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Towards clinical translation of nanomedicines: Formulation scale-up and model systems

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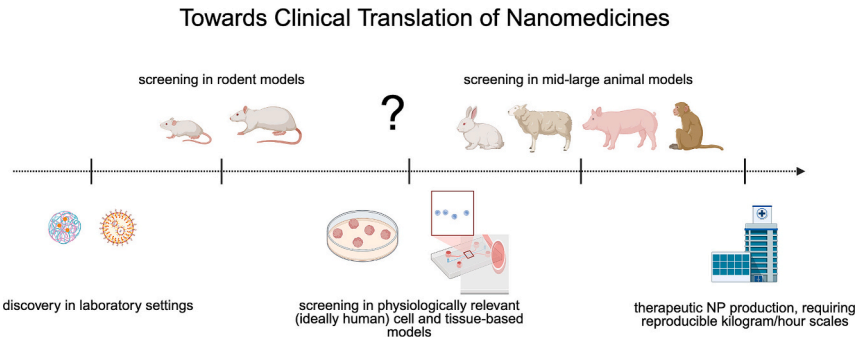
HIGHLIGHTS

- Scale-up from lab to commercial manufacturing remains a key barrier for nanomedicines.
- Formulation methods should be chosen for reproducibility and scalability to meet translational goals.
- Mid- to large-size animal models may be more representative of NP behavior in humans than rodent models.

GRAPHICAL ABSTRACT



Abbreviations: BBB, blood-brain barrier; CARPA, complement activation-related pseudoallergy; CETP, cholesteryl ester transfer protein; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CIJ, confined impinging jets; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; CT, computed tomography; CV, coefficient of variation; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked-immunosorbent assay; GMP, Good Manufacturing Practice; HFF, hydrodynamic flow focusing; HPH, high-pressure homogenization; HSC, hematopoietic stem cells; IV, intravenous; IVT, in vitro transcription; LNP, lipid nanoparticle; NAMs, new approach methodologies; MIVM, multi-inlet vortex mixer; MPS, mononuclear phagocyte system; MRI, magnetic resonance imaging; NOAEL, no observed adverse effects level; NP, nanoparticle; NPC, neural precursor cell; PACE, poly(amine-co-ester); PCLSs, precision cut lung slices; PDI, polydispersity index; PEG, polyethylene glycol; PEI, polyethylenimine; PLGA, poly(lactic-co- glycolic acid); PMMA, poly(methyl methacrylate); PNP, polymeric nanoparticle; QA/QC, quality assurance/quality control; Re, Reynolds number; TPP, sodium triphosphate.

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ABSTRACT

Non-viral nanomedicines, including nanoparticles (NPs) composed of lipids and polymers, represent a transformative approach to drug and gene therapy. However, clinical translation of these technologies is limited by two key barriers: the scale-up of NP formulations and the challenge of conducting predictive preclinical studies in relevant animal models. Efficient upscaling of nanomedicines, from cost and material requirement perspectives, requires manufacturing processes that can reliably provide products across the many orders of magnitude of scale from discovery (<mg of product) to large-scale testing (>kg of product). Additionally, initial preclinical studies are often performed in mouse models for discovery; however, mid- to large-size animal models such as rabbits, pigs, sheep, and nonhuman primates are more relevant to human scale and physiology in the context of evaluating the safety, efficiency, and efficacy of therapeutic strategies proposed for use in humans across age groups. This review summarizes some current strategies to scale-up the production of nanomedicines for translational investigations. Animal models and new approach methodologies are also addressed for NP assessment and screening, including the physiological distinctions when comparing rodent models to larger species that can impact NP delivery. Current challenges are also highlighted in terms of scale-up and preclinical validation with the objective of highlighting scalable, effective nanomedicine platforms that can be considered for translation to human trials.

1. Introduction

Nano-scale vehicles composed of natural or synthetic materials are versatile carriers that can be leveraged to effectively deliver a variety of therapeutic cargoes to cells. Among commonly used non-viral drug delivery systems are lipid- and polymer-based nanoparticles (NPs), often chosen for their physiological biocompatibility and ability to effectively package genetic therapies in the form of nucleic acids. [1] Both polymeric NPs (PNPs) and lipid NPs (LNPs) have unique strengths that have made them attractive choices for encapsulating drug cargoes for delivery. PNPs, for example, can be designed with tunable properties including chemical composition, diameter, surface charge, and controlled cargo release. [2] An example of a polymer-based therapy currently in the clinic is Lupron Depot, which leverages the polymer poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable and biocompatible material, in the form of microspheres to treat endometriosis. [3] PLGA NPs have also been used to encapsulate chemotherapeutic drugs in cancer therapy research. [2] LNPs are similarly biodegradable, biocompatible, and exhibit high nucleic acid encapsulation efficiencies. [4,5] Notably, LNPs were used to encapsulate mRNA for delivery in the clinical application of SARS-CoV-2 vaccines. [6]

In terms of clinical translation, the ability to scale-up production of NP-based therapeutics is critical. Despite the promise of both PNPs and LNPs for therapeutic applications, scale-up of these vehicles from the laboratory to commercial-level production and ultimately to the clinic remains complex. For example, in the context of the LNP-based SARS-CoV-2 vaccines, manufacturers experienced obstacles with scale-up and distribution of the vaccines, which included limited resources and fast production time requirements. [7] Scaling up NP formulations requires optimization of small batch/scale laboratory protocols such that NPs can be mass-produced while maintaining quality control of key characteristics such as diameter/size, surface charge, efficiency of cargo encapsulation, sterility, and safety/toxicity. Additionally, sensitive drug cargoes such as nucleic acids must be protected during formulation to preserve their therapeutic function. Scale-up processes can be difficult to optimize due to the need to balance manufacturing efficiency, maximize production yields, acquire raw materials, minimize costs, quality assurance/quality control (QA/QC), and adequately address any potential environmental hazards simultaneously. A drug development program that reaches the clinic has been reported to cost at least $350 million U.S. dollars. [8] While discovery-scale research can take significant time to perform, the cost of this phase is considerably lower than the scale-up phase where key QA/QC functions must be maintained and meet Good Manufacturing Practice (GMP) requirements. In this review, we highlight some common NP formulation techniques and strategies for scaling the production rate of nanomaterials from the discovery phase to large animal assessments and preparation for a clinical trial. Here, we focus on LNPs and PNPs as these classes of non-viral vehicles are among the most frequently used and fastest growing carriers for biomedical applications. The authors acknowledge other vehicles are also employed that are beyond the scope of this review. Readers are referred to the literature for additional information. [5,9,10]

A key component of the bench-to-bedside translation of nanomedicine-based therapeutics is thorough testing in a range of small and large animal models. Rodent models (e.g., mice and rats) are used for early discovery and to assess the safety and potential efficacy of various drugs and drug delivery systems due to their small size, ease of maintenance, range of genetic modifications available, and variety of well-established disease models. [11] However, there is a significant gap between rodents and humans in terms of physiological features including the immune system. [12] Therefore, it is essential to evaluate the therapeutic potential of nanomedicines preclinically in larger animal models that have qualities more similar to humans prior to the conduct of clinical trials. Mid-size models such as rabbits [13] and larger species such as sheep, pigs, and nonhuman primates, particularly rhesus macaques, have anatomic, developmental, physiologic, genetic, and behavioral similarities when compared to humans. [14–19] Currently, the rate of nanomedicines considered for treating cancer that have advanced from phase 1 clinical trials to market approval is approximately 6%. This highlights the importance of physiologically relevant models prior to commercialization. [20] In this review, we emphasize some key features that provide important insights for future human applications. Coupled with these key models are new approach methodologies (NAMs) that together can provide insights in physiologically relevant *in vitro* culture models with human cells and *in vivo* models to ensure the physiologic complexities are fully explored. These highlights are not intended to be an all-inclusive review; readers are referred to the extensive literature on these topics for further information.

