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Overcoming gene delivery hurdles: physiological considerations for nonviral vectors

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Abstract

With the use of contemporary tools and techniques, it has become possible to more precisely tune the biochemical mechanisms associated with using nonviral vectors for gene delivery. Consequently, nonviral vectors can incorporate numerous vector compositions and types of genetic cargo to develop diverse genetic therapies. Despite these advantages, gene delivery strategies using nonviral vectors have poorly translated into clinical success due to preclinical experimental design considerations that inadequately predict therapeutic efficacy. Furthermore, the manufacturing and distribution processes are critical considerations for clinical application that should be considered when developing therapeutic platforms. This review evaluates potential avenues towards improving the transition of gene delivery technologies from *in vitro* assessment to human clinical therapy.

Gene therapy technology and limitations

Gene therapy is a promising therapeutic strategy predicated on rectifying pathogenic diseases or other chronic ailments through genetic modification (e.g., cancer). At its foundation, gene therapy involves the intentional modulation of gene expression patterns through the delivery of exogenous genetic material such as DNA, messenger RNA (mRNA),

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Conflicts of interest

CHJ, ABH, and BAP are also employees of Abcombi Bioscience Inc.

microRNA (miRNA), RNA interference (RNAi) molecules such as small interfering RNAs (siRNA) or small hairpin RNAs (shRNA), and antisense oligonucleotides (AONs). Due to properties such as negative charge or large size, most biomacromolecules require vectors for delivery [1]. Over the last twenty years, the number of clinical trials evaluating gene-delivery technology has steadily increased [2], but these trials have only yielded five products globally, and none in the United States [3]. These products, none of which were approved prior to 2003, include: Gendicine, Oncorine, Rexin G, Neovascugen, and Glybera. Unfortunately, nearly 95% of clinical trials have failed to proceed beyond Phase II studies [2], illustrating that there are potent challenges to developing clinically-relevant gene therapies.

Of the clinical trials listed in Table 1, ~90% utilized viral vectors such as adenoviruses, adeno-associated viruses (AAVs), lentiviruses, or retroviruses. Although the development of viral vectors has substantially advanced gene delivery technology, they possess several inherent shortcomings: limited DNA packaging capacity, complex production processes, broad tropism, cytotoxicity, immunogenicity, and tumorigenicity [4]. Nonviral vectors possess the potential to address many of these issues, thanks to recent advances in material science, nucleic acid chemistry, and nanobiotechnology [5].

To become clinically relevant, nonviral vectors must better tolerate the challenges of a physiological environment. Conventional research and development of nonviral vectors has been focused on altering the vector's material composition or formulation to improve the efficiency of *in vitro* gene transfer or to prevent premature degradation of the vector during *in vivo* circulation [6-8]. Unfortunately, solutions from this paradigm are typically validated in experimental systems that poorly represent the *in vivo* environment. Most *in vitro* tests are conducted using adherent immortal cell lines, which are typically transfected for long durations under non-physiological conditions. Consequently, such experiments fail to account for complications associated with organism-level gene delivery, such as renal clearance, serum inactivation, off-target delivery, and nuclear translocation in somatic cells. Positive outcomes in oversimplified *in vitro* tests do not guarantee success in animal model validations. Furthermore, even success in animal models does not guarantee clinical success. Current animal testing methods cannot fully address translation to human physiology, and there are economic considerations associated with the clinical setting.

In this review, we summarize the challenges that impede the clinical translation of gene delivery technology and discuss potential strategies for overcoming each barrier. We highlight compositional considerations for nonviral gene delivery vectors, *in vitro* and *in vivo* experimental design strategies that will circumvent, if not eliminate, limitations that have previously stymied clinical success of gene delivery technology.

Compositional considerations

Genetic cargo

Because developing gene delivery strategies often requires testing many combinations of different vectors and cargo, there is a risk of inefficiently applying resources to test less viable options. This can be avoided by evaluating only vector and cargo combinations

ideally suited for the intended application. Careful consideration of the specific advantages and disadvantages associated with a particular genetic cargo and/or delivery vector to reduce the amount of compositional design options to a manageable number.

