vascularized, and have heightened vascular permeability. The extent of macrophage and other immune cell infiltration also significantly affects the accumulation and retention of nanoparticles. All of these factors represent a formidable challenge, and given the changing attitude of the regulatory agencies toward animal testing,[128] more effort is needed in this area.

A critical issue emerging in nanomedicine is identifying patients that would benefit the most from nanodrug delivery. As a case in point, antibody-drug conjugates and chimeric antigen receptor (CAR) T-cell therapies rely on target expression to select patients for the most suitable therapy. It was proposed that a similar approach could be used for targeted nanoparticles.[129] The potential of theranostics for patient stratification is evident in cancer, and there is a precedent for screening patients where positron emission tomography (PET) tracer ligands determine tumor accumulation before administering the ligand with a potent therapeutic radionuclide.[130] The Enhanced Permeability and Retention (EPR) effect is highly variable in human patients,[131] providing a rationale for personalized approaches to estimate the EPR effect by imaging.[132] Using magnetic resonance imaging (MRI) nanoprobes or targeted gold nanoparticles for computed tomography (CT),[133] it may be possible to prescreen and select responsive patients before administering nanomedicines. In this context, FDA-approved iron oxide nanoparticle ferumoxytol is inexpensive and safe (no radiation), and could be employed for patient stratification.[132,134] Dynamic contrast-enhanced (DCE) and dynamic susceptibility (DSC) MRI with gadolinium contrast or ferumoxytol can noninvasively assess perfusion and vascular leakage in cancer lesions that indirectly correlate with nanomedicine accumulation.[135] Ultrasound techniques, including Doppler, B-mode imaging, elastography, super-resolution, and contrast-enhanced ultrasound (CEUS), hold promise for predicting nanoparticle distribution and therapeutic effectiveness in solid tumors. Ultrasound has the benefits of lower cost, safety, portability, high spatial and temporal resolution, and high sensitivity of detection of contrast agents, including nano- and microbubbles.[136] However, regardless of the modality, the imaging via nanoprobes could overestimate or underestimate the accumulation of nanomedicines because the mechanism of extravasation and uptake efficiency of imaging nanoprobes could be different from actual nanomedicines. Another issue with imaging is the difficulties of logistical and financial burdens of obtaining serial imaging sessions. Tumor biopsy biomarkers can potentially fill this gap, and it seems likely that a combination of tissue plus imaging biomarkers would be ideal for identifying patients who would benefit from nanomedicine treatment.[137]

Safety requirements vary across different clinical scenarios, and some medical interventions tolerate lower benefit/risk ratios. Therefore, the criteria for nanosafety must be formulated for each disease application, including its stage and phase and other patient-specific parameters such as age, gender, and current and previous conditions. Interestingly, a recent study suggests that sex and the timing of the menstrual cycle can affect liposome accumulation in reproductive organs and lead to off-target toxicities.[85]

Regulatory frameworks for nanomedicine are still evolving, and despite the recent FDA guidance on nanodrugs and liposomes[20,21] guidelines on safety and efficacy need to be expanded further, especially for novel materials. The safety and efficacy of nanomedicines need to be assessed following the protocols for a "new drug" because encapsulation of a known molecule often results in altered pharmacokinetic, efficacy, and toxicity profiles. Because of the complexity of nanomaterials, alternative strategies should be considered in coordination with regulatory authorities to accelerate the clinical development of nanomedicines with promising preclinical data. As a work-around, short studies at subtoxic doses may allow pharmacokinetic analysis, information on biomarkers (e.g., inflammatory response, complement activation), and even biodistribution and targeting (e.g., using PET-radiolabeled nanomedicines) to help select the best formulation for further development (1).

5. FUTURE DIRECTIONS IN NANOMEDICINE

Nanomedicine is, by definition, an application-based field, and its potential to deliver therapeutics, imaging agents, and nucleic

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Box 1. Education Aspect of Nanomedicine

Education in nanomedicine is often overlooked and was one of the discussion topics. One of the essential pieces of advice for young investigators is to look carefully at questions that arise during experiments. Students were encouraged to find a good mentor to help navigate these questions and support the young investigator in choosing where to focus their energy. For nanomedicine, interactions with the disease microenvironment, understanding the pharmacokinetics of the carrier nanoparticle and the drug payload, and efficient manufacturing are among the top questions for investigation. It was also advised to combine nanomedicine with other research areas to explore uncharted territories and pursue questions that transcend the boundaries of a single discipline. Previously successful examples of interdisciplinary research include but are not limited to nanoimmunoengineering, nanoinformatics, and nanochemical biology.