2. Scale-up of lipid and polymer NPs

Scale-up of nanotherapeutics from discovery to commercialization requires formulation techniques that ensure uniform and consistent production of therapeutic NPs at clinical grade as well as respective cargo molecules. For example, individual laboratories can generate mRNA via *in vitro* transcription (IVT) on the scale of tens of micrograms (~25 mg) per batch. [21] In contrast, on a commercial scale, over one billion SARS-CoV-2 Pfizer vaccines were produced, with each dose containing 30 μg of spike protein mRNA, thus over 30 kg of mRNA were manufactured for these vaccines (as of 2023). [22] Additionally, 0.77 mg of LNP components were used for one dose of the Pfizer vaccine. [23] This corresponds to over 770 kg of LNPs that have been formulated in this example. Here, the transition from laboratory to commercial scale required an increase in production yield by over *one billion-fold*. Several

strategies for the scale-up of PNPs and LNPs are addressed below, with many of the NP formulation techniques applicable to both NP types. Additionally, each formulation strategy may yield varied coefficients of variation (CV%s), polydispersity indices (PDIs), sizes, maximum scales, average production scale ranges, and scale-up hurdles (Figs. 1–3). PDI is a metric used in NP formulation to evaluate the extent of uniformity of NP batches. Fig. 1 was constructed to provide approximate size ranges and CV%s for each formulation technique addressed in the text. [24–33]

2.1. Mixing devices

2.1.1. Turbulent mixing methods

Nanoprecipitation of NPs is a process in which an organic solvent miscible with water (such as dimethyl sulfoxide or DMSO) containing dissolved polymer/lipid is added to water while mixing. The rapid desolvation of the polymer or lipid in the aqueous phase results in the precipitation of NPs. Once the NPs are formed, the organic phase must then be removed via evaporation or dialysis, yielding NPs that can be washed and concentrated by centrifugation. [34] This technique is scalable using various mixers with relatively low production costs. Ethanol injection, a specific type of nanoprecipitation for LNPs, is an excellent choice for the scale-up of LNPs due to its compatibility across various production scales, uniform size range, and high stability. [35]

Confined impinging jet (CIJ) mixing is a method developed in 2003 by the Prud'homme group that enables the flash nanoprecipitation of NPs through the evolution of turbulence. When the two fluids meet at the intersection of the CIJ mixer channels, high shear forces and local turbulence rapidly evolve between impinging flows, resulting in desolvation and the precipitation of NPs at low millisecond timescales. [36] Although it is generally thought that turbulence occurs above a Reynolds number (*Re*; a dimensionless value used to characterize the level of turbulence in a flow) of 2,000-4,000, turbulence can evolve at *Re* as low as 90. [37,38] In order to initiate turbulence at low channel dimensions, high flow rates are required involving large volumes (i.e. milliliter scales or larger), which is impractical for discovery-scale NP formulation. While NP formation using CIJ mixing can be automated using continuous flow controllers through both system inlets, CIJ mixers are limited by their relatively simple geometry with only two inlet streams, typically only allowing for narrow flow rate ratios of both inlet streams which can have downstream effects on NP stability. [39,40] Additionally, these mixers might be limited in their operation time given that NPs can aggregate during flash nanoprecipitation using CIJ mixing, leading to poor flow stability. [41] Despite these limitations, CIJ mixing allows for high-throughput NP production. Zhang and colleagues utilized a T-mixer to produce polystyrene NPs at an extremely high rate of approximately 146 g/h or 3.5 kg/day, a rate far exceeding discovery scale. [42]

Multi-inlet vortex mixing (MIVM) achieves low millisecond mixing of fluids from four inlets in a single chamber. This method allows for the independent tunability of flow rates and has high scale-up potential. [40,43] Feng et al. analyzed NP variations between CIJ and MIVM at the same *Re* [44] and observed an increased production rate of up to ~333 g/h or 8 kg/day via MIVM compared to a reduced mg/day production rate via CIJ (Fig. 2). The size and stability of the NPs remained consistent (Fig. 1) using both CIJ and MIVM. [45] Their findings illustrate the potential for large scale-up of NP production via nanoprecipitation using MIVM as a turbulent mixing device (Fig. 2).

Static mixers, devices that enable continuous and rapid chaotic mixing of fluids under laminar flow through optimized internal component geometry, can also be used to produce uniform NPs on a commercial scale by intensifying the mixing capabilities of various NP formulation technologies. These mixers feature stationary, blending elements that repeatedly mix fluid streams as they flow through the device to accomplish a mixing timescale on the order of milliseconds. [46,47] Dong et al. investigated the number of static mixing elements and the flow rates, concentrations, and volumes of polymer:crosslinker that could be altered and optimized to obtain PNPs with various sizes and PDIs (Fig. 1). In this study, smaller NP size could be obtained by reducing the flow rates of the fluid streams or reducing the concentration of solutes. [46] The static mixer consisted of a channel featuring twisting and intertwining components resulting in repeated disruption of laminar flow, enabling efficient mixing. Dong and colleagues also demonstrated that LNPs can be continuously produced at a rate of 150 g/h or 3.6 kg/day using static mixers (Fig. 3). [47] For use in early discovery, static mixers have also been miniaturized to enable formulation of lower sample volumes. [48]

2.1.2. Microfluidic mixing methods

While often used only in early discovery, microfluidic mixing devices provide control over distributions of NP size and surface charge and have been increasingly leveraged across all scales of production (Fig. 1). These mixing techniques are generally characterized by a lower *Re* than millifluidic mixers given their constrained length scale. Hanna et al. describe the benefits of using microfluidic devices to develop large and diverse LNP libraries that can be used for *in vivo* screening applications. [49] Hydrodynamic flow focusing (HFF), for example, can be used to produce PNPs and LNPs via nanoprecipitation in μm to mm wide microfluidic channels on a microsecond mixing timescale. [50] A stream of organic solvent with dissolved polymer/lipid flows through a microfluidic channel where it meets two streams of aqueous solution,

Classes of NP Formulation Technique

	Method	Size Range (nm)	%CV
Precipitation	Chaotic Mixing	30-100	<0.25
	Turbulent Mixing	20-200	<0.25
Emulsion	Emulsion/ Evaporation	~100	<0.4
	Microemulsion	20-100	<0.3
Extrusion	Membrane Extrusion	40-130	<0.3
	Homogenization	40-120	<0.3

Fig. 1. Classifications of various PNP/LNP formulation techniques and corresponding typical NP size ranges and CV% values measured via dynamic light scattering, transmission electron microscopy, or scanning electron microscopy. Selection of appropriate NP formulation technique is crucial for translation as each method can yield various size ranges and CV%s, which is critical for NP scale-up.

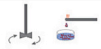
Method	Typical Working Scale (mL)	Max Practical Scale (mL)	Yield	Hurdles to Scaling
	10^{-1}	10^{2}	>95%	• requires parallelization across scales
	1	10^{4}	>80%	• mixing similarity across flow rates
	1	10^{2}	>70%	• size of batch reactor
	10^{-1}	10	>80%	• feed rate
	1	10^{2}	>30%	• intrinsically serial • membrane fouling
	10^{2}	10^{4}	>70%	• damage from shear • material dependent clogging

Fig. 2. Synopsis of various PNP/LNP formulation technique working scales (mL), maximum practical scales (mL), yields, and scale-up obstacles.