Nucleic acid payloads utilized in gene delivery applications can be divided into two groups based on their mechanism of action: i) transiently-active payloads (e.g., DNAs, AONs, and RNAs) and ii) genome editing systems. Transiently-active molecules can be further segmented into expression-dependent or -independent subdivisions. These subgroups differ in traits such as the type of nucleic acid cargo (DNA or RNA), location of activity (nucleus or cytoplasm), and mechanism of action (transgene expression or RNA interference), respectively. The most common expression-dependent systems consist of circular, double-stranded DNA constructs, termed “plasmids” (pDNA), which facilitate gene delivery by driving expression of the transgene encoding the protein of interest (Figure 1). These plasmids contain several basic components: the transgene expression system (i.e., promoter, gene of interest, and terminator), regulatory signals, antibiotic resistance marker, origin of replication, and the remaining bacterially-derived plasmid backbone (BB). The native plasmid systems are only active *in vivo* for only 1-2 months, but derivatives of these systems have demonstrated improved expression durations extended to several months/years while simultaneously boosting the expression magnitude of the transgene by 10- to 1,000-fold [9, 10]. For example, plasmids that are devoid of the BB have demonstrated prolonged and sustained efficacy compared to traditional pDNA [11, 12]. The extension of activity is due to the reduction of a silencing phenomenon that occurs when ~1 kb or more of DNA is placed outside of the transcriptional cargo (between 5' end of promoter and 3' end of poly A site) [13]. Strategies such as these expand therapeutic options for expression-dependent systems, especially within circumstances where traditional plasmids do not provide an adequate therapeutic timeframe or when permanent genetic changes are undesirable.

A second set of transient-active molecules consists of synthetic nucleotide sequences (DNA, RNA, and chemical analogues) that serve to reduce and/or eliminate expression of specific protein(s) (Figure 1). Although these molecules are predominantly expression-independent, they can also be packaged into the plasmid-based systems described previously [14, 15]. The most commonly used types of these molecules are RNAs, (i.e., siRNA [16], shRNA [17], and miRNA [18]). Typically, siRNA and miRNA are employed to facilitate translation-inhibition of mRNA transcribed from the targeted gene(s) by directing RNA-induced silencing complex (RISC) activity [19, 20]. Although RNA possesses considerably lower stability (~1 hour in plasma) compared to DNA [21], the advantage of RNA-based gene therapy is the associated reduction in immunogenicity and mutagenesis [22]. Furthermore, a highly successful gene knockdown using siRNA can reduce targeted gene's expression by 80–95% without reducing cell viability [23]. In addition, RISC's modulation of target gene expression does not require nuclear translocation, which is the bottle-neck of expression-based systems. One drawback, however, is that the expression level of as many as 100 genes may be altered by a single siRNA transcript [24]. Alternatively, unlike RNA-based molecules, AONs interfere with mRNA translation through steric-hindrance of ribosomal binding in a sequence-specific manner, improving targeting specificity [25], but at the cost of decreasing knockdown efficiency (20–80%) [26]. Despite numerous advantages, off-target interactions with non-specific mRNA sequences, insufficient delivery to the targeted

cells, and high manufacturing-costs of practice-grade AONs in sufficient quantities, impairs the wide- scale adoption of particular strategy [27, 28].

Unlike transient-active molecules, genome-editing systems such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs) introduce permanent genomic changes into host cells through gene correction, disruption, and whole-gene insertion, respectively [29]. Of these systems, CRISPR-Cas, has garnered the most attention recently due to its ease of design and implementation; however, improvements in efficiency and specificity are still necessary [30]. For example, a recent study conducted in human zygotes achieved an editing efficiency of only 25%, far below clinically relevant levels [31]. Even though permanent gene modification in humans is a promising avenue of research, ethical concerns will hinder widespread adoption for the foreseeable future.

Selecting the appropriate material for building the Vector

Once the clinical application and therapeutic genetic cargo have been determined, the composition of the delivery vector is the next consideration. Non-viral vectors can be broadly classified as either biomaterial or biologically-derived agents.

Biomaterial-based vectors—Conventional biomaterials are either lipid- or polymer-based and contain a net-positive charge [32-35]. These constructs can encapsulate or attach to the gene expression systems via electrostatic interactions, or other physical/chemical mechanisms [4]. Alternatively, researchers have recently developed inorganic nanoparticle-based nucleic acid delivery systems that act as single- entity agents capable of gene transfection and regulation without the need of auxiliary carriers or cationic transfection agents. Of these, gold nanoshell (NS)-based delivery vehicles are designed to control the release of genetic cargo by light-inducible mechanisms with pulsed laser irradiation [36]. For these NS-based systems, attachment of the genetic cargo is mediated by the electrostatic attachment between the negatively charged phosphate backbone of the nucleic acids and the cationic region of the gold nanoshells. Another system, spherical nucleic acid (SNA) nanoparticle conjugates, (gold nanoparticles covalently functionalized with small interfering RNA duplexes) mediate activity by interacting with scavenger receptors in the cell membrane, thus increasing cellular uptake [37, 38]. Regardless of the inorganic system, the most common attachment mechanism is by gold nanoparticles functionalized with alkylthiolated oligonucleotides through Au-S bonds [39].

