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Nanomaterial Strategies for Delivery of Therapeutic Cargoes

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Over the last decade, much progress has been made in developing nanoparticle-mediated delivery systems to overcome the limitations of existing in vivo delivery technologies. However, the balance between efficacy and safety continues to limit the clinical translation of nanoscale delivery systems. Furthermore, optimizing delivery efficiency requires tuning nanoparticle type and attachment chemistry, both of which are dependent on the cargo being delivered. While the delivery of protein therapeutics is of particular interest, the complexity inherent to protein cargo introduces additional challenges for cargo loading and stabilization. Advances needed for efficient and safe in vivo nanoparticle delivery systems should prioritize design strategies that co-optimize safety, biodegradability, and covalent functionalization. In this review, the most commonly used non-viral nanoparticles are outlined for delivery of small molecule drug, nucleic acid, and protein therapeutic cargoes and potential strategies are discussed for rationally designing nanoparticle-mediated delivery systems.

1. Introduction

Challenges in drug delivery include poor biodistribution, lack of specificity, and off-target effects. Therapeutic efficacy is often limited by the presence of biological barriers, such as the immune system and endosomal escape, that hinder drug transport and result in less than 1% of the drug administered being delivered to the target site.[1,2] Nanoparticles (NPs) are nano-scale tailorable platforms that range in size from 1 to 100 nm and can be constructed from different materials such as polymers, metals, and lipids.[3] NP-mediated drug delivery provides a promising solution to the problems addressed above by protecting drugs from degradation, enhancing cellular internalization and transport properties, increasing drug solubility and loading capacity, providing spatiotemporal control through controlled drug release, and reducing side effects, thus improving drug delivery efficacy.[2,4,5]

Despite their potential advantages, current NP drug delivery systems are still limited by the trade-off between efficacy and safety. For example, lipid NPs are biocompatible with low toxicity, but often show low drug loading capacity. Conversely, carbon nanotubes (CNTs) have a high aspect ratio and thus surface area for drug loading but are non-biodegradable and are still being investigated for toxicity. Advancements in NP-based delivery will need to consider multiple design criteria including biodegradability, functionalization, internalization, and toxicity and provide a thorough assessment of long-term biological fate to inform the regulatory aspects over these technologies.[6] In this review, we first describe common nanocarriers for small molecule, nucleic acid, and protein-based cargoes, followed by the emerging trends for pairing NPs and cargoes. We then discuss functionalization techniques for NP-mediated drug delivery systems, focusing on the chemistry for cargo attachment and functional groups used. Lastly, we consider the safety implications of NP-based delivery of therapeutics and suggest considerations for NP usage in vitro and in vivo.

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2. Nanoparticles for Therapeutic Cargo Delivery

A broad range of NPs are available for therapeutic cargo delivery, but each comes with limitations. Such limitations should be considered when constructing a NP delivery system for a particular cargo and these limitations can be overcome by leveraging the distinct advantages each NP class offers. The main classes of NPs used for delivery and favorable pairing options for cargo delivery are summarized below.

2.1. Classes of NPs

An ideal NP system for cargo delivery should employ facile cargo conjugation, protect its cargo from degradation in vivo, exhibit low toxicity and immunogenicity, be effectively uptaken by cells, provide stability and endosomal escape, biodegrade on a timescale that avoids accumulation, and release its cargo at the target location.[7] Simultaneously achieving all figures of merit is challenging, thus NP design is typically approached

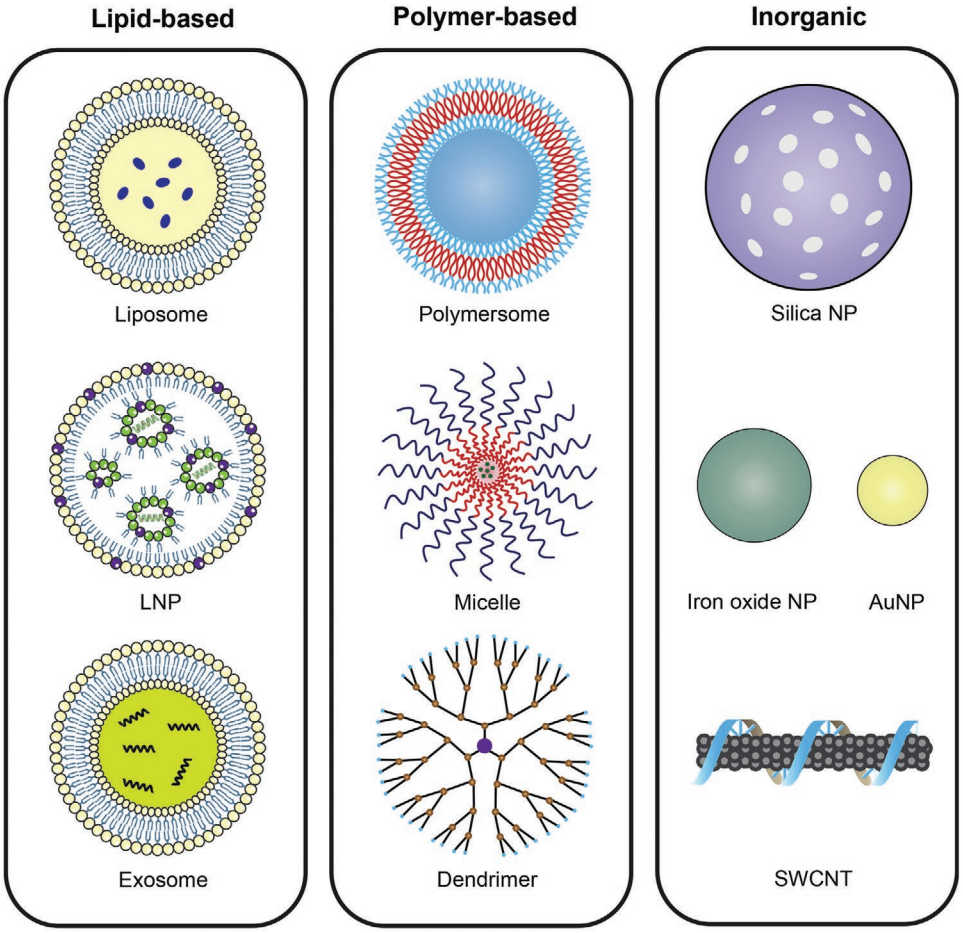

Figure 1. Schematic structures of NPs used for the delivery of small molecule drugs, nucleic acids, and proteins. Some images in this figure are adapted under the terms of a Creative Commons Attribution 3.0 Unported License. Copyright Servier Medical Art by Servier (http://smart.servier.com).

by considering the application and optimizing for the features most important therein. Because the structure of a nanocarrier is closely related to its advantages, in this section, the most commonly used NP classes are outlined by discussing their structures, advantages, and limitations (**Figure 1**).