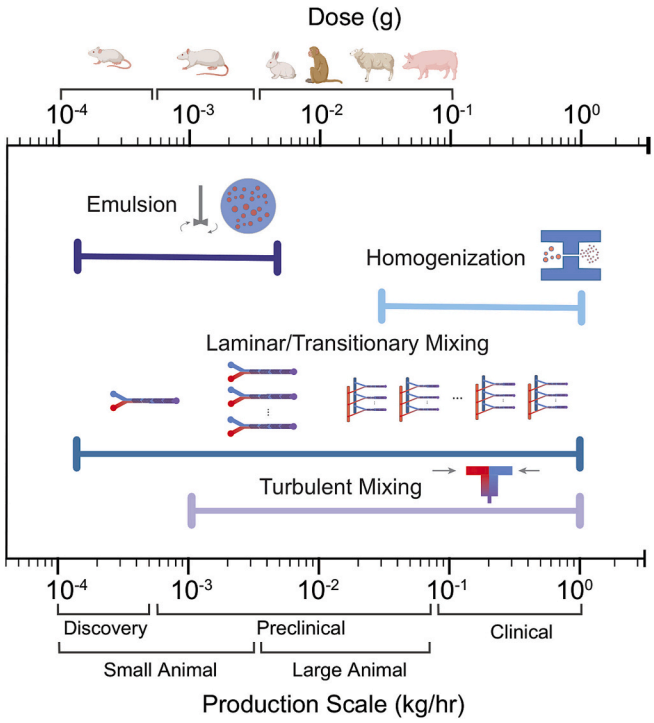


Fig. 3. Animal model selection and typical production scale for the main classes of polymeric/lipid NP formulation techniques. NP formulation method should be carefully considered as it is directly related to NP production scales needed for specific scale-up stages such as discovery, preclinical, and clinical. Additionally, choice of animal model for NP testing is crucial as large animal models are more likely to be representative of human physiology.

initiating nanoprecipitation of PNPs/LNPs that can be collected via the outlet channel. [51] A variety of parameters can be tuned to increase the rate of NP production in these systems. Baby et al. showed that increasing the height and flow rate of the microfluidic device four-fold increased the NP production rate from 72 mg/h or ~ 1.7 g/day up to 288 mg/h or ~ 7 g/day without significantly affecting diameter or PDI (Fig. 3). [52] Furthermore, 3D HFF has been proposed to further increase production scale (Fig. 2). In this case, polymer and organic solvent streams flow from a reservoir on top of the water streams to the base of the microfluidic device where the streams meet. This incorporates a third dimension as part of the mixing and nanoprecipitation process, which can prevent NP clogging during formulation. [53] Rhee et al. demonstrated that when using 3D HFF compared to bulk nanoprecipitation or 2D HFF at the same formulation parameters, 3D HFF produced PLGA-polyethylene glycol (PLGA-PEG) NPs with smaller sizes and the lowest PDI. The corresponding *Re* for the precipitation outlet channel was 16.6. [53] In an alternative strategy, Shepherd et al. produced a microfluidic chip to scale-up the production of LNPs encapsulating mRNA by 256-fold by incorporating 256 mRNA/lipid mixing chambers all on the same chip. The production rate of LNPs increased from ~0.1 L/h or 2.4 L/day with the 1× device to ~17 L/h or 408 L/day with the 256× device, capable of producing ~34,000 mRNA vaccines/h. Scale-up did not affect NP quality as no significant changes in mixing efficiency, encapsulation efficiency, LNP hydrodynamic diameter, or LNP concentration were observed (Fig. 1). [26]

It is important to note that there is extensive work in progress by commercial entities that develop microfluidic technologies and devices that allow for multiple production scales of mRNA-encapsulated LNPs. For example, NanoAssemblr™ formulation systems from Cytiva include devices that can manufacture mRNA-LNPs at the laboratory, preclinical, and clinical scales. Specifically, the NanoAssemblr™ Spark™ formulation system is designed for discovery scale LNP production on the order of microliters. In addition, the NanoAssemblr™ Ignite™ system is optimized for preclinical LNP production scales on the order of milliliters. Advancement of these devices has revolutionized LNP scale-up and accelerated clinical translation.

2.1.3. *Electrostatic complexation*

Electrostatic complexation takes advantage of electrostatic interactions between a polymer/lipid and a solution with molecules of an opposite charge. Ionic gelation, a type of polymeric electrostatic complexation, typically uses chitosan, alginate, or another highly charged polymer (ex. polyethylenimine or PEI). [54] Calvo et al. added chitosan to a solution of sodium triphosphate (TPP) in which the strong electrostatic interactions between chitosan, which is positively charged, and TPP, which is negatively charged, created a network of crosslinked chitosan under magnetic stirring. Performing this reaction while stirring the solution enables chitosan NPs of a specific size to form, depending on the stirring speed and concentration of polymer. [55] The formulation of polyplexes (a class of PNPs) also requires electrostatic interactions; here, positively charged polymers in an acidic buffer interact with negatively charged nucleic acids, effectively condensing the latter and forming a complex. Polyplexes have demonstrated high transfection efficiency for several nucleic acid delivery applications. [56] Lipids can also be used to incorporate mRNA into NPs using electrostatic complexation, where ionizable lipids can interact with negatively charged mRNA during LNP formulation. [57] Electrostatic complexation has key advantages for scale-up in that various classes of therapeutics can be encapsulated. Generally, stronger electrostatic interactions between the therapeutic cargo and the cationic lipid or polymer will lead to higher encapsulation efficiencies. [58] Additionally, this technique does not require

specialized equipment. [59]

2.2. Extrusion

2.2.1. Precipitation extrusion and membrane emulsification

Precipitation extrusion for PNPs involves two miscible solutions separated by a nanoporous membrane. The solute-containing layer is extruded through the nanoporous membrane into the solvent layer, where the polymer precipitates immediately. Guo et al. demonstrated that size control of PNPs is achievable by extruding a chitosan-based solute into sodium hydroxide solvent to formulate chitosan NPs with a narrow size distribution (Fig. 1). [60] Membrane emulsification involves a dispersed phase, which contains the polymer, and a continuous phase where the emulsification will take place. The dispersed phase with the polymer can enter through membrane pores where the polymer is forced into a uniform size and emulsified in the continuous phase. The key distinction between membrane emulsification and precipitation extrusion is that membrane emulsification features two layers that are immiscible and therefore requires an additional precipitation step to produce NPs, namely evaporation of the solvent. [60]

Some key benefits of precipitation extrusion and membrane emulsification include precise control of delivery vehicle size and the versatility of different emulsions that can be used to accommodate various polymers and drug cargoes (Fig. 1). [60,61] Albisa and colleagues demonstrated that membrane emulsification is highly tunable by altering types of organic solvents and flux rates, limiting energy consumption while maintaining PNP uniformity. [62] As both formulation techniques require NPs to be extruded through pores with specified sizes, the distribution of NP diameters is very narrow, enabling uniform NP production. Additionally, it is possible to continuously produce NPs using these methods at larger scales by increasing the flow rates of continuous/dispersed phases (Figs. 2 and 3). For example, Yang et al. showed that continuous production of poly(methyl methacrylate) (PMMA) NPs is possible using membrane emulsification while maintaining an extremely narrow PDI (Fig. 1). [63]

Membrane-based extrusion methods also have disadvantages, including membrane fouling (Fig. 2). Fouling refers to the aggregation of NP components along the pores of the membrane during extrusion, which can hinder or block the flow through those pores and reduce NP batch size significantly. [64] Fortunately, the tunability of membrane-based extrusion techniques allows for optimization of the process to limit NP accumulation in the membrane during extrusion. Parameters such as pore size, membrane composition, hydrophilic coatings, and transmembrane pressure can be altered to limit the stickiness of NPs in membrane pores. [65]