The chemical structure of vectors can be altered through tuning of electrostatic interactions in order to reduce the risk of aggregation and ineffective delivery [54]. Specifically, vector stability can be increased through the formation of a hydrophilic shield via grafting of polyethylene glycol (PEG) [55, 56]. PEGylation inhibits premature clearance by masquerading vectors as water particles [57]. For example, PEGylated liposome with 15 mol % modification exhibited a higher blood circulating concentration (ID/mL) at 6 h after intravenous administration. The extent of improved stabilization was positively correlated with the length of PEG, ranging from 1 kDa to 40 kDa [58]. Furthermore, the size and shape of gene delivery vector can impact its blood clearance rate, tissue distribution, endothelial

uptake, intracellular transport, accumulation after endocytosis [59] [60]. Generally, vectors with sharp corners and edges have an increased probability of cytoplasmic translocation and a decreased probability of cellular excretion, which is a preferable trait for achieving high transfection efficiencies [61]. Although vector shape is a critical gene delivery parameter, its effects on biological interactions are not as clear as its physical behavior (e.g. degradation profiles). Finally, ligand-receptor modifications can be introduced to target specific cells and limit unintended off-target effects [62].

Biologically-derived vectors—Unlike biomaterial-based vectors, nonviral biologically-derived agents have unique features for mediate and enhance gene delivery efficacy. Engineered bacteria (e.g., *Escherichia coli*), for example, can deliver a diverse range of biomolecules in addition to nucleic acids [42-45]. Furthermore, *E. coli* possesses several traits that are advantageous for gene delivery: i) economically-feasible cell-growth kinetics, ii) established molecular biology techniques, iii) general size- restricted targeting, iv) adjuvant-like physical composition, v) superior innate or engineered endosomal escape mechanisms compared to biomaterials and vi) greater stability than biomaterial vectors [46, 47]. However, biologically-derived vectors often require complex synthesis and purification schemes and extended lead times are required when reengineering a vector for new situations. Hybrid combinations of biomaterial and biological vectors have been explored to harness the innate advantages associated with both delivery vector classes, further expanding the capabilities of gene delivery by combining two powerful experimental areas (i.e., polymer chemistry and molecular biology) [47-49].

Taking into account physiological factors—Gene delivery outcomes are mediated by cellular entry, achieved through either non-specific or receptor-mediated uptake mechanisms. However, some gene delivery strategies can bypass the uptake process entirely to gain immediate access to the cytosol [40]. For example, bactofection of mammalian cells (through either active invasion or passive uptake by phagocytic cells) can deliver pre-made mature mRNA to the cytoplasm of the target cell for immediate translation by the host cell machinery [41]. Upon uptake, genetic cargo is then transported intracellularly in endosomal vesicles prior to release into the cytosol through vector-specific mechanisms. These include lipid mixing or flip-flop mechanisms facilitated by cationic lipids or the proton sponge effect prompted by cationic polymers [1]. Alternatively, controlled release of the genetic cargo from inorganic nanoparticle- based nucleic acid delivery systems is triggered by light through either a thermal or non- thermal mechanism [36]. Other issues to be taken into account include vector stability in physiological media, circulation residence time, cytotoxicity, immunogenicity, off-target cellular uptake, and poor activity in non-dividing cells [50, 51]. Furthermore, if gene delivery vectors are to be administered directly into circulation, their residence time can be reduced through various environmental factors such as serum inactivation, enzymatic degradation, complement-mediated clearance, and reticuloendothelial system recognition [52, 53]. Therefore, vector composition must be considered to enable optimal gene delivery potential since the chemical, physical, and biological structure will primarily dictate the final gene delivery outcomes (Figure 2).

Experimental Strategies

In vitro setup and assay design

Efficacy—One limitation associated with *in vitro* testing of gene delivery pertains to the assays used to determine delivery efficacy (Table 2). For expression-dependent systems, transfection efficiency is typically determined using transgenic cargo driving expression of fluorescent or luminescent protein such as enhanced green fluorescent protein (EGFP) or firefly luciferase respectively, due to the ease of measuring and quantifying the expression level of these signal proteins. Although, these reporter protein techniques are often used interchangeably to validate gene delivery strategies, each separately describes only one metric of efficacy. For example, EGFP (and other proteins assessed via fluorescence-activated cell sorting [FACS]) are excellent at describing population-based expression metrics such as the percentage of cells expressing a desired transgene (i.e., gene delivery efficiency), whereas luciferase is better equipped to quantify gene expression magnitude within a population (i.e., the total protein expressed). Individually, these metrics provide an incomplete look at gene delivery, but when coupled with an assessment of cytotoxicity, characterization of gene delivery efficacy is improved and has been used as a predictor for *in vivo* success [46, 47, 65].