2.1.1. Lipid-Based NPs

Lipid-based NPs are the most broadly used NPs for delivery in mammalian systems, with several lipid NP-based drugs on the market.[8] Their advantages include high bioavailability, biocompatibility, biodegradability, low toxicity, self-assembly, ease of surface modifications, and ability to carry a wide range of cargos that vary in size. However, the main drawbacks associated with lipid-based NPs include low encapsulation efficiency and limited systemic delivery to locations other than the liver.[8]

Liposomes, one of the most widely used types of lipid-based NPs, measure ≈30 nm through several micrometers. Liposomes are composed of a spherical assembly of phospholipid bilayers, providing the ability to simultaneously encapsulate hydrophilic, hydrophobic, and lipophilic cargo. Thus, liposomes are favorable for co-delivery of a broad spectrum of cargo such as proteins, oligonucleotides, and small molecules.[7,9] Their in vitro and in vivo stability can be improved using surface modifications involving the incorporation of ligands or polymers such as polyethylene glycol (PEG)[10] or chitosan.[11]

Lipid nanoparticles (LNPs), another subset of lipid-based NPs, range in size from 50 to 1000 nm and have structures similar to that of liposomes, but the main difference lies in the micellar structures formed in their cores. LNPs are typically composed of cationic or ionizable lipids that facilitate conjugation with cargo, phospholipids and cholesterol that provide membrane stability, and PEGylated lipids that improve stability and extend circulation.[8,12] Although permanently-charged cationic lipids were developed in early systems to overcome low cargo encapsulation limitations, their permanent positive charge was found to cause high toxicity and immunogenicity.[13] More recently, ionizable lipids have been developed and offer a modular alternative to permanently-charged cationic lipids. The ionizable lipid core of LNPs remains neutral at physiological pH but becomes protonated in the acidic environment of endosomes, thus allowing both endosomal escape and reduced toxicity.[12–14] Such advantages make ionizable LNPs especially promising for nucleic acid delivery, including the co-delivery of functionally distinct nucleic acid therapies.[8,12,15,16]

Exosomes are nanosized (30–120 nm) extracellular vesicles that are naturally secreted by cells to facilitate intercellular

communication. Exosomes are composed of a cell-derived lipid bilayer membrane that surrounds their hydrophilic core, which allows for encapsulation of hydrophobic and hydrophilic cargoes ranging from small molecules to oligonucleotides.[17] Exosomes are promising in vivo delivery vehicles due to their high biocompatibility, low immunogenicity, and intrinsic ability to cross biological barriers. However, the clinical translation of exosomes is limited by the lack of standardized techniques for their purification and challenges in their isolation from biological fluids.[17–19]

2.1.2. Polymer-Based NPs

Polymeric NPs, a class consisting of natural and synthetic polymers, range in size from 1 to 1000 nm and are widely used as delivery vehicles. Additionally, some polymers are also used as surface modification tools. Cargo can be encapsulated in their core, entrapped in the polymer matrix, or chemically conjugated to the NP surface or to the polymer itself.[8] Therefore, the facile synthesis and functionalization of polymers enables control over the NP's characteristics and flexibility in the types of cargo that can be loaded and delivered. Similar to lipid-based NPs, polymeric NPs can load a wide range of cargo, including hydrophilic and hydrophobic compounds that vary in size from small molecules to macromolecules like proteins.[8] Other advantages of polymeric NPs include biocompatibility and biodegradability.[20] However, polymeric NPs are limited by toxicity risks that are caused by aggregation or the interaction of highly positively charged polymers with blood.[8,21]

Two common forms of polymeric NPs are nanocapsules, which encapsulate cargo within their core, and nanospheres, which distribute cargo within their polymer matrix. Micelles are a type of polymeric NP that self-assemble to form nanospheres with a hydrophobic core and hydrophilic coating to protect cargo and provide stability.[8] Dendrimers are hyperbranched globular polymers consisting of an atomic or molecular core in the center and tunable branches and functional groups on the surface, which allow precise control over size, shape, and surface chemistry for cargo conjugation.[7] Cargo can be loaded either by encapsulation or conjugation to the dendrimer surface. One type of dendrimer that is well-studied is polyamidoamine (PAMAM). PAMAM has protonatable amine groups that enable escape from endosomal degradation, simple and flexible encapsulation, non-immunogenicity, and suitability for oral delivery owing to enhanced penetration of the gut epithelial barrier.[7]

Other polymers can be used to form NPs for delivery, such as chitosan, polyethylenimine (PEI), polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA). Chitosan is a natural polymer that is mucoadhesive, highly biocompatible and biodegradable with low toxicity, has high affinity to cell membranes, facilitates endosomal escape, and can load a wide range of cargos such as nucleic acids, anticancer agents, proteins, and antibiotics. However, chitosan is limited by poor solubility due to its low protonation percent at physiological pH.[7,22] PEI is a cationic synthetic polymer with a high positive charge that makes it favorable for the delivery of negatively charged drugs, nucleic acids, and bioactive molecules, and allows efficient cellular uptake. The main drawback associated with PEI is that its high positive charge leads to toxicity because negatively charged components in blood could form aggregates with PEI. Therefore, to find the balance between efficiency and toxicity, a trending approach is to coat PEI surfaces with PEG, chitosan, or other passivating molecules.[7] Other synthetic polymers that can form NPs include PLA, PGA, and PLGA, which have similar properties and advantages including high biocompatibility and biodegradability.[7] In particular, PLGA offers flexibility through adjusting its lactic acid to glycolic acid ratio.[23] Another polymeric NP type is polymersomes, which are artificial vesicles composed of amphiphilic block copolymers, resembling the structure of liposomes. Polymersomes are reported to have improved stability and cargo-retention efficiency, making them effective vehicles for the delivery of therapeutics to the cytosol.[8]

2.1.3. Inorganic NPs

Inorganic NPs are mostly used for research purposes in sensing and imaging and are sparsely used in clinical applications due to their inability to biodegrade. Inorganic NPs typically measure between 2 and 100 nm, with advantages including tunable optical, magnetic, and electric properties in addition to facile modification of their size and geometry.[8] Major drawbacks associated with inorganic NPs besides their non-biodegradability include low as-synthesized solubility and toxicity risks arising from the nature of their constituent heavy metals. Due to the toxicity risks associated with inorganic NPs, only biodegradable and nontoxic inorganic NPs are suitable for in vivo delivery purposes, whereas the unique optical properties of each inorganic NP can be explored in imaging and sensing. Iron oxide NPs, silica NPs, gold NPs (AuNPs), and carbon-based NPs such as carbon nanotubes (CNTs) are the most common nanoparticles of this class.