Publishing and reporting negative results in nanomedicine is essential, and many investigators felt that negative data could present an interesting start/pivot point. It was noted that all lab researchers encounter failures, and strategies to circumvent these failures often reveal counterintuititve directions of research and therapeutic approaches. The attendees also discussed the challenges of publishing negative results. Admittedly, one must be sure that the negative result is due to the science rather than the individual running the experiment. The student attendees expressed concern about where to publish negative data given the "publish or perish" and "significant impact or nothing" climate, but the advice from the established researchers was to pursue publication regardless. Several attendees were involved with various journals and reiterated the importance of sharing negative findings so that others avoid repeating the same mistakes or attempting projects with poor outcomes. It was pointed out that registered clinical studies must ensure that negative results are published. However, some counterarguments included that focusing on failures could cause readers to abandon attempts that achieve different results.

One issue noted was that the field needs more standardization to facilitate publication. Standardizing sections such as "Materials and Methods" would make submitting, reviewing, and reading articles easier for everyone involved. Essential details are often omitted, intentionally or not, and reviewers can miss such omissions. In this context, the information needed to reproduce experiments should be reported in the Methods section to alleviate reproducibility issues.[138] An example includes nanoparticle synthesis, where the standard should include protocols for volumes, flows, the geometry of the microfluidic chip, etc., to ensure accurate replication.

acids in the disease context is well acknowledged. **However, as in every other scientific field, combining good old-fashioned, hypothesis-driven basic research with application-driven engineering is critical to finding better clinical solutions.** This consideration should guide the future directions of basic and translational nanomedicine research discussed in the following section.

Computational modeling should be utilized more in developing better nanoformulations and optimizing the colloidal and pharmacological properties of existing nanomedicines. Coarse-grained molecular dynamics models can be developed to predict and elucidate the mechanisms regulating the release of small molecules from complex lipid and polymeric matrices and map the adsorption of proteins, antibodies, and molecules of the complement system on blood-borne nanomedicines.[139−141] Continuum mechanics and mesoscale models have been extensively used to predict the vascular and extravascular transport of nanoparticles and their interactions with blood cells.[111,142] However, it would be impractical to use purely mechanistic models to predict the formation of nanoparticles resulting from the self-assembly of various organic and inorganic elements, including lipids and polymers with various head groups, chemical terminations, and molecular weights. Machine learning tools, possibly instructed by physicochemical principles, could more efficiently handle the large number of independent variables governing such a problem and facilitate the identification of optimal therapeutic agent−material combinations to maximize efficacy.[143,144] Proper training of machine learning algorithms requires large data sets providing information on the size, polydispersity, loading, release, and cytotoxic performance of various nanomedicines under multiple configurations. In this context, high-throughput fabrication and characterization of nanomedicines using microfluidic-based systems, multiwell plate-based characterization tools, automated microscopy, and robotic platforms for sample handling are crucial as the amount of data required for machine learning cannot be easily generated with traditional manual operations.

High-throughput and massively parallel screening approaches (e.g., using CRISPR-Cas9 libraries) can identify pathways and receptors of nanoparticle uptake by immune and tumor cells.[145,146] Applying omics to nanomedicine remains a largely unexplored area and offers exciting opportunities in precision cancer therapy, rational nanoparticle engineering, and controlled nanoparticle distribution. The results of these findings may lead to the rational engineering of nanoparticles to interact with specific cells, which could alter their *in vivo* distribution for improved targeting.[129,147,148] In this context, pooled screening can identify genes involved in nanoparticle cell trafficking and toxicity, providing specific molecular mechanisms to guide the design of safer nanomaterials. One key advantage to pooled screening is the ability to simultaneously evaluate the impact of hundreds or thousands of perturbations (cellular, genetic, chemical), allowing for a rapid and unbiased approach to biomarker identification. There are also some caveats to pooled screening, including relatively high initial costs based on required time and reagents and the need for computational expertise to interpret results rigorously. Next-generation DNA sequencing of phage libraries combined with advanced proteomics may lead to the identification of panels of high-quality homing and penetrating peptides.[149] This, in combination with quantitative comparative studies on peptides as affinity targeting ligands, may provide the nanomedicine community with a broad spectrum of validated targeting ligands.