Alternatively, gene delivery can be utilized to modulate gene expression therapeutically by decreasing expression of a target gene (e.g., RNAi). The efficacy RNAi to inhibit expression of target genes is generally assessed by detecting reduced levels of mRNA and proteins. This typically involves assays such as quantitative PCR (qPCR) or RNA sequencing (RNA-Seq) for RNA measurements or western blots for protein measurements. However, while conducting these experiments, it is important to evaluate potential off-target effects [66, 67]. This is especially important considering that such off target effects can reduce cellular viability by nearly 80%, potentially compromising cytotoxicity tests [24]. The benefit of using qPCR and RNA-Seq is that they can also evaluate the expression of housekeeping genes to provide a baseline for expression. One important consideration in delivery of RNAi cargo is that the level of gene expression does not directly indicate that the desired phenotypic change has been elicited. This may occur in situations where only a small amount of protein is necessary to perform a specific cellular function or when the function of the target gene can be achieved through alternative means, thus targeting multiple genes for RNAi-based gene therapy is often desirable [68]. This was demonstrated in a study which increased repression of tumor growth from two-fold using individual siRNAs to seven-fold using three different siRNAs [69]. It is therefore necessary to supplement gene expression assays with additional tests that determine phenotypic impact of the delivered genetic cargo.

Cytotoxicity—One of the important properties evaluated with *in vitro* gene delivery is cytotoxicity, generally assessed by the MTS and MTT assays, which determine cell viability through a colorimetric measurement of cellular mitochondrial activity (Table 2). Within *in vitro* confines, stress-induced increases in mitochondrial activity can lead to underestimations of cytotoxicity [70]. Furthermore, conditions such as pH and the presence of metal ions can interfere with the assay, further confounding results [71]. To address these

limitations, it is desirable to confirm the results with additional experiments. For instance, techniques such as microscopy or the growth of colony forming units on a plate can enable visual estimations of cell viability to support information gleaned from MTS and MTT assays. Ultimately, the conventional settings for such tests represent an artificial environment that cannot fully imitate the complexity of an *in vivo* system. However, *in vitro* cytotoxicity experiments represent a critical aspect of preclinical research and substantial cell damage measured with these assays is an initial indicator of vector-associated drawbacks. Alternatively, low levels of cytotoxicity are a promising start to gene delivery therapies that must be tested further in more physiologically-relevant settings [72, 73].

Integrity of the vector—Once delivery vectors are introduced *in vivo*, they are subjected to various forms of inactivation or clearance that are not accounted for in *in vitro* tests (Table 2). In addition to mechanisms for bodily clearance, there are various components of human serum that interfere with gene delivery through interactions with the delivery vector [74]. Typically, when the binding affinity of the vector to albumin is low ($<10^6$ M) the kinetics for such processes are often relatively slow compared the rates of clearance and tissue distribution [74]. However, when the binding affinity is higher (10^7 - 10^9 M), binding to serum proteins becomes significant, and should be considered in *in vitro* models [74]. These limitations can be accounted for with accommodations to current *in vitro* tests to improve their simulation of blood composition (e.g., adding serum) and circulatory flow.

To protect the cargo from degradation through mechanisms such as macrophage uptake, approaches like PEGylation can be used [75]. Unfortunately, the PEGylation of vectors often interferes with effective gene delivery *in vivo* by impairing membrane interactions [76]. It is therefore desirable to design modified cationic polymers that improve gene delivery by protecting their cargo, until preferentially degrading near the desired delivery target [77, 78]. The initial *in vitro* experiments conducted should ensure that both processes are performed in series, rather than in parallel. For instance transfection experiments using vector that has been previously subjected to stability testing helps ensure that the surface modification is capable of both providing protection and degrading to enable cellular uptake. Alternatively, transfection efficiencies could be determined by using a microfluidic device that subjects the vector to physiological conditions prior to reaching the target cells. Upon successful delivery to the targeted cell or tissue, the foreign expression system must successfully navigate additional barriers including escape of endocytosis ($<2\%$ success rate) [79, 80]. Upon endosomal escape, the expression system may require successful nuclear translocation, which is dependent on the type of cell being targeted. Nuclear translocation is considered to be a highly inefficient process that depends upon disruption of the nuclear membrane, which typically occurs during cell division [81]. This becomes problematic when the cellular target for gene delivery is a slowly growing or non-dividing cell line. In such cases, translocation must occur through nuclear pores, which have size- and signal-related (i.e., nuclear localization signal) constraints [82]. Therefore, it is necessary for *in vitro* experiments to employ a cell type that displays similar metabolic and mitotic rates as the intended *in vivo* target.