2.2.2. High-pressure homogenization

In the high-pressure homogenization (HPH) process, a lipid contained in a medium is forced through a nozzle to create uniform LNPs of a desired size. [66] Lipids are initially melted into liquid form with or without a drug to be encapsulated. During hot homogenization, the lipid-drug phase is emulsified into an aqueous phase where both mixtures are kept at the same high temperature (above the melting point of the lipid). The emulsion is then forced through a nozzle to create uniform LNPs with an encapsulated drug. A caveat of this technique is that encapsulated drugs can be degraded or denatured during extrusion at high temperatures. Cold homogenization, on the other hand, is a gentler process for the drug cargo, as extrusion occurs at a much lower temperature. In this case, the lipid is initially heated above the lipid melting point. Then, the lipid-drug solution is cooled below its melting point, after which milling is performed on the lipid-drug solution to create small particles. Subsequently, the lipid-drug particles are added to an aqueous phase, and the resulting solution is extruded through a nozzle. A benefit of HPH is control over LNP size (Fig. 1). [33] Using HPH, drug-loaded LNPs can be produced on the kilogram scale at high efficiencies; further, it is possible to encapsulate nucleic acids using cold HPH, although high shear and cavitation may limit the stability of the cargo in this case. [67] Variations in HPH equipment offer a wide range of LNP batch sizes. Some homogenizers can produce greater than 10,000 L/h or 240,000 L/day (Figs. 2 and 3) containing thousands of kilograms of LNPs, and some can produce as little as 5 L/h or 120 L/day or less containing less than a few kilograms of LNPs in a small laboratory scale setting. [68]

2.3. Emulsion

2.3.1. Emulsion evaporation

Emulsion evaporation can be used to formulate PNPs or LNPs with either hydrophilic or hydrophobic encapsulants. In the case of PNPs, polymers are dissolved in an organic phase and then emulsified with a surfactant solution while vortexing. Subsequently, the emulsified NPs are reduced in size via ultrasonication. The organic solvent is then evaporated, and NPs are washed and concentrated via centrifugation. Both hydrophobic and hydrophilic drugs can be encapsulated via single (oil-in-water) or double (water-in-oil-in-water) emulsion processes, respectively. [69] In a single emulsion process, a hydrophobic agent is added to the polymer solution before being emulsified in the aqueous phase. A double emulsion involves first dissolving a hydrophilic drug or nucleic acids in an aqueous solution and emulsifying the aqueous phase with the polymer-containing organic solvent solution before a second emulsion of the organic phase into another aqueous phase. [70] A disadvantage of this technique is that scalability is hindered by the lack of reproducible NP sizes and limited drug loading ability. Rotary evaporators can be used to expedite the evaporation of organic solvents, but evaporation is difficult to scale-up. [71] Bulk stirring of NPs for this formulation process has been explored, but is challenging to control and therefore impacts the reproducibility of NP characteristics. [51,72] As such, emulsion evaporation is typically used on a smaller, laboratory scale and remains difficult to recapitulate on a larger production scale (Figs. 2 and 3).

2.3.2. Microemulsion

Microemulsion is a LNP formulation technique similar to emulsion evaporation, except this process does not require dissolving lipids in organic solvent. Instead, melted lipids are directly emulsified in the aqueous phase with a surfactant. Sonication is then performed to break up the particles into smaller sizes. This method requires large amounts of surfactants, which can be expensive. [73] However, an advantage is that microemulsions do not require organic solvents, which can be hazardous. Further, the process can be scaled up using a linear workflow. Kakkar et al. described the scale-up of sesamol-loaded LNPs by 100× to a 500 mL microemulsion batch size. Experimental components were scaled 100× using the same process for 1× LNP batches, with NP sizes conserved. [74]

3. Animal models

Rodent models have enabled major advances in NP development, but numerous physiological and immunological differences constrain the predictive value of these models for clinical translation. Therefore, prior to human clinical trials, testing in more anatomically and physiologically relevant animal models can offer critical advantages in bridging the translational gap for nanomedicine development, ensuring studies are well-designed and consider the strengths of the species selected. In the following section, we address some of the major features of rodents, rabbits, sheep, pigs, nonhuman primates (specifically macaques), and humans relevant to nanomedicine screening and product development.

3.1. Mice and rats

Mice (*Mus musculus*) and rats (*Rattus*) are widely used small mammalian models in biomedical research due to the range of models

available and low cost. [75,76] Rodent models of specific diseases can be produced with genetic modification, dietary alteration, or drug treatments as examples. Thousands of well-established rodent disease models are publicly available from repositories and commercial vendors such as the Jackson Laboratory and Charles River Laboratories. In contrast, there are more limited disease models available in large animal species. Rodent reporter models have proven useful for screening delivery efficiency and biodistribution of NPs and LNPs, but there are significant limitations in the translation of these studies due to differences between rodent and human physiology. [77–79] The body size differences between adult humans (~60–80 kg), rats (~200–700 g), and mice (~15–30 g) impacts the way these models can be applied for translation to human studies. Species-specific differences exist in the immune system and organ weight in relation to total body weight (e.g., heart, lungs, liver, spleen to name a few) (Table 1). [12] These differences can lead to variation in biodistribution and delivery efficiency of NPs across species *in vivo*. Further, on average the resting heart rates and glomerular filtration rates are higher in rodents than other animal models and humans, which can result in significant differences in outcomes of NP biodistribution and clearance (Table 1). Notably, the liver to body mass ratio in rodents is large (up to 5% [80] compared to 2% [81] in humans) and can lead to increased NP uptake in the liver. In rodent models, this may significantly reduce NP biodistribution and increase clearance from the blood after intravenous (IV) administration. For example, Hatit et al. observed species-specific differences in hepatocyte uptake of LNPs between wild-type, primatized, and humanized mice following IV delivery. [82] This study noted that wild-type mice were somewhat predictive of human hepatocyte delivery, but primatized mice were more representative of human hepatocyte delivery than wild-type mice. Comparative studies such as these illustrate the value of large animal models for preclinical validation of NPs, as they are likely to be more representative of human biodistribution, delivery, and safety.

In addition to physiological differences between rodents and other animal models that could impact NP delivery, rodents may not always represent the extent of disease observed in humans or other animal models. For example, Cardenas et al. observed that mice lack key characteristics of venous thrombosis that are present in humans. [136] Additionally, cystic fibrosis (CF) mouse models do not fully recapitulate the airway pathophysiology that is seen in humans with CF, such as severe mucus accumulation. This is due to unique ion transporters in mouse lungs that can still regulate the ion and mucus balance even with CF, a phenomenon not seen in people. [137] Such disease pathology differences support the use of other animal models to complement studies in rodents, providing research platforms with key features relevant to human anatomy and physiology. [15,138,139] Excellent models exist in pigs and macaques that are more comparable to human pulmonary and cardiovascular systems and can also address health conditions such as metabolic syndrome and diabetes, as well as neurodegenerative and neurodevelopmental disorders. Issues related to the lifespan cannot be fully studied in rodents [140] because of the short lifespan compared to these other species. Testing NPs in larger species can provide important insights on the outcome of delivery of NPs in humans and biological differences (e.g., males, females) with the essential rigor and reproducibility. [141]

3.2. Rabbits

Rabbits (*Oryctolagus cuniculus*) occupy a critical "middle ground" in the translational spectrum, bridging the gap between small rodents and larger species such as sheep, pigs and macaques. Rabbits are relatively cost-effective and can be integrated into existing research facility infrastructure. They are recognized as a well-established, laboratory species that is phylogenetically closer to humans than rodents. [142,143] The size of rabbits can allow complex surgical procedures, serial blood sampling, and repeat tissue biopsies, which are technically challenging in murine models. Further, their relatively longer lifespan enables longitudinal studies extending over several years, which is essential for evaluating the long-term durability and safety of emerging NP-based therapies.

3.2.1. Notable physiological features relevant to NP studies

The anatomy of the rabbit allows for the use of clinical-grade instrumentation, such as intravascular catheters and high-resolution multimodal angioscopy, to evaluate the targeted delivery of NPs to specific lesion sites. This capability enables the real-time monitoring of NP accumulation and therapeutic effect within a vessel size relatively comparable to human coronary arteries, which is technically prohibitive in mice due to their sub-millimeter vessel diameters. [144] The relatively longer lifespan as noted above is useful, for example, to assess NP mediated gene editing over a longer time course.