Iron oxide NPs comprise the majority of FDA-approved inorganic NP-based therapeutics.[8] Favorable properties of iron oxide NPs include biodegradability, biocompatibility, and unique magnetic properties.[23–25] Iron is an essential trace element, so iron transport pathways and iron homeostasis are well-understood, which leads to greater confidence in its low toxicity relative to other inorganic NPs whose toxicity and biodegradability are less understood.[23]

Silica NPs also exhibit biodegradability and low toxicity, but the unique advantage of silica NPs lies in their excellent tunability in physical features such as size (surface area and volume), shape, porosity (mesoporous silica NPs), and surface modifications such as conjugation to targeting ligands or imaging agents. Such flexibility in the potential physical and chemical modifications make silica NPs favorable for carrying various types of cargo.[24,26,27] However, the main limitation of silica NPs include a lack of understanding of their long-term stability in vivo.[24]

AuNPs are among the most studied inorganic NPs because of their unique optical and photothermal properties (arising from free electrons on the surface) that can be tailored through modifications in size, shape, structure, and composition.[8] While AuNPs are not biodegradable,[28] their inherent inertness

is believed to result in low toxicity.[24] Concerns regarding long-term biocompatibility and accumulation-based toxicity limit the use of AuNPs.[24,29]

Single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) are composed of cylindrically rolled graphene sheet(s) (one plane of covalently bound sp^2 hybridized carbon atoms) and have unique properties such as intrinsic fluorescence, high surface area, high mechanical strength, and electrical and thermal conductivity. Carbon nanotubes also have a high cellular uptake efficiency and high flexibility in the range of cargos that can be loaded onto their surface.[1,4] However, limitations for implementing carbon nanotubes include an incomplete understanding of their biocompatibility and their non-degrading nature that causes concern with regards to their bioaccumulation.

Other emerging inorganic NPs include metal-organic frameworks (MOFs) and quantum dots (QDs), particularly graphene QDs. MOFs are crystalline porous coordination polymers that are composed of inorganic metal subunits linked to organic ligands. As a result of their large surface area, highly ordered structure, and easily tunable pore size and shape, nano-sized MOFs offer flexibility in loading a wide range of cargoes.[30,31] QDs are inorganic semiconducting nanocrystals with high fluorescence intensity and photostability as well as broad excitation and narrow emission spectra, which make QDs especially promising for in vivo imaging. When integrated into drug delivery systems, QDs can provide real-time tracking in vivo. However, like most inorganic NPs, they are limited by their non-biodegradability and toxicity.[32] Graphene QDs are an attractive alternative to conventional QDs for delivery purposes because they maintain the favorable optical properties of QDs while simultaneously offering improved mechanical strength, greater biocompatibility, and lower toxicity.[33]

2.2. Cargo Types and Favorable Nanoparticle Pairings

Therapeutic cargoes vary in key properties such as size, shape, charge, and hydrophobicity. For a given cargo to be delivered efficiently, it should reside firmly within or onto its nanocarrier and the cargo-nanocarrier complex should be stable in vivo. Previous approaches in drug delivery relied upon the structural properties of both the cargo and potential nanocarriers to engineer a delivery platform that loaded cargo efficiently and provided adequate cargo-carrier stability. As a result, these systems leveraged noncovalent interactions between cargo and nanocarrier to ensure cargo stability. In this section, such approaches will be illustrated, and the previously discussed NP delivery options for each type of cargo will be discussed in the order of increasing cargo size, with which delivery becomes more challenging.

2.2.1. NPs for Small Molecule Delivery

The delivery of small molecules, organic compounds with low (<1 kDa) molecular weight, is well-studied and relatively well-understood because of their small size, ease of conjugation to the delivery system, higher cargo-carrier stability, and easily validated cellular uptake. For example, PEGylated liposomal doxorubicin (Doxil/Caelyx) was among the first FDA-approved nanomedicines (1995).[8] PEGylated PLGA NPs are also effective in delivering small molecules such as doxorubicin and paclitaxel.[21] Mesoporous silica NPs[26] and chitosan NPs[22] also attracted considerable attention owing to their advantages outlined in Sections 2.1.2 and 2.1.3.[34]

2.2.2. NPs for Oligonucleotide Delivery

The delivery of oligonucleotides is more challenging than delivery of small molecules because oligonucleotides cannot readily enter cells efficiently as they are hydrophilic, polyanionic, and large. As an additional consideration, oligonucleotides are highly susceptible to degradation in vivo. The challenges in the delivery of RNA therapeutics exemplify the oligonucleotide delivery challenge. RNA therapeutics have become increasingly important because of their easy design and ability to target any gene for post-transcriptional modification. RNA therapeutics range from smaller sizes such as antisense oligonucleotides (ASO), small interfering RNAs (siRNA), and microRNAs (miRNA), to larger messenger RNAs (mRNAs).

For the delivery of RNA therapeutics, LNPs are the most commonly used NP delivery system, and a trending approach involves ionizable LNPs that are positively charged at acidic pH and neutral at physiological pH to increase transfection efficiency as well as reduce toxicity.[13,14,35] Recently, LNPs played an important role in the clinical development of COVID-19 mRNA vaccines. Two FDA-approved mRNA-based vaccines BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) utilize LNPs to encapsulate and deliver mRNA encoding the spike protein of SARS-CoV-2.[36] Briefly, synthetically-produced mRNA coding for the SARS-CoV-2 spike protein is encapsulated with ionizable cationic lipids that assemble into a nanoparticle as detailed in a comprehensive review by Kathryn Whitehead and colleagues.[37] PEGylation of phospholipids enhances biocompatibility, and the lipid nanoparticles load mRNA via electrostatic attraction and promote endocytosis and subsequent cytosolic release of the mRNA cargoes.

Another delivery platform for RNA therapeutics is cationic polymers due to their ability to stabilize the negative oligonucleotide charge.[7] In particular, PEI is widely studied for its high transfection efficiency.[13] However, the emergence of anti-PEG antibodies[38] and the toxicity risks associated with PEI motivate the use of lower toxicity polymers for in vivo RNA delivery.[13] Polymer surface modifications were also found to increase transfection efficiency and stability in vivo as detailed in Section 3.