Replicating in vivo environments—An additional consideration is to ensure that the physical design of *in vitro* experiments accurately represents *in vivo* conditions. For instance, many *in vitro* studies evaluate gene delivery using cells that are adhered to a surface [83, 84]. A limitation associated with this design is that in the undesirable case of vector aggregation, aggregates can still interact with cells and demonstrate gene delivery [1, 85]. This can lead to the detection of false-positives that will likely be unsuccessful *in vivo* [1]. Furthermore, experiments often utilize long-duration transfections containing high concentrations of DNA not realizable under physiological conditions. More specifically, a several-hour long transfection, chosen to optimize expression within cells in a static closed environment [86], fails to account for the convective transport associated with circulatory flow. As such, transfection efficiencies determined under such *in vitro* conditions will likely be dissimilar to *in vivo* performance since the vector's ability to deliver its cargo to the desired tissue while also rapidly flowing through the circulatory system is not being challenged (Figure 3A). An attractive alternative would be to utilize microfluidic technology to more accurately represent the kinetics and transport conditions relevant for physiological gene delivery [87, 88]. Furthermore, utilizing 2D or 3D microenvironments can affect gene delivery by modulating cellular physiology [89]. Cells cultured in 3D environment interact and remodel their microenvironment and can develop into complex structures, effectively recapitulating *in vivo* phenotypes. Gene delivery experiments utilizing 3D environments provide the ability to assess challenges such as mass transport, physical forces, and cell microenvironment interactions that are important in *in vivo* systems. Yet, 3D cultures have not become the standard approach in the field of gene delivery due to difficulty in the cellular and molecular analyses [90].

Furthermore, if treatment is intended for specific targets (e.g., tumor masses), *in vitro* models using direct delivery to layered or suspended cells cannot accurately predict the risk of off-target effects (Figure 3) [91]. It is also important to balance the need for selective targeting of the desired tissue with the clearance rate of the vector itself. These conditions therefore suggest a mechanism for delivery that must be both target-specific and possess a clearance rate that is low enough to ensure that the cargo can be delivered effectively. However, if the clearance time for the gene therapeutic is too long, significant off-target effects can be observed [92]. These concerns can be addressed by avoiding the use of monoclonal cell lines for *in vitro* tests and determining the specificity of gene delivery in a diverse cellular population in which a specific cell type is the desired target. In addition, if delivery to a mass of cells such as a tumor is desired, it is also important to recognize the transport-limitations of the genetic material into the cell mass [93]. Typically, such processes benefit from the enhanced permeability and retention (EPR) effect *in vivo*, which results in better transport of macromolecules across the tumor endothelium due to its structural properties [94]. This can enable accumulation of therapeutics at tumor sites that is 10-50 times higher than background [95]. However, not all tumors are equally permissive; it is therefore necessary to possess an understanding of the tumor architecture to inform the design the delivery vector's particle size [94] and to conduct *in vitro* tests using tumors with similar physical properties.

Cell type—Another key difference between typical *in vitro* experiments used to assess gene delivery efficacy and the vector's actual physiological application is the cell type used in such *in vitro* tests (Figure 3). Immortal cell lines are often used in *in vitro* experiments due to their indefinite capacity for cellular division from monoclonal populations. This has the advantage of providing genetically identical cellular populations that can be evaluated over extended durations since they are not prone to senescence. These advantages come at a cost, however, since the cells in human and animal models possess different regulation and unrestrained cellular division is abnormal behavior (and usually only present in cancerous instances). In addition to this inaccurate representation of *in vivo* cellular behavior, immortalized cell lines further confound *in vitro* testing due to their continuous and potentially rapid ability to divide and therefore disrupt the nuclear membrane. This can serve to enhance nuclear translocation of transgene elements in immortal cell lines compared to somatic cells present in *in vivo* models, leading to overestimations of the efficiency of gene delivery [96]. Consequently, the use of immortal cell lines in *in vitro* gene delivery experiments can yield false- positive results that subsequently fail in *in vivo* delivery. One potential solution is to complement tests using immortal cell lines with primary cell lines (serving as a better mimic of *in vivo* cell types) in side-by-side *in vitro* analyses.

Clinically predictive animal models

Animals are commonly used in biomedical research as models for human disease in the context of gene delivery, as well as drug discovery and development. Furthermore, use of animal models allows for extensive testing to estimate and refine dosage regimens, and predict human safety profiles [97-102]. Arguably, their most prominent role is in the therapeutic assessment (toxicology and activity) of potential gene and drug candidates, with positive results representing the gold-standard of preclinical research [97]. Despite their heavy usage, animal models imperfectly predict human responses, and results from animal models cannot always be replicated in clinical trials. Paradoxically, successful validation in animal models is an entry-requirement for all human-based clinical trials [103]. Therefore, it is important to conduct experiments using animal models in a manner that consistently translates to clinical success.

