Nonviral gene delivery: techniques and implications for molecular medicine

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Medical research continues to illuminate the origins of many human diseases. Gene therapy has been widely proposed as a novel strategy by which this knowledge can be used to deliver new and improved therapies. Viral gene transfer is relatively efficient but there are concerns relating to the use of viral vectors in humans. Conversely, nonviral vectors appear safe but inefficient. Therefore, the development of an efficient nonviral vector remains a highly desirable goal. This review focuses on the numerous challenges preventing efficient nonviral gene transfer in vivo and discusses the many technologies that have been adopted to overcome these problems.

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The appeal of gene therapy lies in its promise to provide elegant cures for serious disease, for treatment at both the somatic-cell and germline level. One of the major benefits of the Human Genome Project will be the correlation of specific genetic mutations and the disorders to which they contribute, enabling identification of a host of new targets for gene supplementation therapy. However, the major factor limiting successful gene therapy is not the identification of candidate genes, but the difficulty of expressing new copies of therapeutic genes adequately within target cells and tissues. Some of these problems were addressed in a recent article in *Expert Reviews in Molecular Medicine* on the use of gene therapy in cancer (Ref. 1).

Most patients treated so far in gene therapy trials have had advanced disease that is not amenable to other treatments. The small number of these studies reflects the numerous technical challenges experienced by investigators and disappointment arising from the therapeutic effect achieved. To date, the majority of gene therapy clinical trials performed have used viral vectors for gene delivery. However, there are major practical limitations to viral gene delivery systems, including difficulties of scale-up production, lack of selectivity, inadequate infectivity in vivo, susceptibility to neutralisation by serum antibodies (particularly on second and subsequent administrations) and inflammatory responses raised against successfully transduced cells. Although intense efforts are under way to overcome these problems, some are hard to address and may be insurmountable. Consequently, considerable attention is turning towards the development of nonviral systems for gene delivery, using basic molecular engineering to design vectors capable of adequate gene delivery without the baggage inherent in millions of years of evolution as a niche pathogen.

The purpose of this review is to describe some of the agents being evaluated as alternatives to viral gene delivery and to identify the major challenges and opportunities facing the field. Several nonviral gene delivery systems are under development, with varying levels of sophistication (Ref. 2). Some of these involve design of vectors capable of delivering genes to target cells in biological environments, whereas others are processes or techniques that can be applied to tissues or cells to improve efficiencies of gene expression using established vectors.

Systems for nonviral delivery of nucleic acids

Use of naked DNA

The simplest nonviral gene delivery vector is naked plasmid DNA. Until recently, systemic gene delivery with naked DNA was considered unrealistic, since plasmid vectors can be rapidly degraded and neutralised by endogenous DNases. Additionally, the phosphate group on the deoxyribose rings of DNA confers a net negative charge to the molecule, limiting the potential for electrostatic interaction with the anionic lipids in the cell membrane. Understandably, this has been considered a prerequisite for cell entry, and therefore a sine qua non for transfection.

However, the potential for correction of genetic defects by direct use of plasmid DNA might be greater than previously thought. Zhang et al. (Ref. 3) demonstrated successful transgene expression in hepatocytes following rapid intravenous administration to mice of large volumes of transfectate (up to ~2.5 ml) via the hepatic vein. A similar strategy using a mouse tail vein injection has also been used (Ref. 4). These hydrodynamic-based techniques have efficiently delivered therapeutic genes coding for growth hormone (Ref. 5) and human factor IX (Ref. 6) to the liver. The mechanism of action is thought to involve transient overload of the right side of the heart, leading to retrograde flow of undiluted injectate (without serum protein) via the portal vein into the hepatic microvasculature. The increased pressure expands pores in the endothelial layer, increasing extravasation of the injectate and leading to hepatocyte transfection (Ref. 7). Other investigators have adopted similar techniques to deliver naked DNA to skeletal muscle using arterial catheters (Ref. 8), or to ischaemic myocardium by retro-infusion of cardiac veins (Ref. 9). Although levels of gene expression using this approach have been impressive (absolute percentage of cells expressing transgene product = 1–40%), the clinical practicalities of this approach are uncertain and scale-up problems might restrict the possibility of achieving an effect in larger-animal models.