Table 1
Physiological features of adult animal models.

species	mouse	rat	rabbit	sheep	pig	rhesus macaque	human
average adult weight	15–30 g [83]	200–700 g [84]	2 kg [85]	70 kg [86]	35–350 kg [87] (large variation between minipigs and domestic pigs)	5–10 kg females, 8–15 kg males [88]	60–90 kg
average gestation period	20 days [89]	20–25 days [90]	30 days [91]	150 days [92]	115 days [93]	165 ± 10 days [94,95]	280 days
breeding cycle	year round	year round	year round	seasonal breeders	seasonal breeders	seasonal breeders	year round
typical # fetuses per pregnancy	1–13 [96]	8–10 [97]	3–7 [98]	breed-dependent; Dorper sheep: 1 [99]	~10–15 [100]	1	1
blood volume (% body weight)	7% [101]	6.5% - 8% [102]	5.5% [103]	6% [87]	6% [87]	6% [104,105]	7% [106]
resting heart rate (beats/min)	500–700 bpm [107]	300–500 bpm [108]	100–350 bpm [109]	65–80 bpm [110]	80–150 bpm [111,112]	150–220 bpm [113,114]	60–90 bpm [115]
glomerular filtration rate (mL/kg/min)	10 mL/kg/min [116]	8–10 mL/kg/min [117]	4–4.5 mL/kg/min [118]	1–2 mL/kg/min [119]	1.5–2.5 mL/kg/min [120]	2 mL/kg/min [121,122]	1.5–2 mL/kg/min [123]
liver mass (% body weight)	2–5% [80]	2–5% [80]	4.5% [124]	1.6% [87]	3% [87]	2–3% [125]	2% [81]
lung mass (% body weight)	1–2% [80]	1–2% [80]	0.6% [124]	1% [126]	1–2% [127]	1% [125]	1–2% [128,129]
heart mass (% body weight)	0.5% [130]	0.5% [131]	0.25% [124]	0.4% [126]	0.5% [16]	0.5% [125]	0.5% [128,129]
spleen mass (% body weight)	0.2% [132]	0.2% [132]	0.08% [124]	0.3% [126]	0.2% [133]	0.1% [125]	0.1–0.2% [134]
kidney mass (% body weight)	0.75–1% [80]	0.5–0.75% [132]	0.8% [124]	0.3% [126]	0.5% [133]	0.1–0.25% [135]	0.25–0.5% [128,129]

3.2.2. *Disease models for potential NP delivery applications.*

Rabbits serve as an excellent model for human cardiovascular research due to their unique metabolic profile. [142] Unlike mice, rabbits naturally express cholesteryl ester transfer protein (CETP) and possess a lipoprotein profile that closely mimics that of humans. [145] When maintained on a high-cholesterol diet, rabbits develop human-like atherosclerotic plaques, providing a robust platform for testing NP-based imaging probes and targeted drug delivery systems aimed at plaque stabilization or regression (Fig. 4). In cancer research, the rabbit is uniquely valued for the VX2 tumor model. [146] The VX2 is a highly aggressive squamous cell carcinoma that can be transplanted into various organs to study primary tumor delineation and lymphatic metastasis. The rabbit's size allows for the use of imaging platforms such as computed tomography (CT) and magnetic resonance imaging (MRI) to monitor the delivery and efficacy of antitumor NPs. Multimodal NPs have been successfully utilized in rabbit models of head and neck cancer to precisely map lymphatic spread, providing a level of translational detail that is not attainable in rodent models. [147] The rabbit is also a classic model species for ocular diseases. [143] Anatomically and physiologically, the rabbit eye is much more similar to the human eye when compared to rodents, particularly regarding vitreous volume, globe size, and the internal structure of the retinal layers, which are important for evaluating the pharmacological distribution and safety of intravitreal or subretinal NP injections. [148] Recent research has leveraged these advantages to deliver CRISPR/Cas9 components or therapeutic transgenes via viral and non-viral NP vectors to treat inherited retinal degenerations and age-related macular degeneration. [149] Additionally, rabbit models have been used in studies focused on cerebrovascular diseases and brain injuries. [150] In the context of NPs, neonatal rabbit models of cerebral palsy have been considered to characterize the manner in which hydroxyl dendrimer NPs are taken up by activated microglia in the brain (Fig. 4). [151] These studies offer critical insights into the development of targeted neuroinflammatory therapies, allowing investigators to monitor how NPs cross the blood-brain barrier (BBB) and localize within specific inflammatory niches that are more complex when compared to rodents. Recent advancements in CRISPR/Cas9 technology have led to new human-relevant rabbit models of genetic diseases as noted above including primary immunodeficient diseases and Usher Syndrome. [152–154].

3.3. *Sheep*

Domestic sheep (*Ovis aries*) exhibit many similarities in body size and organ systems when compared to humans. Sheep weigh approximately 3–5 kg at birth and 70–90 kg as adults. This species has similar pulmonary physiology and neurodevelopment when compared to humans, providing valuable models including those related to prematurity. [155] CRISPR/Cas9-based gene editing approaches have also been assessed with the efficient generation of genetically engineered sheep models. For example, models of human disease can be produced by somatic cell nuclear transfer or zygote micromanipulation approaches. [156–158] It should be noted that there are less reagents available (e.g., antibodies for flow cytometry or immunohistochemistry) when compared to other species including mice and nonhuman primate species.

3.3.1. *Notable physiological features relevant to NP studies*

Lung development and cellular and size composition in sheep correlates well with humans, thus providing relevant models for local airway NP delivery (Fig. 4). [159,160] Additionally, sheep respond to allergens and particulate matter inhalation by related mechanisms (e.g.,