An ideal model associated with developing gene delivery strategies is one that replicates the manifestation and end-phenotype of a specific human disease while responding similarly to established standard of cares. Specifically, an ideal model should possess all of the following features compared to humans: i) similar biological makeup (anatomy and physiology; genetic context), ii) similar disease causation and pathological response(s) (especially phenotypic end-points of clinical studies), and iii) predictive validity (i.e., humanized mice). However, in most cases, the progression of a disease is not fully understood, which results in non-orthogonal observations and conclusions. Even when disease progression is similar, dissimilarities in the model and patient reactions to chemical entities challenge the context and validity of animal usage. For example, genetically engineered mice (*Mus musculus*) are the most sophisticated animal models of human cancer, mimicking the pathophysiological and molecular features human malignancy. Though mouse cancer models have contributed to our understanding of cancer biology, several limitations remain, such as restricted subset of tumour types, limited recapitulation of *de novo* human tumour development, and drug

response, thus inhibiting our capacities to develop corresponding gene therapies [104]. Various animal models have been developed to study particular aspects of the disease; however, they must be used in collection to have any predictive validity in potential gene therapy [105- 107]. These shortcomings demonstrate the challenge with animal models beyond providing basic scientific studies and preliminary screening procedures.

Another major pitfall in establishing clinical validity of animal models is the use of genetically identical inbred animal strains in some disease models. Using transgenic animal models enables unprecedented insight into phenotypically-linked disease progression, but fails to properly represent the notoriously diverse genetics, nutrition, and lifestyles present in the human population. Going forward, inbred animal strains may still be utilized to establish or initially reduce the number of potential therapeutic candidates, but use of outbred animal strains or multiple animal strains and/or species would improve the fidelity of translation to human clinical applications. Similarly, evaluation of both animal genders will likely increase predictive validity. Given the substantial shortcomings of any one animal model, researchers are advised to use a battery of individually-diverse animal models. When evaluated collectively, they can be utilized at various stages in the preclinical process to answer specific experimental questions. For example, testing of late-stage pre-development cancer therapeutics in a select array of complementary animal models will better represent the genetic and phenotypic heterogeneity of human cancers that will provide more robust data sets and stronger clinical recommendations [108].

Manufacturing considerations

Several criteria must be met when designing gene delivery vectors that will eventually be produced at industrial scale and used in a clinical setting. Namely, the manufacturing pathway should possess the following features to become feasible: i) ease of fabrication/ uniformity control, ii) inexpensive synthesis, iii) facile purification, iv) noninvasive administration, and v) cheap storage for maintaining stability [109]. Aqueous complexation of lipid/polymer vector and nucleic acid cargo is the most common preparation procedure to form nanoparticles used for administering gene therapy [110]. However, despite the simplicity associated with aqueous formulation, this process demonstrates batch-to-batch variability that is unacceptable in a clinical setting. One potential solution is the application of mechanical preparatory methods, such as PRINT (particle replication in non-wetting templates), that confer particle uniformity [111].

The requirement for commercialization of any gene therapy strategy that is most overlooked in preliminary stages is the need for inexpensive synthesis and formulation. Most nonviral vectors are generally incapable of becoming clinically relevant without extensive chemical modifications that usually require multiple rounds of engineering to perfect. Successful clinical development requires that sufficient gene delivery performance is achieved without incurring expensive synthetic schemes. Interestingly, even when a particular nonviral vector requires an expensive synthesis scheme, the cost is marginal when compared to viral alternatives [3]. More specifically, the development of robust and scalable processes for mass production of viral vectors (which include labor- intensive cell culture and purification steps) remains an economic challenge that impedes potential therapeutic use [112].

With respect to practical application, traditional (needle-based) administration of gene therapeutics is hampered by the following disadvantages: refrigeration-costs for liquid formulations, personnel training requirements for administration, needle-based injuries, and patient compliance. To overcome these, Irvine and coworkers developed a polymer film tattooing technology, which offers a dry skin-patch administration platform that is pain-free and self-administrable [113-116]. The solid phase *in situ* polyplex formation is based on a layer-by-layer (LBL) formation approach, successfully avoiding the disadvantages of traditional administration methods.

Lastly, the robustness and stability of gene therapy formulations is strongly associated with storage methods. For instance, freeze-drying and vacuum-drying-based storage [117], as well as sugar-based formulations [118], can provide efficient preservation for most genetic materials and vector complexes via water replacement. Unfortunately, however, these processes have not been thoroughly optimized to maintain conformational stability [119], retain biological activity [120], and elongate dose shelf life [121, 122]. The ideal production and distribution process would provide a cost-effective gene therapy agent capable of non-cold storage maintenance to enable global access to therapeutics.