2.2.3. NPs for Protein Delivery

Proteins are arguably the most difficult molecular biology cargoes to deliver into cells, owing to their large size, nonuniform structure, and fragile tertiary structure needed for protein function, making it difficult to load, stabilize, and deliver proteins efficiently. The most widely pursued protein cargoes for delivery include antibodies for therapeutics, and

ribonucleoproteins (RNPs) such as Cas9/gRNA complexes for genome editing applications. These proteins demonstrate the difficulties that accompany large protein cargo sizes and complexities. For intracellular antibody delivery, lipid-based NPs are widely used because of their high biocompatibility and in vivo cargo stability, but the main drawback of using lipid-based NPs for protein delivery is their low encapsulation efficiency.[39] Among polymeric NPs, PLGA NPs are among the most used polymeric delivery platforms for antibody delivery due to their biocompatibility, biodegradability, and controlled cargo release. Chitosan NPs are especially promising for delivering anticancer antibodies because they exhibit antitumor activity by disrupting the cell membrane and inducing apoptosis.[39] For Cas9/gRNA RNP delivery, although commercial cationic lipids are available to deliver Cas9/gRNA RNP, their drawbacks have limited certain applications for gene editing.[40] Therefore, modifications such as incorporating permanently cationic lipids (e.g., DOTAP) into traditional ionizable LNPs have been employed to improve transfection efficiency and reduce toxicity.[40,41] Polymeric NPs such as PAMAM dendrimers and chitosan NPs have also been developed for intracellular RNP delivery.[42] Surface-loading of RNPs on nanoparticles for delivery is an alternative to RNP encapsulation. To this end, CRISPR–Gold was engineered by conjugating AuNPs with DNA, which were complexed with donor DNA, Cas9 RNP and the endosomal disruptive polymer poly(N-(N-(2-aminoethyl)- 2-aminoethyl)aspartamide)(PAsp(DET)) for in vivo genome editing.[43]

3. Attachment Chemistries for Nanoparticle Cargo Delivery Systems

NPs leveraged in recent years to deliver therapeutic cargo have harnessed various functionalization strategies to improve cargo delivery efficiency. As detailed in Section 1, early systems relied on noncovalent adsorption onto or encapsulation within nanoparticles to increase delivery efficiency compared to free cargo. Though these systems demonstrated the beneficial effects of nanoparticles as cargo carriers, they showed modest delivery efficiencies coupled with unpredictable in vivo properties that depended heavily on the properties of the nanoparticle and cargo in question.[44] Recently, researchers have shifted toward rationally designing NP-cargo systems to improve their delivery efficacy.[45] Incorporating surface chemistry modifications such as carboxylation to covalently attach cargo of interest prevents premature cargo release and negative off-target effects. Attaching functional groups such as targeting moieties, cell-penetrating peptides, and PEG can increase target site accumulation, enhance cellular uptake, and lengthen in vivo circulation times, respectively. Researchers have also developed environment-responsive nanoparticle systems to take advantage of the dynamic nature of in vivo systems, responding to stimuli to modulate the aforementioned functionalization strategies as necessary to release therapeutic cargo, alter NP properties like size, or otherwise benefit delivery. Compared to earlier passive systems that rely on nonspecific adsorption of cargo and intrinsic material properties to promote biocompatibility, rationally-designed nanoparticles can be more efficient and effective as therapeutics when responsive attachment chemistries are applied to covalently attach both cargo and functional groups (**Figure 2**). Recent studies towards these ends are summarized in the sections below.

3.1. Nanoparticle-Cargo Conjugation Chemistry

Recent methods for covalent cargo attachment to nanoparticles have been as diverse as the nanoparticles themselves. Potential therapeutic cargoes delivered through covalent attachment chemistries include small molecule drugs like doxorubicin, oligonucleotides including siRNA, and proteins such as Cas9 RNP. The variability in cargo properties such as hydrophobicity, size, and available functional groups has motivated researchers to use a wide range of chemistries to effectively attach and deliver therapeutics. Additionally, the nanoparticle surface may lend itself towards a certain type of attachment chemistry if its structure favors certain modifications, as discussed in Section 2. NPs applied in biological systems are also exposed to complex environments that vary in pH, redox potential, and biomolecule concentration and composition. Researchers have leveraged these properties by functionalizing nanoparticles to respond to environmental and external stimuli to delay cargo release and thus reduce off-target effects. This section highlights these recent trends in cargo and responsive attachment chemistries for nanoparticle-based drug delivery systems.

3.1.1. Small Molecule Drug Attachment

Small molecule therapeutics typically exhibit limited half-lives in circulation which can require high systemic doses to achieve therapeutic effects.[46] Researchers have sought to solve this problem by developing nanoparticle systems that both shield the drug from clearance and degradation and deliver it to its cellular target.[47,48] Early efforts focused on synthesizing nanoparticles in solution to encapsulate the drug in the interior of the particle, or by noncovalently passivating the drug on the nanoparticle's surface.[46,49,50] Although these methods successfully shielded cargo from degradation and rapid clearance, they also suffered from off-target effects due to premature cargo release and low efficacy stemming from inefficient target site accumulation. Further, the hydrophobic nature of most small molecule therapeutics limits the types of nanoparticles that can be used as delivery vehicles to those with hydrophobic interiors or surfaces such as liposomes[51] and graphene oxide,[52] as detailed in Section 2.2.3.

Recent work has moved to mitigate these issues by rationally designing nanoparticles to covalently conjugate cargo, resulting in better spatiotemporal control over drug release and increased target site delivery efficiency. N-hydroxysuccinimide (NHS) conjugation chemistry has been used the most in recent years to conjugate small molecules to nanoparticles.[53,54] For example, You et al. conjugated the anticancer drug doxorubicin (DOX) to carboxylated mesoporous silica nanorods via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/NHS conjugation.[53] They showed substantial improvement in key parameters such as DOX half-life, maximum observed concentration, and clearance rate in in vivo mouse models, favorable