Lipoplexes

Theoretically, cationic carrier molecules might complex with DNA and neutralise its electrostatic charge, thereby promoting cell-membrane–DNA interaction and increasing transfection efficiency. Complexation of cationic lipids with DNA

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(Ref. 10) was first described in 1987, and 'lipofection' was reported to be 5- to >100-fold more efficient than the earlier calcium phosphate or the DEAE (diethylaminoethylene)-dextran transfection techniques. Cationic lipids are highly soluble in aqueous solution, forming positively charged micellar structures termed liposomes. Contemporary liposome preparations contain cationic lipids but are dependent on a neutral or helper lipid [usually dioleoylphosphatidylcholine (DOPE)] to provide effective transfection. Complexes formed by self-assembly of DNA with liposomes are generically known as lipoplexes.

It was initially suggested that several liposomes might associate with a single plasmid molecule to render it charge neutral, condensing the DNA to form a small dense lipoplex (Ref. 11). However, electron micrograph studies have produced images of lipoplexes with a range of macromolecular structures (Refs 12, 13, 14). Immediately after complexation or at low DNA concentrations, multiple liposomes appear to abut with DNA sandwiched between. Different complexes emerge later, which might vary depending on charge ratio, lipid formulation and mode of preparation. Condensed lipoplexes are seen with diameters of 100–200 nm, and also elongated, 'spaghetti'-shaped, lipoplexes, which are thought to represent DNA surrounded by a lipid uni- and/or bilayer. Large aggregates or 'meatball' lipoplexes are also observed, and thought to comprise numerous lipid and DNA molecules. Precisely which of these represents the most transfection-efficient fraction is not clear. Lipoplexes have been clearly seen close to endocytotic pits and within endocytotic vesicles just below the cell membrane (Ref. 12). Lipoplexes are thought to be internalised by endocytosis (Refs 15, 16), although fusion with the cell membrane (Ref. 17) or disruption of the cell-membrane lipid bilayer have also been proposed (Ref. 18). Larger aggregated lipoplexes might be internalised by phagocytosis (Ref. 19).

Once the lipoplex has been internalised to the endosomal system, rapid mixing of cationic (liposome) and anionic (endosome) lipids might disturb the endosomal membrane (Ref. 20). The presence of the neutral lipid DOPE in the liposome is thought to promote endosomal rupture, purportedly by a mechanism involving transition from a bilayer phase to an inverted micellar structure (Ref. 21). The vast majority of the time, the endosome will mature and fuse with lysosomes, where DNA will be degraded, and no gene expression will occur. Rarely, during lipid mixing the endosomal wall will rupture, and although most of the encapsulated DNA will remain bound to the lipid, some will manage to escape into the cytoplasm and traffic to the nucleus, culminating in gene expression. For a summary of lipoplex gene delivery see Figure 1.

Lipoplexes are powerful systems for introducing plasmids into target cells; however, their hydrophobic and positively charged surface frequently leads to interactions with plasma proteins and other extracellular proteins, which bind nonspecifically to the lipoplexes and might inactivate them (Ref. 22). In this regard, protein-resistant lipoplexes have been developed (Ref. 23). The effect of cell-membrane-associated and extracellular-matrix-associated proteoglycans on lipoplexes has been neglected for a long time. Many of these proteins are sulphated, and therefore are negatively charged, allowing them to interact with positively charged lipoplexes. Gene expression is abolished or markedly inhibited by the most heavily sulphated of these proteins, including the glycosaminoglycans (GAGs) heparin, heparan sulphate and chondroitin B (Ref. 24). There is some evidence that these proteins can compete with DNA contained within the lipoplex, causing it to dissociate away from cationic carrier molecules (Ref. 25). Most of the GAGs appear to adhere to the complexes and are involved in the process of internalisation. Individual proteoglycans might dictate the efficiency of internalisation and the intracellular compartment into which the DNA is delivered, since different proteoglycans appear to inhibit (or even enhance) gene expression to a varying degree (Ref. 26). It is also feasible these proteins regulate intracellular transport and exert an affect on gene transcription/translation by a pathway that is as yet unknown.

Although lipoplexes often show high levels of transgene expression following direct administration or injection into target tissues (Refs 27, 28, 29, 30), their nonspecific membrane activity usually precludes cell-selective targeting. This leads to indiscriminate transgene expression in cells present at the site of administration. Furthermore, a major problem with the application of most nonviral systems, including lipoplexes, is their poor efficiency at transfecting nonproliferating cells. This is thought to be mainly a result of the integrity of the nuclear membrane providing a physical barrier to entry.