Concluding remarks and future perspectives

In recent years, substantial technological advancements in nonviral delivery vectors and nucleic acid chemistry have reinvigorated exploration of gene therapy as a potent clinical application. Coupled with the maturation of genomics and recombinant DNA technology, these technological advancements may enable gene therapy to provide an avenue for remedying diseases considered “untreatable/undrugable” by conventional approaches. However, despite these strides, the broad clinical application of nonviral vectors has yet to come to fruition. The lack of clinical success is partially caused by the experimental models implemented in preclinical research. When these models inaccurately represent relevant physiological conditions, translation into clinical success is unlikely. Consequently, with revisions to contemporary *in vitro* and preclinical models, novel gene therapy strategies can be developed that have significant clinical potential.

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Outstanding Questions

Can current *in vitro* gene delivery methodologies be further optimized to result in clinically-predictive results that can expedite the screening process of potential therapeutics?

What will be the best gene expression strategy (i.e., transiently-active vs genome-editing) to target diseases derived from epigenetic switches, which control gene action and have an obscure effect upon disorders like cancer and Alzheimer's disease?

Can the concept of cellular-targeting or controlled release via rationally designed delivery vectors limit potentially off-target effects and conversely, can these strategies enable the targeted delivery to rare cell types that may be dispersed in various anatomical sites and may also be defined by more than one surface marker?

Even if the all technical barriers are overcome, companies will still face substantial financial concerns: What is a suitable price and payment structure for gene therapy products? Will single administration therapies (i.e., genome-editing) require a different structure? Will radical solutions such as the 'pay-for-performance model' disincentivize large pharmaceutical companies to develop new, potentially life-saving products?

Trends

The rise of CRISPR-mediated genome editing provides a powerful tool for application in genetic engineering applications. Although recent studies using human embryos demonstrated off-target effects, CRISPR may eventually demonstrate utility in gene therapy applications.

Within complicated human systems, there is a greater potential for unintended side effects that may not be observed in preclinical studies. An example of this is the occurrence of leukemia in patients receiving treatment for X-linked Severe Combined Immune Deficiency using adeno-associated viral vectors.

Clinical translation is often impaired by limitations associated with the in vitro experiments conducted to validate the vector formulations. These assays often assess only one aspect of gene delivery in a non-therapeutic context. For instance, many clinical trials have demonstrated limited efficacy due to the loss of transgene expression over time.

Another impediment is the tendency for in vitro experiments to poorly represent physiological conditions. Vector circulation and clearance, as well as nuclear translocation of the genetic cargo are critical aspects of gene delivery that are not adequately addressed using conventional experimental design. For instance, immune responses to treated cells impaired the efficacy of gene therapy approaches for treating haemophilia B and lipoprotein lipase deficiency.

Figure 1.

General gene delivery mechanism. Upon assembly of the chosen nucleic acid cargo with the delivery vector construct, the composite particles must traverse various extracellular barrier (e.g., serum endonucleases) followed by gaining cellular entry through endocytosis (depicted in the figure) or by other means. Following uptake, particles modulate gene expression by either in the cytosol (expression-independent) or in the nucleus (expression-dependent). Regardless of the strategy selected, a gene delivery vector must successfully navigate *in vitro* and *in vivo* testing and GMP manufacturing prior to entering clinical testing.

Figure 2.

Compositional design considerations for three representative properties of nonviral gene delivery vectors. Genetic cargo is surrounded by a coating (green outline) that provides protection and/or cell-specific targeting (top left). Furthermore, the vector itself can be designed from a variety of different chemical compositions (top right) and constructed into various shapes (bottom left). This flexibility in compositional design allows researchers to tune gene delivery vectors for specific clinical applications.

Figure 3.

Physical design considerations of *in vitro* systems vs. *in vivo* conditions. Gene therapy studies typically incubate delivery transfection agents (red [A] and blue [B]) under conditions that are not representative of systemic *in vivo* circulation. One such condition is the use of long incubation times (A), which are physiologically unrealizable and can be avoided by using microelectromechanical systems (MEMS). Additionally, *in vitro* measurements of preferential cell uptake are usually validated using homogenous cell populations in media lacking relevant levels of potentially interfering serum proteins (green particles) (B). Transfection studies can be improved by determining delivery specificity metrics in physiological media with heterogeneous cell populations to accurately predict non-specific interactions with serum proteins and off-target delivery respectively.

Table 1

Breakdown of US gene therapy clinical trials.