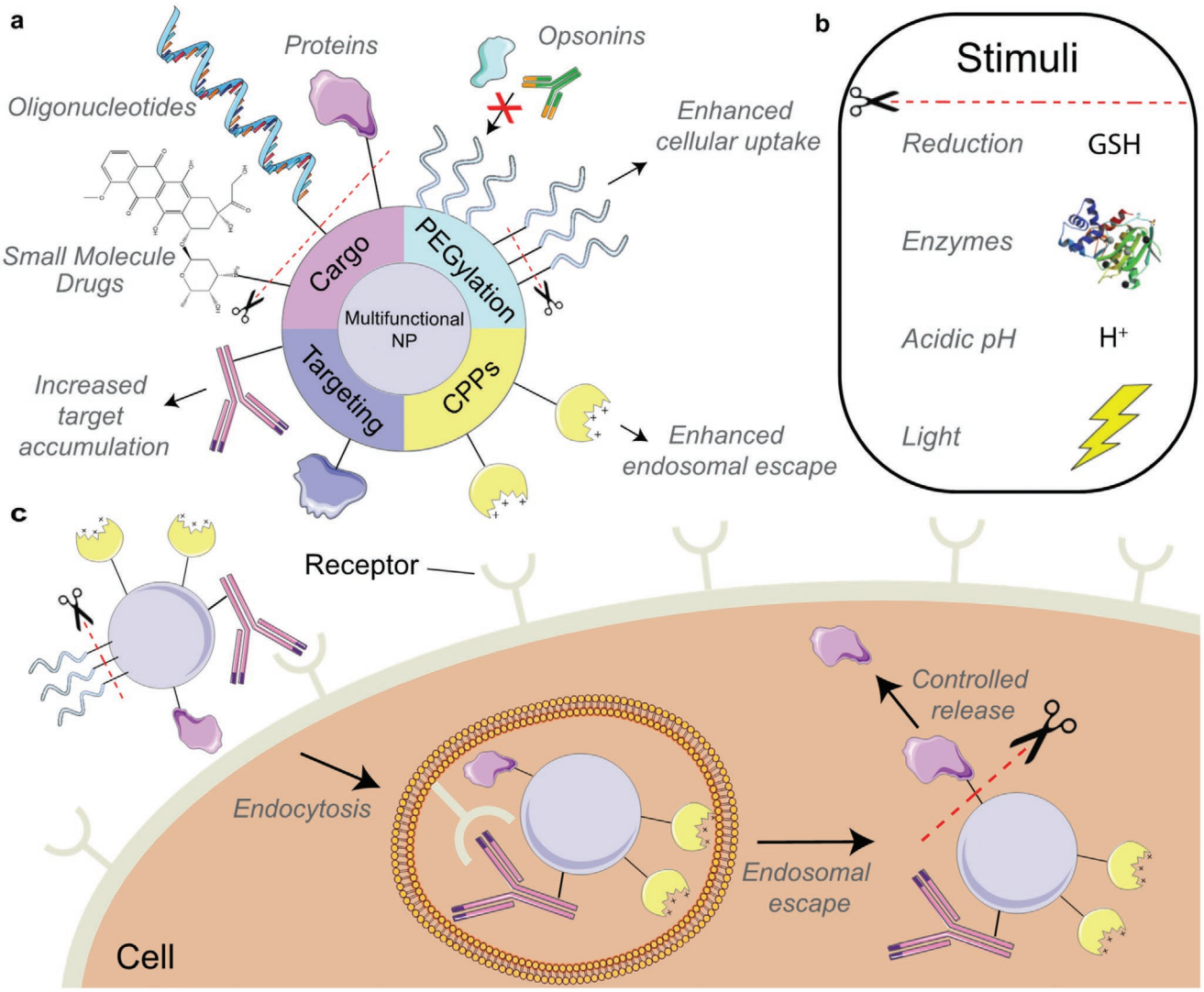

Figure 2. Multifunctional nanoparticles for cargo delivery. a) Strategies for covalent functionalization of nanoparticles with PEG, targeting moieties, and CPPs for enhanced delivery efficiency. b) External and internal stimuli for controlled release of indicated functional groups. c) Schematic of multifunctional nanoparticle cargo delivery. The nanoparticle is targeted toward specific receptors overexpressed on target cells and controlled shedding of PEG groups increases cellular uptake. Once endocytosed, CPPs can enhance endosomal escape, and controlled release chemistry enables cargo to be secured until delivery to the cytosol. Overall, delivery efficiency is increased compared to nonfunctionalized nanoparticles. Some images in this figure are adapted under the terms of a Creative Commons Attribution 3.0 Unported License. Copyright Servier Medical Art by Servier (http://smart.servier.com).

biodistribution and accumulation in tumors, and reduced tumor volumes compared to free DOX. These benefits were attributed to the covalent conjugation of the drug to the nanoparticle to prevent premature drug release and incorporation of folic acid as an effective targeting molecule towards carcinoma cells to increase cellular uptake.

Though widely used, NHS conjugation chemistry to link drugs to nanoparticle carriers is limited to a select group of drugs since it relies on availability of a non-essential primary amine in the small molecule structure to serve as a conjugation handle. Functionalizing small molecules with other reactive groups such as thiols circumvents the need for direct NHS conjugation.[55,56] Aires et al. functionalized the cancer therapeutic gemcitabine with a reactive pyridine-disulfide group, which enabled conjugation to thiol-functionalized iron oxide magnetic nanoparticles, enabling drug release within and selective uptake by CD44-positive cancer cells.[55] Thiol chemistries provide the added benefit of being responsive to glutathione (GSH), which is a reducing agent present in abundance in cellular cytosol. This concept was used by Huang and coworkers to conjugate DOX to a mPEG-PLGA linker adsorbed to graphene oxide, which showed increased DOX release in vitro in the presence of dithiothreitol (DTT), a strong reducing agent.[56] Other examples of stimuli-responsive attachment of small molecule drugs have been demonstrated in recent years,[57–59] highlighting the improved control over delivery these chemistries can provide.

It is possible that such chemical modifications to other small molecule drugs might alter the drug's key properties such as hydrophobicity and interfere with the drug's mechanism of action, but further studies are needed to determine the possible modifications for each drug to improve drug delivery without negating therapeutic effect.

3.1.2. Oligonucleotide Attachment

Delivery of oligonucleotide therapeutics such as plasmid DNA, siRNA, and mRNA has drawn much attention in recent years to leverage existing cellular machinery for gene therapy and transient protein expression.[60] However, free oligonucleotides are subject to rapid enzymatic degradation when used in vivo. Further, the negative charge inherent to the phosphate backbone of oligonucleotides results in poor cellular entry, degradation by nucleases, and toxic side effects when delivered to cells alone.[61] Researchers have used this negative charge to complex oligonucleotides to nanoparticles that are either inherently cationic or functionalized with cationic moieties such as PEI.[62–64] However, the presence of other ions in the highly dynamic in vivo environment could interfere with the stability of this electrostatic bond, and cationic molecules themselves show toxicity and induce an immune response in vivo, limiting their use for oligonucleotide delivery strategies.[65]

Covalent modifications to oligonucleotides can aid their delivery by providing a more stable linkage between the nanoparticle and oligonucleotide cargo. Oligonucleotides are large compared to small molecule therapeutics and thus provide more possible conjugation sites for delivery. Unfortunately, traditional chemical modifications to the nucleotide bases of siRNA could adversely affect downstream gene silencing efficiency by interfering with siRNA binding to the RNA-Induced Silencing Complex (RISC), preventing mRNA cleavage.[66] One solution to this problem involves sulfhydryl labeling of the 5' end of siRNA.[67–69] Conde and colleagues leveraged the natural affinity of thiol groups for gold nanoparticles (AuNPs) to deliver thiol-modified siRNAs to colon cancer cells.[67] By modifying the terminal end of the siRNA with a thiol group, the integrity of the siRNA sequence is preserved and enables silencing of a gene responsible for signal transduction in colorectal cancer. Although proven with reduction-responsive chemistries, covalent conjugation of siRNAs to nanoparticles for controlled release via other stimuli is an unexplored and promising direction toward improved oligonucleotide therapeutics.