Alternative (non-i.v.) routes of administration for specific indications should be explored. Besides the obvious case of nanoparticle vaccines for subcutaneous, intradermal, and intramuscular administration, other examples include intracerebrospinal fluid injections for leptomeningeal metastases, intra-arterial injection for improved regional delivery to the brain and other organs, oral delivery for gastrointestinal pathologies, inhaled delivery for pulmonary infections and diseases, vaginal delivery for obstetric or gynecologic

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applications, intrauterine delivery for fetal therapies, topical application for skin conditions, and intranasal delivery for CNS therapies. These indications will require completely different design criteria, e.g., stability in solutions with varying salt, acid, and protein concentrations and under various fluid dynamics. These tissue microenvironments will also affect drug release, and the canonical physicochemical rules of nanomedicine optimization for intravascular delivery may not apply.

Regarding oncology applications, the fact that primary tumors are often resected suggests that the focus of nanoformulations should be redirected to models of metastatic disease to achieve improved efficacy in a low EPR environment. In specific clinical settings with poor outcomes or without an accepted standard of care, nanomedicine should also be tested in early disease, referred to as primary or neoadjuvant therapy, to obtain quick insight into efficacy and curative potential. As discussed above, a promising approach is to exploit the uptake of nanoparticles by immune cells for reprogramming and remodeling the tumor microenvironment, including TAMs, MDSCs, neutrophils, dendritic cells, cancer-associated fibroblasts (CAFs), endothelium, and extracellular matrix. Combining nanomedicines with therapeutics that modulate the tumor microenvironment (e.g., amino-bisphosphonates such as alendronate, stimulator of interferon genes protein (STING) agonists, toll-like receptors (TLR) agonists, and immune checkpoint inhibitors) is highly promising. For example, angiotensin receptor blockers normalize the tumor microenvironment and inhibit CAFs, thereby reducing collagen levels in the tumor microenvironment and improving the efficacy of chemotherapeutic drugs, nanoparticle-based drug carriers, and immune checkpoint inhibitors.[150]

Many ailments lack effective treatments, e.g., infectious diseases, ischemic heart disease, and neurodegenerative diseases. Organ transplantation represents a very attractive arena for the delivery of agents to prevent rejection and delayed graft failures, including inflammatory, autoimmune, thrombotic, and ischemia-reperfusion mechanisms. This situation would allow the administration of drug delivery systems prophylactically into a donor or in the procured organ, thereby acting during the period of highest vulnerability without the need for chronic dosing after the transplantation. Additionally, some chronic conditions involving fibrosis, chronic inflammation, autoimmune diseases, and neurodegenerative diseases lack effective treatment and might present good opportunities for nanomedicine. However, nanomedicines for chronic use may face challenges, including diminishing therapeutic activity and the progressive development of side effects, as shown for Doxil in aged mice as opposed to young mice.[39] Thus, using nanoparticles involving repetitive administration in the elderly, chronically ill patients must be approached with caution.

There is a currently unrealized potential for using nanomedicines in obstetrics.[151,152] The placenta is an organ and biological barrier that mediates the exchange of nutrients, oxygen, and waste between maternal circulation and the growing fetus. One of the main challenges in treating chronic and acute conditions during pregnancy is the fetal toxicity of drugs, which generally have low molecular weight and penetrate the placenta mainly by passive diffusion. Incorporating low molecular weight drugs in nanoparticles enables retention in the maternal circulation and minimizes fetal exposure.[153] While this provides tremendous potential for nanoparticle technology, work in this field requires appropriate models, including nonhuman primates, to mimic the human placenta accurately. Cells that comprise the placenta (trophoblasts, endothelial cells, immune cells) can be directly targeted using LNPs to deliver mRNA encoding vascular endothelial growth factor to treat pregnancy disorders such as pre-eclampsia.[154] Another application is *in utero* delivery of mRNA for correction of fetal diseases,[155] but the safety of this approach needs to be carefully considered.