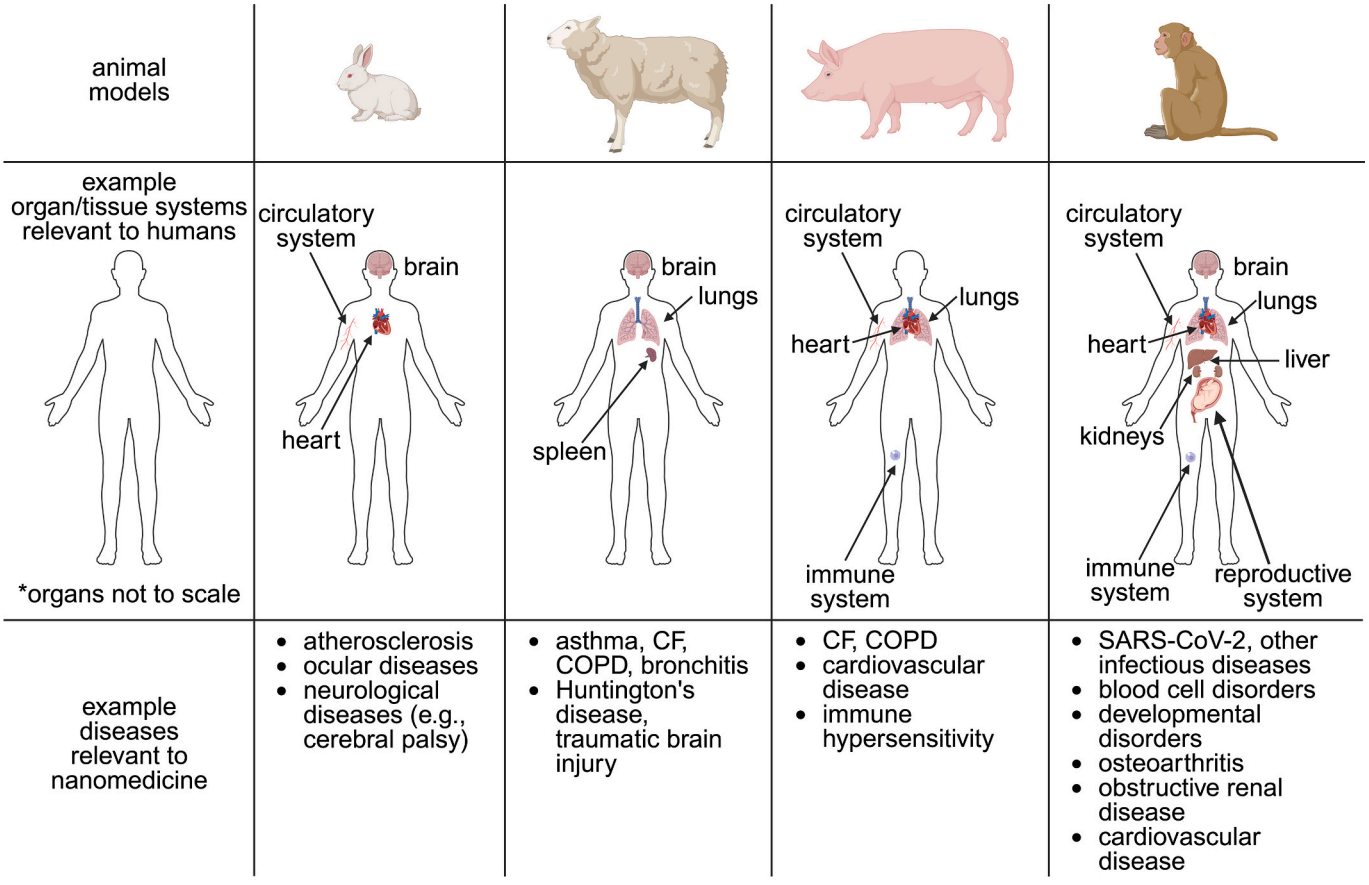


Fig. 4. Schematic detailing physiological similarities between humans and large animals used in NP research. Rabbits, sheep, pigs, and primates each have distinct physiological features that are relevant to NP delivery in humans.

mucociliary clearance) when compared to humans. [161] However, sheep harbor a relatively large population of lung intravascular macrophages. [162] Relevant to systemic IV delivery, sheep have a similar liver mass to body mass ratio (1.6%) [87] when compared to humans as well as a sinusoidal spleen when compared to mice. [163,164] Splenic sinusoids may allow for greater retention and uptake of NPs, which can impact biodistribution. [165] While sheep and humans share many physiological similarities, a key difference relevant to *in vivo* NP studies is the immune system. Elkhamary et al. studied inflammatory responses with regard to immune cell presence in humans and sheep in cell culture, where they observed differences in immune cell composition. [166] Additionally, the proteome following inflammatory stimulation consisted of many species-specific proteins and only some proteins were shared by both species, which could lead to differing immune responses *in vivo* following NP administration. [166]

3.3.2. Disease models for potential NP delivery applications

Sheep are relevant models for many human respiratory diseases including asthma, chronic bronchitis, chronic pulmonary hypertension, emphysema, bronchioloalveolar carcinoma, CF, chronic obstructive pulmonary disease (COPD) [161,167], and topics related to prematurity. [168] The pathophysiology of CF is representative of human disease in sheep. [169–171] They are good models for the study of the physiological effects that COPD may induce, such as a decrease in ventilation rate and an increase in inflammatory responses. [159,172] Sheep are also used to study neurological disorders including Huntington's disease and traumatic brain injury. [173,174] Sheep and human brains share structural features including cerebral geometry as well as the same configuration of neural components, such as the cortex and neural cell types. [17,19] This species has been used as a delivery model for multiple disease applications (Fig. 4). For example, Ducournau et al. tested an intranasal NP-based vaccine in a sheep model of congenital toxoplasmosis. [175]

3.4. Pigs

Similar to sheep, pigs (*Sus domesticus*) share many physiological features with humans, and are commonly used in cardiovascular research. They are also valuable models for testing the safety, efficiency, and efficacy of nanotherapeutics. Depending on the species, adult pigs weigh between 35 and 350 kg; there is a large variation between adult minipigs and domestic pigs. [87] Common organ size, especially in minipig lines, and the ability to use gene editing technologies has permitted the development of animals that lack many of the major antigens stimulating xenogeneic-based rejection in whole organ transplantation by innate and adaptive components of the human immune system. Such genetic modifications have been combined with human transgenes that serve to further inhibit complement activation and coagulation, along with promoting localized immunosuppression. [176]

3.4.1. Notable physiological features relevant to NP studies

The porcine heart closely resembles the human heart in size, including a similar weight to body weight ratio of ~0.5% and blood flow hemodynamics. [16] Furthermore, blood distribution to the heart via coronary arteries is comparable to human physiology. [18,177] As such, pigs provide valuable models to study pharmacokinetics of intravenously administered therapeutics (Fig. 4). Similar to sheep, physiological similarities to humans are also observed in the porcine lung, such as the number of bronchial branches and architectural structure. [177,178] Pigs and humans share related immune responses to infectious agents, in contrast to mice, [179] and both have strong similarities in the immune system proteomes and genomes. [180] Further, humans and pigs exhibit innate and adaptive immune responses that function through similar mechanisms. [179] It has been noted that the pig immunome resembles over 80% of the human immunome, whereas murine and human immunome only overlap by 10%. [181] Particularly relevant to systemic NP delivery, pigs have a similar mononuclear phagocyte system (MPS) when compared to humans (Fig. 4). [182] The MPS is the body's defense system to guard against foreign matter via phagocytosis by immune cells which have a major role in the biodistribution of NPs. Notably, pigs are also highly sensitive to complement activation following NP administration via complement activation-related pseudoallergy (CARPA). This phenomenon has been observed in humans in response to some PNP and LNP therapeutics in rare cases of immune hypersensitivity. CARPA in humans can cause potentially life-threatening anaphylaxis. [183]

3.4.2. Disease models for potential NP delivery applications

Pigs have been widely used to model various respiratory diseases such as CF and COPD. In the context of CF, pigs have a comparable quantity of cystic fibrosis transmembrane conductance regulator (CFTR) protein when compared to humans [184]; human CF-associated pathology including mucus buildup and increased susceptibility to lung infection is also observed in this species. Liu et al. studied methods to improve local delivery of LNPs encapsulating fluorescent reporter mRNA in pig lungs by a charge-mediated stabilization of the LNPs upon aerosolization. [185] Various porcine models for testing NPs for cardiovascular diseases are also under development. For example, Nakano et al. developed a platform for NP-eluting stents in pig coronary arteries. [186] This study confirmed effective and prolonged delivery of PLGA NPs into local cells while maintaining high biocompatibility. In other similar applications, researchers have also explored the delivery of NPs to coronary arteries to prevent restenosis. Zago et al. studied the delivery of sirolimus NPs via a balloon catheter to coronary arteries in pigs, where the sirolimus NPs were able to reduce neointima and could therefore provide a potential therapy for restenosis. [187]

Pigs are known to be prone to potent complement activation and immune system reactions in response to NP administration. As such, porcine models are very valuable for studying complement activation / immune system hypersensitivity of nanotherapeutics prior to human use. For example, similar to humans, pigs may experience severe cardiovascular and skin effects. [183] However, it should be noted that CARPA reactions in pigs do not represent a common response to nanotherapeutics.