Another oligonucleotide of interest for therapeutic applications is plasmid DNA (pDNA) delivery. Since pDNA is typically circular and thus contains no terminal end to serve as a conjugation handle, the most common delivery methods rely on either cationic moieties[62,70,71] or encapsulation.[72,73] However, Beals et al. demonstrated a strategy for plasmid delivery by incorporating a biotin group into the sequence of an EGFP plasmid for covalent conjugation to streptavidin-functionalized gold nanoparticles.[74] Since the specific sequence and location chosen to incorporate the biotin group did not interfere with functional gene elements, effective adaptation of this system for other plasmids would require the identification of a similar sequence/plasmid region. Though difficult, this advancement could enhance the delivery of plasmids that express proteins of interest such as the CRISPR/Cas9 gene editing system.

3.1.3. Protein Attachment

Protein therapeutics such as monoclonal antibodies, cytokines, and peptide hormones have been developed in recent years for disease treatment and transient manipulation of cellular function.[75] Protein delivery can circumvent the limitations of oligonucleotide delivery such as permanent genetic alteration by genome insertion, off-target effects due to sustained gene expression, and carcinogenesis.[76] Despite these advantages, delivery of protein therapeutics suffers from limitations stemming from the complexity of proteins including fragile tertiary structure, susceptibility to enzymatic degradation, and poor endosomal escape once internalized.[77] These limitations have been overcome through nanoparticle-mediated delivery as reviewed extensively elsewhere.[42,78–80] However, similar to oligonucleotide and small molecule drug delivery, nanoparticle-based protein delivery systems have relied upon noncovalent protein adsorption or encapsulation.[43,81] Though proteins have been shown to adsorb well to many exposed nanoparticle surfaces in vitro, proteins are likely to rearrange, denature, and desorb when subjected to the dynamic and complex protein environment in vivo.[82] Covalent protein conjugation to nanoparticles via a hydrophilic spacer such as PEG has been shown to be an effective way to preserve protein structure during nanoparticle attachment.[67]

Unlike oligonucleotides, proteins are more amenable to covalent conjugation due to the presence of amino acid side chains like lysine and cysteine that can serve as conjugation handles to react with NHS or maleimide, respectively. However, the number and location of these side chains must be considered before employing covalent chemistries. For example, if a lysine is positioned too close to the substrate binding pocket of a protein, its use as a covalent conjugation handle would render the protein inactive once released from the nanoparticle by unexpected steric hindrance. Furthermore, many proteins such as human monoclonal antibodies rely on disulfide bridges within their tertiary structure for stability. Maleimide chemistry could interfere with these disulfide bonds and lead to the loss of binding or enzymatic activity by denaturation. Site specific conjugation has been implemented to covalently bind proteins for analyte sensing,[83] but similar applications for protein delivery are less developed and should be emphasized in the future.

Due to the importance of maintaining protein tertiary structure for enzymatic activity once delivered, there is much potential for improvement in protein delivery strategies by rationally designing covalent protein-nanoparticle conjugates. For most applications, there must be an accompanying release mechanism to ensure the protein can function without additional steric hindrance from a still-attached nanoparticle. Stimuli-responsive chemistries have thus been employed for controlled protein release.[84–86] Tian et al. demonstrated this concept by conjugating bovine serum albumin (BSA) to aldehyde-functionalized mesoporous silica nanoparticles. The reaction between primary amines on BSA and the aldehydes on the nanoparticle

surface formed an acid-sensitive imine bond which enabled protein release at pH 6.[84] This principle could be extended to conjugate other proteins with linkers responding to different stimuli such as reducing environments, near-infrared light, and enzymatic cleavage.[87] Taken together, these design parameters of using site-specific, covalent, and stimuli-responsive chemistries for nanoparticle conjugation could enhance the delivery efficiency of proteins with unusual characteristics such as large molecular weight, high net charge at physiological pH, or complex quaternary structures.[42,88,89]

3.2. Functional Groups for Improved Delivery

Nanoparticles designed and optimized in vitro are presented with an array of challenges when used in vivo. In particular, biomolecules such as peptides and proteins are shown to adsorb to the surface of nanoparticles and form a layer referred to as the protein corona. This phenomenon can alter predicted properties such as stability and half-life, which in turn negatively affects nanoparticle in vivo cargo delivery efficiency.[87] Additionally, non-functionalized nanoparticles can be subject to rapid clearance by the immune system and unintended cargo release, leading to insufficient accumulation of cargo at the target site. Even if the nanoparticle system efficiently reaches the target site and is taken up by the intended cells, poor endosomal escape remains a barrier to cargo delivery of all kinds.[77]

To mitigate the detrimental effects of protein corona formation, researchers have conjugated PEG to their nanoparticles. However, PEGylation negatively affects cellular uptake due to the resulting large increase in NP size, and there is also evidence that PEG polymers can induce immunogenicity.[38,90] Since PEG primarily functions in circulation by preventing adsorption of immune system marker proteins called opsonins, PEG is of limited utility once the nanoparticle has reached its target to be uptaken by cells. It is thus advantageous to conjugate PEG to nanoparticles with controlled release chemistry, allowing researchers to shed the PEG layer and aid cell uptake. Useful extracellular release triggers include the acidic tumor microenvironment (pH ≈ 6.5)[91] and enzymes such as matrix metalloproteinase-9.[92] When combined with controlled release of cargo, as exemplified by Han and coworkers,[93] PEG shedding can greatly improve nanoparticle cargo delivery.

Researchers have similarly sought to functionalize nanoparticles with targeting moieties including small molecules like folic acid,[53,56] hyaluronic acid,[94,95] galactose,[96,97] peptides[67,72] like RGD,[58,59,93] and proteins such as antibodies.[55] These groups bind to receptors that are overexpressed on the surface of their target cells compared to normal cells, leading to preferential uptake within the target cell. Like the cargo they are helping deliver, these targeting groups benefit from covalent conjugation to nanoparticles to prevent unintended desorption, which renders them ineffective. Since these groups vary widely in size and available conjugation handles, there is no single chemistry most commonly used for attaching targeting groups.