The early phases of the conception, design, and assembly of nanomedicine candidates are usually driven by the material and pharmaceutical sciences, as well as chemical and biological engineering. The researchers tend to focus on the potential desirable features, while toxicities are sometimes overlooked. The individual components and the assembled particles must pass the safety barrier for use in humans. Testing nanoparticle toxicity in healthy animals and animal disease models is critical. Recent studies revealed unexpected and undesirable pro-inflammatory side effects of nanoparticles injected in animals with pre-existing low-scale inflammatory conditions.[156] These findings indicate that the topics of nanotoxicology, nanosafety, and nanoimmunology need more attention and provide an impetus to conduct animal studies investigating approaches to boost the safety features of nanomedicines.[157]

Due to extensive interactions with the body's internal milieu, some researchers are convinced that nanocarriers should be considered an active ingredient rather than an excipient.[158] In some cases, nanomaterials can exhibit drug-like properties,[159] such as anticancer properties and anti-inflammatory of unmodified gold[160] and silver[161] nanoparticles. In an entirely different application, the high hydration of the phospholipid head groups in liposomes improves lubrication and reduces cartilage wear in osteoarthritis. For this indication, the efficacy is highly dependent on liposomal physicochemical properties: large multilamellar liposomes (MLV) stay on the surface of the cartilage, but single laminar vesicles penetrate deep into cartilage due to 20−90 nm pores in the synovial membrane.[162] Another exciting example of nanomaterial repurposing is that of ferumoxytol, an iron oxide nanoparticle approved for treating iron deficiency.[163] The Fe_3O_4 nanocrystals are readily taken up by macrophages, which enables their use in off-label applications such as imaging agents for MRI and theranostics. As mentioned above, stimuli-responsive gas-core nano and microparticles can help with delivery issues and modify the disease microenvironment for therapeutic benefit. Considering the myriad interactions between nanoparticles and the physiological environment noted above, repurposing existing nanomedicine platforms presents significant opportunities.

While many groups focus on lipid-based drug delivery systems (such as liposomes and LNPs), the translation of other systems, such as dendrimer, protein, and polymer-based nanoparticles, as well as inorganic nanoparticles (e.g., gold and iron oxide), is in its infancy. These definitely should be explored for clinical translation. To pursue productive translational research in the realm of nanomedicine, clinicians have to be involved from the early conceptualization stages. This is essential to set forth the clinical need, medical goals, and mechanisms to intervene. Additionally, multidisciplinary teams of experts in material sciences, physics, computer modeling, biomarker discovery, and other fields must engage while paying equally meticulous attention to both the intended and unintended effects of the constructs under development.

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6. CONCLUDING REMARKS

Nanomedicines can reduce toxicity, stimulate the immune system, alter the disease microenvironment, and improve the delivery of multiple drugs in a disease setting. Additionally, nanoparticles have proven essential for the effective delivery of mRNA vaccines, representing a clinical market share that is rapidly evolving given the broad range of unmet clinical needs that may benefit from nucleic acid–based therapies combined with appropriate, patient-specific companion diagnostics. Different medical conditions offer unique challenges, and developing methods to selectively identify patients most likely to benefit from nanomedicines would improve clinical outcomes. **We should continuously promote basic research in nanomedicine, even without immediate patient benefits, and recognize that the foundation of translational research has a long path.**[164] After all, with liposomal doxorubicin, it took more than 20 years from the initial animal experiments with PEGylated liposomal doxorubicin until the clinical proof of the drastic reduction in cardiotoxicity of Doxil compared to free doxorubicin.[13,165] Understanding the mechanisms underlying the observed suboptimal clinical efficacy is critical to moving beyond incremental advances in safety and realizing the full therapeutic potential of nanoparticle-mediated therapeutics. The field continues to progress and learn lessons that will benefit future drug development.

AUTHOR INFORMATION

Corresponding Author

Dmitri Simberg − *Department of Pharmaceutical Sciences, The Skaggs School of Pharmacy and Pharmaceutical Sciences, the University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States;* orcid.org/0000-0002-5288-6275; Email: dmitri.simberg@cuanschutz.edu

Authors

Thomas Anchordoquy − *Department of Pharmaceutical Sciences, The Skaggs School of Pharmacy and Pharmaceutical Sciences, the University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States*

Natalie Artzi − *Brigham and Woman's Hospital, Department of Medicine, Division of Engineering in Medicine, Harvard Medical School, Boston, Massachusetts 02215, United States; Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02215, United States; Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts 02215, United States;* orcid.org/0000-0002-2211-6069

Irina V. Balyasnikova − *Department of Neurological Surgery, Feinberg School of Medicine, Northwestern University; Northwestern Medicine Malnati Brain Tumor Institute of the Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, United States*