3.5. Nonhuman primates (Rhesus macaques)

Rhesus macaques (*Macaca mulatta*) are one of the more common nonhuman primate species for modeling human diseases and testing therapeutic strategies across a range of age groups (prenatal, infant, juvenile, adult, geriatric). [14,94,95] They share many essential features with humans due to their close phylogenetic relationship. Parallels in genetics, anatomy and physiology, as well as reproduction, development, immunology, social complexity, and cognition aid in overcoming the barriers to clinical translation. Of the large animal models highlighted, rhesus and other macaque species are most closely related to humans in terms of organ to total body weight mass ratios in adults (Table 1), and represent a gold standard for preclinical testing. Macaques share many physiological features, particularly in relation to reproduction [188], development [94], and organ ontogeny and maturation (e.g., cardiovascular, pulmonary, renal) [15,189–194] including the hematopoietic and immune systems. The menstrual cycle as well as menopause only occur in primate species, which therefore serve as ideal models for female reproduction and women's health across the lifespan. [95,188] From a developmental perspective, placental structure is most similar to humans when compared to other large species. [14,195] The development of the kidneys and lungs in rhesus macaques follows the same stages and occurs at similar points during gestation as in humans. Both human and monkey lungs continue to mature before and after birth. [15] At birth, the lungs of both species are at similar developmental stages; the most distal conducting airways have not yet formed, and only a small proportion of adult alveoli are present. Humans and rhesus possess unique cell types not observed in other species, and the

overall pattern of tracheobronchial epithelial differentiation is remarkably similar in both species. [15,139] Rhesus also have similar segmental arrangement, branching patterns, mucosal surfaces, and arterial structure when compared to humans. The structural arrangement, branching design, mucosal linings, and arterial organization of rhesus monkey lungs are also comparable to those in humans. For these reasons, rhesus monkeys have been essential models for studying airway diseases such as asthma and COPD, as well as the effects of wildfire and tobacco smoke. [138] The importance of this species in metabolic syndrome [196] and aging research, including topics such as immune senescence, have been highlighted in the literature. [197–203] It has been shown that when gravid rhesus macaques are fed a high-fat diet, their offspring are at greater risk for developing type-2 diabetes, alterations in leptin sensitivity, nonalcoholic fatty liver disease, high blood pressure, and obesity. [204]

3.5.1. Notable physiological features relevant to NP studies

Some examples of reported NP applications are highlighted. Kim et al. studied an LNP formulation that delivered mRNA to murine hematopoietic stem cells (HSCs) *in vivo*, primary human HSCs *ex vivo,* and $CD34^{+}$ cells *in vivo* in young rhesus monkeys without mobilization or conditioning. [205] Slayden et al. administered NPs IV to rhesus macaques with the goal of *in vivo* targeting of endometriosis in spontaneous models. The particles carried various payloads, including fluorescent dyes and magnetic cargo with the goal of use for imaging, as well as thermal ablation of diseased tissues. [206] Kulhankova et al. described treatment strategies for airway diseases based on gene editing. [207] Following intratracheal aerosol delivery of dye-labeled peptide cargo in young rhesus macaques, CT scans were used to assess delivery across lung lobes immediately post-administration. *CCR5* was targeted and editing efficiencies in airway epithelia up to 5.3% were achieved with no inflammatory responses detected systemically. Schmidt et al. performed a preliminary study where NPs containing plasmid DNA encoding for hIGF1 driven by the PLAC1 promoter (placenta-specific) was delivered via a catheterization approach used for uterine artery embolization to a mid-gestation gravid rhesus macaque. This preliminary study demonstrated successful NP delivery to the maternal circulation close to the placenta. [208] Preliminary investigations have also focused on biodistribution of PNPs shortly after ultrasound-guided delivery via the portal vein in early second-trimester fetal rhesus monkeys. Here it was demonstrated that PLGA NPs and poly(amine-*co*-ester)-polyethylene glycol (PACE-PEG) NPs distributed to multiple fetal tissues when assessed 24 h post-administration with no evidence of adverse findings. [209] Wilson et al. developed ultrasound-responsive NPs that could be used to release drugs such as propofol in deep brain visual regions safely and effectively. [210] Musunuru and colleagues demonstrated that LNPs encapsulating CRISPR/Cas base editors can modify genes relevant to diseases in cynomolgus macaques. [211] For example, after a single infusion of LNPs, significant knockdown of PCSK9 in the liver was achieved; treatment resulted in reductions in PCSK9 and LDL blood cholesterol of ~90% and ~ 60%, respectively, with a duration of 8 months.

3.5.2. Disease models for potential NP delivery applications

Opportunities include naturally occurring disease models [212–214], experimentally induced models (e.g., infectious agents, obstructive renal disease, blood cell disorders), and aging-related models (e.g., osteoarthritis) (Fig. 4). [95,201,215–217] Considering the large range of approaches and applications available (Table 2), macaque models are widely applicable for non-viral vector delivery such as NPs. Macaques have been critically important for the development of vaccines including LNP-based platforms for prevention of SARS-CoV-2 infection (Fig. 4). [218,219] As noted in a prior review, studies in macaques have provided key insights into SARS-CoV-2-associated vascular disease and thrombosis mechanisms, for example, including inflammatory and thrombotic mediators, demonstrating interactions between these two pathways. [95] Much baseline coronavirus knowledge was known before the pandemic and relied on nonhuman primate models. [95]

3.6. Practical considerations for NP studies across animal models

In Table 1 **and** Fig. 4, we highlight key physiological features across species, including average body weight and blood volume in adults, and some relevant disease models. One key observation is the difference in relative liver size in rodents compared to larger species and humans; the liver of rodents is typically much larger in terms of percent organ weight to total body weight. As the primary organ responsible for drug metabolism, the impact of liver processing is a critical consideration for interpreting results derived from therapeutic delivery by NPs in translational models and future human applications. Furthermore, the higher rodent resting heart rate and glomerular filtration rate when compared to large animal models are factors that can impact biodistribution, pharmacokinetics, and pharmacodynamics of therapeutic NPs during preclinical testing.

Large animal models serve as a key translational bridge for assessing the safety, pharmacokinetics, efficiency, and in some cases efficacy of NP therapeutics prior to considering human trials. Body weight, extended lifespan, and species-specific physiological features must be carefully considered for the intended clinical application and NP platform to maximize translational success. In Table 2, we provide some examples of different routes of administration and treatment targets for PNP/LNP delivery *in vivo* that can be considered in parallel with the design of the vector under consideration. Vector design and disease target are important to consider when assessing the best route of administration.

3.7. New approach methodologies (NAMs): Human cellular model systems

While animal models remain essential for preclinical evaluation of nanomedicines, NAMs are increasingly important to pursue and include as part of the experimental paradigm. These *in vitro* human-based model systems (e.g., 3D organoids, organ-on-a-chip), as well as computational and artificial intelligence-based methods are under study for a range of screening applications and uses, although validation is needed. [220]. Physiologically relevant cell culture and *ex vivo* models can be very informative for the study of NPs in human tissues. Integrating human-based models into NP screening workflows is key to early discovery. However, these models require validation and are best used in parallel

Table 2
Examples of tissue/organ system targets and *in vivo* routes of therapeutic administration.