Another category of functional groups used to improve delivery efficiency are cell-penetrating peptides (CPPs). The use of these peptides has been reviewed extensively,[98–100] and researchers are continually discovering and synthesizing new CPPs to aid cargo delivery. Most CPPs are cationic and function by disrupting the endosome, which is formed after endocytosis of the nanoparticle. Without the aid of CPPs, most nanoparticles struggle to escape from the endosome and are either trafficked to the cell surface and expelled or degraded when the endosome matures into the highly acidic lysosome.[101] Since the cargo discussed in this perspective mainly function intracellularly, CPPs are a key component in increasing their delivery efficiency.[67,73,92] Taken together, nanoparticle functionalization with PEG, targeting ligands, and CPPs can lead to a new generation of nanoparticles with better cargo delivery capabilities.

4. Safety

Despite the advances in NP-mediated delivery as discussed previously, cytotoxicity and genotoxicity still prevent the widespread application of NP delivery systems. NPs can enter into the body through inhalation, ingestion, skin, or by clinic injection, therefore affecting biological environmental responses such as cellular uptake and stimulation of the immune system (**Figure 3**).[87,102] Functionalization is one approach to reduce toxicity, but potentially comes with the trade-off of decreased efficacy or loss in activity.[1] Based on all of the considerations above, we highlight basic ethical considerations for the use of NP-based delivery systems in terms of their clinical versus experimental use and discuss the public perception of their safety.

4.1. Safety Concerns

One main limitation that hampers NP-based drug delivery systems is safety. Highly toxic cargo, such as anticancer agents that are used to kill tumor cells, are commonly delivered by nanocarriers, in which case nano-encapsulation may aid the drug's safety profile. In other cases, the nanocarrier itself may generate more toxicity than the free cargo, as a trade-off for better cargo delivery and therapeutic efficacy. Furthermore, cytotoxicity and genotoxicity of the NPs themselves could lead to accumulation in tissues, induce oxidative stress, increase inflammatory factors, mitochondrial stress, DNA damage, and structural changes in membrane proteins thus disturbing substance transport.[1,7,102] For biomedical reasons, both the NPs and the degraded products need to be biocompatible and nontoxic to reduce the inflammatory response from the immune system.[21]

The immune system induces a response when it recognizes foreign objects such as bacteria and viruses to remove them from the system. It is divided into the innate (non-specific) immune system and the adaptive (specific) immune system.[104] When used as drug carriers, nanoparticles can interact with the immune system through 1) complement activation, in which NPs activate the complement innate immune system by the classical, lectin, or alternative pathway; 2) immunostimulation, in which NPs are used to stimulate beneficial immune responses to harness the immune system to combat diseases or deficiencies and 3) immunosuppression, in which NPs are functionalized to reduce inflammatory effects.[87]

In vivo experiments on lab animals and in vitro experiments in cell culture are the main approaches for studying NP toxicity

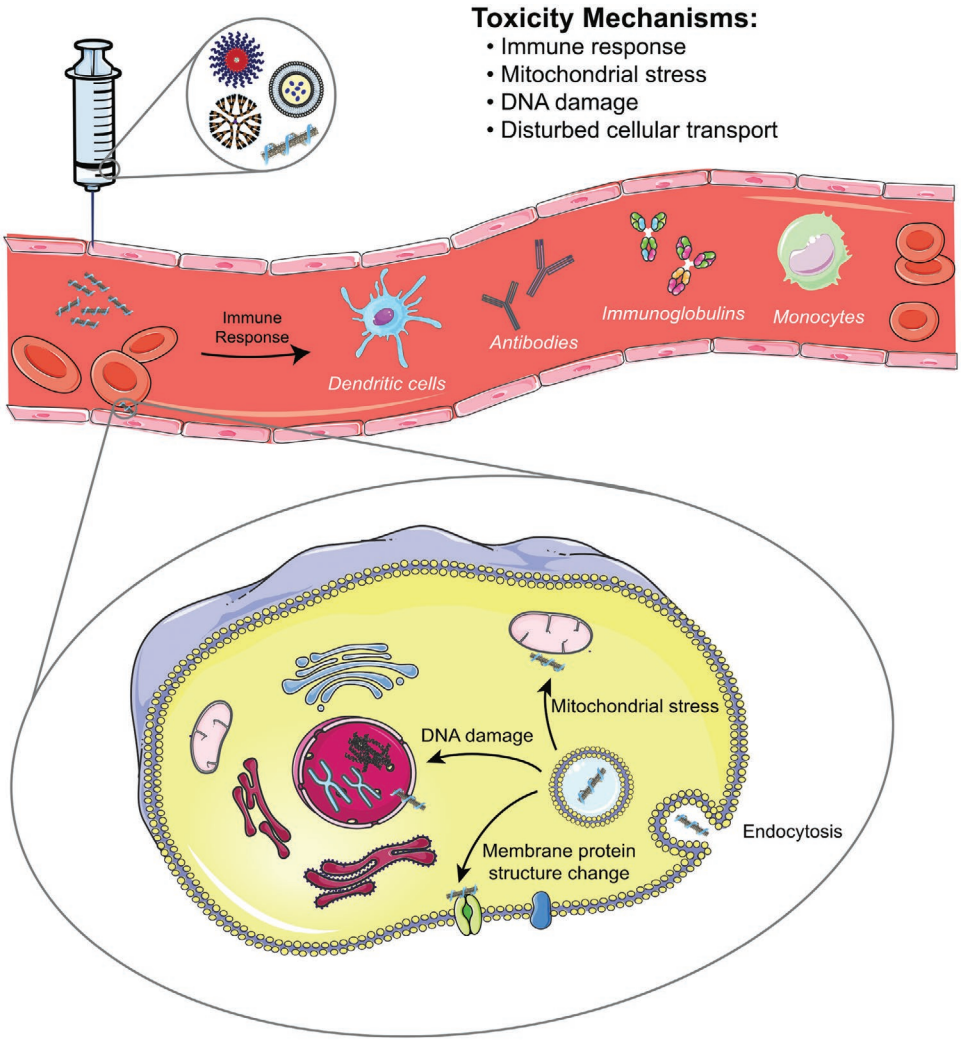

Figure 3. Safety considerations for nanoparticles. After administration, NPs can induce immune responses in the human body through interactions with components in the blood such as immunoglobulins, antibodies, dendritic cells, and monocytes.[103] NPs can also elicit an immune response by damaging cells through mitochondrial stress, DNA damage, and disrupting the structure of cell membrane proteins.[6] These inflammatory responses are unwanted and could be suppressed by rational nanoparticle design. Some images in this figure are adapted under the terms of a Creative Commons Attribution 3.0 Unported License. Copyright Servier Medical Art by Servier (http://smart.servier.com).