Yechezkel Barenholz − *Membrane and Liposome Research Lab, IMRIC, Hebrew University Hadassah Medical School, Jerusalem 9112102, Israel*

Ninh M. La-Beck − *Department of Immunotherapeutics and Biotechnology, Jerry H. Hodge School of Pharmacy, Texas Tech University Health Sciences Center, Abilene, Texas 79601, United States;* orcid.org/0000-0002-1956-3263

Jacob S. Brenner − *Departments of Medicine and Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Warren C. W. Chan − *Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario M5S 3G9, Canada; Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada;* orcid.org/0000-0001-5435-4785

Paolo Decuzzi − *Laboratory of Nanotechnology for Precision Medicine, Italian Institute of Technology, 16163 Genova, Italy;* orcid.org/0000-0001-6050-4188

Agata A. Exner − *Departments of Radiology and Biomedical Engineering, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, United States;* orcid.org/0000-0003-3913-7066

Alberto Gabizon − *The Helmsley Cancer Center, Shaare Zedek Medical Center and The Hebrew University of Jerusalem-Faculty of Medicine, Jerusalem 9103102, Israel*

Biana Godin − *Department of Nanomedicine, Houston Methodist Research Institute, Houston, Texas 77030, United States; Department of Obstetrics and Gynecology, Houston Methodist Hospital, Houston, Texas 77030, United States; Department of Obstetrics and Gynecology, Weill Cornell Medicine College (WCMC), New York, New York 10065, United States; Department of Biomedical Engineering, Texas A&M, College Station, Texas 7784,3, United States*

Samuel K. Lai − *Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0003-4721-528X

Twan Lammers − *Department of Nanomedicine and Theranostics, Institute for Experimental Molecular Imaging, Center for Biohybrid Medical Systems, University Hospital RWTH Aachen, 52074 Aachen, Germany;* orcid.org/0000-0002-1090-6805

Michael J. Mitchell − *Department of Bioengineering and Penn Institute for RNA Innovation, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States;* orcid.org/0000-0002-3628-2244

S. Moein Moghimi − *School of Pharmacy, Newcastle University, Newcastle upon Tyne NE1 7RU, U.K.; Translational and Clinical Research Institute, Faculty of Health and Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4HH, U.K.; Colorado Center for Nanomedicine and Nanosafety, University of Colorado Anschutz Medical Center, Aurora, Colorado 80045, United States;* orcid.org/0000-0003-0836-926X

Vladimir R. Muzykantov − *Department of Systems Pharmacology and Translational Therapeutics, The Perelman School of Medicine, The University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Dan Peer − *Laboratory of Precision Nanomedicine, Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel; Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Center for Nanoscience and Nanotechnology, and Cancer Biology Research Center, Tel Aviv University, Tel Aviv 69978, Israel;* orcid.org/0000-0001-8238-0673

Juliane Nguyen − *Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0002-8578-7396

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Rachela Popovtzer − *Faculty of Engineering and the Institute of Nanotechnology & Advanced Materials, Bar-Ilan University, 5290002 Ramat Gan, Israel*
Madison Ricco − *Department of Pharmaceutical Sciences, The Skaggs School of Pharmacy and Pharmaceutical Sciences, the University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States*
Natalie J. Serkova − *Department of Radiology, University of Colorado Cancer Center, Aurora, Colorado 80045, United States*
Ravi Singh − *Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27101, United States; Atrium Health Wake Forest Baptist Comprehensive Cancer Center, Winston-Salem, North Carolina 27101, United States;* orcid.org/0000-0003-0750-1804
Avi Schroeder − *Department of Chemical Engineering, Technion, Israel Institute of Technology, Haifa 32000, Israel;* orcid.org/0000-0003-2571-5937
Anna A. Schwendeman − *Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48108; Biointerfaces Institute, University of Michigan, Ann Arbor, Michigan 48108, United States;* orcid.org/0000-0002-8023-8080
Joelle P. Straehla − *Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, Massachusetts 02115, United States; Koch Institute for Integrative Cancer Research at MIT, Cambridge, Massachusetts 02139, United States;* orcid.org/0000-0002-9446-1718
Tambet Teesalu − *Laboratory of Precision and Nanomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu, 50411 Tartu, Estonia*
Scott Tilden − *Department of Pharmaceutical Sciences, The Skaggs School of Pharmacy and Pharmaceutical Sciences, the University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.4c00182

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