Disease	Vector	Gene Target	Trial Number
Phase I			
Leber's Hereditary Optic Neuropathy (LHON)	Adeno-Associated Virus	G11778G ND4	NCT02161380
Late-Onset Pompe Disease	Adeno-Associated Virus	α-Glucosidase	NCT02240407
Late Infantile Neuronal Ceroid Lipofuscinosis	Adeno-Associated Virus	CLN2	NCT01161576
Parkinson's Disease	Adeno-Associated Virus	Aromatic L-Amino Acid Decarboxylase	NCT01973543
α1-Antitrypsin Deficiency	Adeno-Associated Virus	α1-Antitrypsin	NCT02168686
Leber Congenital Amaurosis	Adeno-Associated Virus	RPE65	NCT00821340
MERTK-Associated Retinal Disease	Recombinant Adeno-Associated Virus	MERTK	NCT01482195
Sickle Cell Disease	Lentivirus (<i>ex novo</i>)	Anti-Sickling (βAS3)	NCT02247843
Fanconi Anemia	Lentivirus	Fanconi Anemia Complementation Group A	NCT01331018
Recurrent Malignant Glioma	Retroviral Replicating Vector	Cytosine Deaminase	NCT01156584
Solid Tumors	Liposome	RB94	NCT01517464
Phase II			
Heart Failure	Adeno-Associated Virus	Sarcoplasmic Reticulum Calcium ATPase	NCT01966887
Blindness Caused by Choroideremia	Adeno-Associated Virus	Rab-Escort Protein 1	NCT01461213
Hemophilia B	Adeno-Associated Virus	Human Factor IX	NCT02484092
Malignant Pleural Effusion	Recombinant Adenovirus	p53	NCT02429726

Disease	Vector	Gene Target	Trial Number
Malignant Melanoma and Other Solid Tumors	Adenoviral serotype 5	CD40L	NCT01455259
β -Thalassemia Major	Lentivirus (<i>ex novo</i>)	β -A(T87Q)-Globin	NCT01745120
HIV-1	Lentivirus	shRNA For CCR5, HIV-1 Fusion Inhibitor, C46	NCT01734850
Transfusion Dependent β -Thalassemia	GLOBE Lentivirus	Human β -Globin	NCT02453477
Wiskott-Aldrich Syndrome	Retrovirus	Wiskott-Aldrich Syndrome Protein (WASP)	NCT01410825
Severe Combined Immunodeficiency, X-linked (SCID-X1)	Self-Inactivating (SIN) Gamma-retrovirus	IL-2RG	NCT01129544
X-linked Chronic Granulomatous Disease (X-CGD)	SIN Gamma-retrovirus	gp91phox Subunit of NADPH Oxidase Complex	NCT01906541
Leukaemia	GMP Grade Retrovirus	Wilms Tumor 1	NCT01621724
Non-Hodgkin Lymphoma	Autologous T Cells	CD19 CAR	NCT02134262

Phase III

Myocardial Ischemia	Adenovirus Serotype-5	Fibroblast Growth Factor-4	NCT01550614
Duchenne Muscular Dystrophy	Lentivirus	AON for Exon 51 of Dystrophin	NCT02255552
Painful Diabetic Neuropathy	Non-Viral	Hepatocyte Growth Factor	NCT02427464

Table 2

General gene delivery clinical translation hurdles.

Conventional Approach	Limitations	Potential Solutions
Cytotoxicity		
MTT, MTS assay	<ul style="list-style-type: none"> Stress-related changes in activity Interference from pH, etc. 	
Transfection efficiency		
Fluorescent reporter (e.g., EGFP)	<ul style="list-style-type: none"> Variation of expression levels Global expression changes not tested 	<ul style="list-style-type: none"> Couple with an assay that compares transgene expression to housekeeping genes (e.g., qPCR)
Use of layered or suspended cells	<ul style="list-style-type: none"> Typically in static culture that doesn't account for circulation Aggregation of vector can give false-positive results 	<ul style="list-style-type: none"> Use microfluidic device to simulate blood-flow
Use of immortalized cell lines (e.g., HeLa cells)	<ul style="list-style-type: none"> Cell lines continuously divide, improving the efficiency of nuclear translocation 	<ul style="list-style-type: none"> Use somatic cells that accurately reflect division rates of target cells
Long duration of transfection (~4 h)	<ul style="list-style-type: none"> Probability that a vector could circulate through the body four hours in a constant volume is likely inaccurate. 	<ul style="list-style-type: none"> Design transfection apparatus to simulate physiological flow of the vector.
Vector stability		
Tests are run in simple formulation	<ul style="list-style-type: none"> Lacks proteins present in blood that can interact with vector 	<ul style="list-style-type: none"> Conduct desired experiments in serum
Protection of cargo and subsequent release at target site.	<ul style="list-style-type: none"> Protective coating needed to ensure stability in blood Coating can interfere with cellular uptake if gene not released 	<ul style="list-style-type: none"> Test under conditions which require both stability away from target and specific release at it.