Target	Routes of Administration	Cells; Specialized Procedures
Brain/Spinal Cord	Intravenous (IV), intrathecal, intracranial, intranasal	Neurons, astrocytes, microglia, endothelial; image-guided
Liver	Intrahepatic, intraportal, IV	Hepatocytes, Kupffer cells, endothelial; image-guided
Kidney	Intrarenal (parenchyma-cortex vs. medulla), IV, renal artery	Epithelial, endothelial, podocytes, mesangial; image-guided
Muscle	Intramuscular, IV	Myocytes, satellite cells
Heart/Cardiovascular	IV, arterial, myocardium	Cardiomyocytes, endothelial, vascular smooth muscle; image-guided
Lung	IV, intrapulmonary (parenchyma), intratracheal	Epithelial, airways, parenchyma; aerosol, nebulization

with relevant animals models in order to simulate the complexity of the *in vivo* environment. For example, organism-wide responses to NP administration and the complex interplay between immune, lymphatic, physiologic, genetic, and disease processes, as well as interactions across multiple organ systems, cannot be captured by human *in vitro* model systems alone. For example, while lung organoids or precision cut lung slices (PCLSs) can be useful for assessing local NP delivery and specific cellular responses in the airway epithelium, they are not able to reflect whole-body biodistribution and interaction across organ systems, systemic and local immune response dynamics, or provide comprehensive insights into the critical need to address the potential for adverse events. Evaluating immune responses to new vectors (viral and non-viral) remains a major priority and requires extensive assessments including T-cell trafficking that cannot be addressed in cell-based systems. Careful selection and integration of human-cell based approaches in parallel with the best choice of animal model while ensuring rigor and reproducibility are significant and key to NP delivery, efficacy, and safety studies. [141]

4. Key challenges for clinical translation of nanomedicines

4.1. Reproducibility and QA/QC

One of the greatest challenges in the scale-up of NPs is the reproducibility of vehicle quality and characteristics; on a commercial scale, significant modifications to laboratory scale processes are often necessary. The modifications needed to produce therapeutic NPs on a large scale can introduce inconsistent production compared to smaller discovery-scale production. Changes to the size, surface charge, and cargo loading of NPs at these higher production scales jeopardize the efficacy of NPs for therapeutic applications, as all parameters are needed to maintain desired delivery properties. As described above, NP formulation techniques can play a large role in altering the NP physiochemical properties; an ideal choice would result in a more reproducible and narrower NP size and surface charge distribution. There are additional challenges when working with polymer and lipid biomaterials, such as batch-to-batch variations of raw materials and NPs. This can occur in early discovery as well as on a commercial scale, where NPs have variable characteristics from separate formulation batches. This challenge can be difficult to mediate particularly when the variations are not due to user error but are related to the inherent complexities within synthesis and formulation protocols. The U.S. and European Pharmacopeia have recognized QA/QC attributes of mRNA LNPs for human use. Specific LNP parameters that are addressed in these guidelines are LNP diameter and PDI (determined by dynamic light scattering (DLS)), lipid identity, lipid content percentages, and encapsulation efficiency. Furthermore, there are extensive qualities of mRNA that are distinguished as key for QA/QC including mRNA sequence, purity, content/concentration, and integrity. [221,222] Together, the extensive efforts by the U.S. and European Pharmacopeia aim to regulate and develop QA/QC guidelines for mRNA-LNP vaccines for human use.

4.2. Therapeutic degradation

Another consideration for scale-up is the preservation of NPs and drug cargo from degradation or inactivation during formulation. Nanomedicines are used to encapsulate, protect, and deliver sensitive drug cargo for delivery to intended cell types. Heat, which is generated in several formulation processes, can be detrimental to these fragile drug cargoes, especially nucleic acids. [223] Additionally, as part of the commercialization process, many steps are required to bring nanomedicines to the clinic including formulation, storage, transport, and distribution, increasing opportunities for NP or therapeutic cargo degradation. In terms of nucleic acid cargo types, DNA is more stable than mRNA as it is double stranded and less prone to hydrolysis. While less stable than DNA, small interfering RNA (siRNA) tends to be more stable than mRNA due to its double stranded nature. [224] Micro RNAs (miRNAs), another type of gene modulating RNA, are also typically more stable than mRNAs due to their much smaller length (~20 nucleotides), decreasing the likelihood of phosphodiester bond cleavage. [225] Modifications can be made to less stable mRNA molecules to protect them from degradation such as the addition of a 5′ cap and a 3′ poly(A) tail and increasing the percentage of guanine and cytosine content. [226] Additionally, circular mRNA can be synthesized by joining the 5′ and 3′ ends of an mRNA molecule and is much more resistant to degradation than linear mRNA. [227] In the context of mRNA, pseudouridine can be substituted for uridine for increased stability and reduced immunogenicity. [228] The ability of pseudouridine to form an extra hydrogen bond during base pairing and evade immune system recognition contributes to these advantages. [228]

4.3. Comparability of studies in different species

As highlighted in this review, rodent studies are often not predictive of NP behavior in larger species or humans. [82] Hatit et al. reported that nonhuman primates were more predictive of human responses compared to mice. [82] Despite the convenience of small animal models for initial testing, the optimal formulations in rodents are unlikely to be the optimal formulations for preclinical evaluations in larger animal models or humans. Appropriate dosing calculations are necessary for all species to ensure the optimal dose of therapeutic NPs for efficiency and safety, considering body weight, surface area, organ system differences, age, biological differences, species selection, pharmacokinetics, and pharmacodynamics. [229] The FDA currently recommends dosing humans based on an algorithm utilizing no observed adverse effects levels (NOAEL) in animal models prior to translation to human studies. [230] The algorithm accounts for a safety factor and the human equivalent dosing, typically based on body surface area. [231]

Performing meaningful studies in animal models requires screening prior to selection. Similar to humans, the potential exists for animal models intended for genome-editing studies that employ a microbe-derived editing protein to have pre-existing antibodies. [232] Humoral responses to *Staphylococcus aureus* (*Sa*Cas9) and *Streptococcus pyogenes* (*Sp*Cas9), two commonly used editors, are generally assessed by enzyme-linked immunosorbent assay (ELISA). In addition, NP formulations frequently utilize PEG which can result in immune responses if screening animals for pre-existing PEG antibodies has not been performed. [233] Other screening protocols include the target site for the gene to ensure that the expected target sequences are present. Following immune responses to components of the NPs employed is also essential to address safety. Thus, screening animals to ensure those selected do not have pre-existing antibodies to components of the vector construct, as well as monitoring immune responses post-administration is key for preclinical studies that can effectively translate findings to future human clinical trials.

5. Conclusions

Scaling up NP formulation for commercial use is currently a primary bottleneck for clinical translation. PNPs and LNPs can be produced in laboratory settings using many different formulation techniques, but these will not be suitable for large-scale production due to various manufacturing complexities. Several NP formulation scale-up strategies can be employed to ramp-up NP production including mixing device-, extrusion-, and emulsion-based techniques. While emulsion-based formulation techniques typically render NPs with a uniform size distribution and are very effective on small laboratory-grade scales, the production rate of NPs using this method remains lower than commercially acceptable standards. Alternatively, mixing and extrusion-based techniques have been shown to develop nanomedicines on greater mass/time scales, highlighting the usefulness of these techniques to mass produce NPs while maintaining uniform size distributions. Various

technologies using these techniques have also shown that formulating NPs in parallel within the same device can significantly increase throughput while maintaining consistent NP characteristics. Looking forward, continued innovation in NP production—including automation, integration of artificial intelligence, and adoption of standardized cell culture and *in vivo* protocols—will further streamline scale-up and enhance rigor and reproducibility.

Beyond formulation scale-up, translating nanomedicines from bench to bedside is a complex, time-intensive process. Before clinical trials, therapeutic NPs require rigorous testing in animal models that closely mimic human physiology to assess both efficacy and safety. As noted, mid-size and large animal models such as rabbits, sheep, pigs, and rhesus macaques are more predictive of NP outcomes in humans than rodents. Physiological relevance and carefully designed studies that include NAMs are expected to improve translational research success. Demonstrating nanomedicine effectiveness and scalable formulation in these models (e.g., maintaining size, zeta potential, drug loading at high-throughput rates) can also enhance scalability prospects. As non-viral nanomedicine technologies continue to transform drug delivery and disease intervention, careful attention to scale-up strategies, model selection, and study design will be critical to optimize future clinical impact.

Declaration of competing interest

A.S.P. is a founder of Xanadu Bio, Inc. and holds equity. D.I. is a founder and holds equity in InfiniFluidics.

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Data availability

No data was used for the research described in the article.

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