profiles.[105] Specifically, the toxicity and immune activation induced by NPs are quantified by cytotoxicity assays. It has been reported that cell viability assays, apoptosis quantification, cell cycle analysis (MTT, LDH, Trypan Blue, CCK-8), and ROS production assays are useful methods to assess cytotoxicity of nanoparticles.[106] However, limitations of the above assays include: 1) traditional cell viability assays such as MTT can produce false positive results; 2) nanoparticles are reported to interfere with assays and lead to inconsistency between in vivo and in vitro responses. To improve the accuracy of the assays, it is important to include both positive and negative controls, repeat multiple technical and biological replicates, and run several orthogonal assays.[4,87]

One solution to the toxicity problem is NP functionalization as discussed in Section 2. The cytotoxicity of nanoparticles is related to size, shape, charge, chemical composition, and crystal structure,[105] and those properties are tunable with different functionalization techniques. Literature reports various examples of successful functionalization strategies that both increase therapeutic efficacy and reduce toxicity, but it is also possible that cytotoxicity increases following functionalization.[1] For example, PEGylation can enhance therapeutic efficacy and reduce cytotoxicity by masking charge-based toxicity and thus prolonging blood circulation of the cargo,[7] but can also lead to the development of anti-PEG antibodies that help clear PEGylated NPs from the body faster.[38] PEGylated biopharmaceuticals have been well-studied and summarized elsewhere[107,108] with key takeaways including a call for genotoxicity studies of PEG and emphasis on the stability of covalent bonding between PEG and NPs.[108]

Additionally, biocompatible and biodegradable materials are good alternatives for toxic nanomaterials that can lead to inflammatory effects. For example, polymers such as liposomes, chitosan, and dendrimers all share properties of

high biocompatibility, biodegradability, and low toxicity, and are widely used in drug delivery systems.[7] For instance, Pandey and Sawant reported that Polyethyleneimine-graft-chitosan (PEI-g-chitosan) demonstrated significantly lower toxicity than PEI in HeLa cells with an MTT assay.[109] Synthetic, non-biodegradable polymers such as poly(methyl methacrylate) can also be replaced with biodegradable ones such as PLA and PLGA.[110]

4.2. Ethical Considerations

From previous sections, we observe the trade-off between efficiency and safety/biocompatibility of nanoparticle-based delivery systems. Natural polymers such as chitosan are biodegradable but potentially less-efficient due to low mechanical strength and exposure to biofouling.[6] We propose that, despite limitations of low stability, low drug-loading capacity, and limited potential for functionalization, biodegradable nanoparticles remain more suitable for clinical use than potentially non-degradable nanoparticle systems. While the latter may show increased efficiency, they are best utilized in laboratory experimental studies and in vivo in model organisms until their long-term safety profiles are fully investigated. It is worth mentioning that both chitosan and CNTs are reported to display varying degrees of toxicity with different chemical modifications and biodegradability does not necessarily guarantee non-toxicity.[110] Nanoparticles that have less well-understood toxicity profiles, show potential for accumulation, and induce inflammatory responses should be limited to lab use where their generation and disposal can be controlled in a closed lifecycle.

4.3. Public Perception of NP-Based Therapeutics

Adoption of nanotechnology in healthcare and clinical practice has been met with excitement and trepidation. An important factor in the successful development and deployment of NP-based medicine is the public's perception of their safety. This relies on the degree to which officials both keep the public informed of the technology and identify the public's concerns and their driving factors. A common misconception is that the use of nanoparticles is a new concept; in reality, nanoparticles have contributed to environmental exposures through the air, water, and food. Furthermore, there are currently over 50 FDA-approved nanomedicines, most of which are based on liposomes as cargo encapsulants.[111] Most recently, with the emergence of the COVID-19 pandemic, the FDA granted emergency use authorization (EUA) to several vaccines, including the two lipid nanoparticle-based mRNA vaccines from Pfizer-BioNTech and Moderna discussed in Section 2.2.2. Despite their efficacy, there has been hesitancy from populations across various countries throughout the European Union and North America, with vaccine hesitancy and resistance representing between ≈30% and 50% of these populations.[112,113] Estimates for achieving herd immunity prior to the rise of the Delta variant were estimated at 67%,[114] which may be difficult to achieve with current vaccine hesitancy statistics and viral strain evolution. In the United States, the perception of the Pfizer/BioNTech and Moderna vaccines as "new" and nanoparticle-based could be driving hesitancy, which may be assuaged by the recent FDA approval of the Pfizer vaccine. Public concerns remain that drive vaccine hesitancy, including efficacy against SARS-CoV-2 viral variants and largely unfounded concerns over the long-term side effects of these vaccines. These unfounded concerns are largely driven by a lack of understanding over the rigor that has gone into the development of COVID-19 vaccines, with social media disproportionately propagating erroneous information on the lack of vaccine safety.[115] Such misinformation negates efforts from healthcare experts to assuage public concerns and correctly inform the public. Both in the context of the ongoing pandemic, and more broadly with the development of future nanoscale therapeutics, public perception of nanoscale clinical technologies will require more consistent and open communication between the scientific community and the public.

5. Conclusion

Nanoparticles are attractive as vehicles for delivering therapeutic cargoes such as small molecule drugs, oligonucleotides, and proteins. To efficiently explore and develop these systems, researchers can narrow the large parameter space by adhering to the principles outlined in this perspective, which can be optimized based on each intended application. Specifically, depending on the properties of the cargo being delivered, nanoparticles can be chosen to pair with cargo that interact well with their intrinsic material characteristics including charge, size, and surface chemistry. Nanoparticles made of biodegradable or biologically inert materials are generally preferred for clinical applications while materials with less well understood toxicity profiles should be investigated prior to their use in vivo.

Researchers can further focus on covalently functionalizing nanoparticles with functional groups including targeting moieties to mitigate off-target effects, PEG to improve pharmacokinetic properties, and CPPs to enhance endosomal escape, all of which can increase cargo delivery efficiency. Additionally, leveraging stimuli-response chemistries to attach groups such as PEG or therapeutic cargo can provide beneficial spatiotemporal control over nanoparticle activity, further enhancing cargo delivery efficiency. In sum, rational design of nanoparticle-mediated cargo delivery systems can improve their function and accelerate the development of safe and efficient therapeutics for human health.

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Conflict of Interest

The authors declare no conflict of interest.

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