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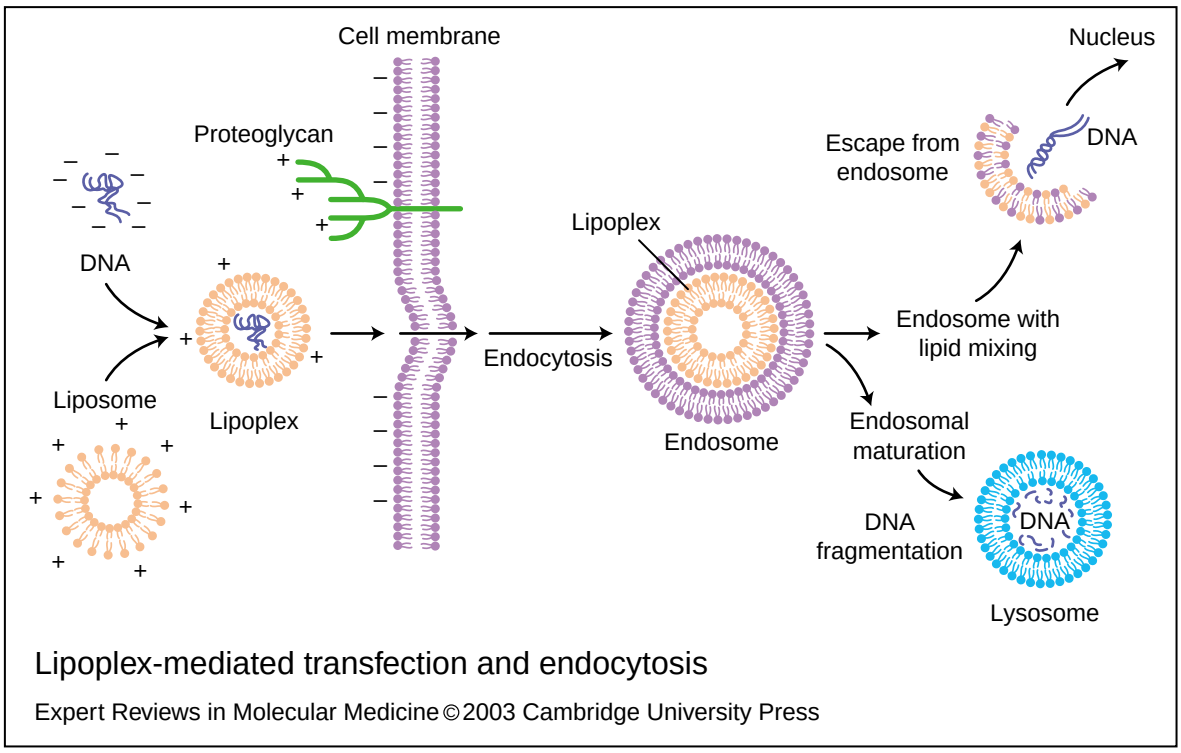

Figure 1. Lipoplex-mediated transfection and endocytosis. Cationic lipids forming micellar structures called liposomes are complexed with DNA to create lipoplexes. The structures fuse with the cell membrane, at least sometimes after interactions with surface proteoglycans. The complexes are internalised by endocytosis, resulting in the formation of a double-layer inverted micellar vesicle. During the maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained DNA into the cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression. Alternatively, DNA might be degraded within the lysosome **(fig001cns)**.

Polyplexes

Polyelectrolyte complexes formed by self-assembly of DNA with cationic polymers are referred to as polyplexes. When plasmid DNA is mixed with cationic polymers, in appropriate ratios and solvents, the resulting complexes are usually nanoparticulate (<100 nm) and surprisingly homogenous (cf. lipoplexes). Several cationic polymers have been evaluated for their ability to form nanoparticles with DNA, the most significant being poly-lysine (pLL) and poly-ethylene-imine (pEI) (Refs 31, 32, 33). It is clear that the properties of the complexes formed are largely determined by formulation conditions (Ref. 34) as well as polymer parameters (Ref. 35), including molecular weight and charge density. Although the majority of studies have involved pLL, probably the most promising complexes under development are those based on pEI, since they often exhibit remarkably high transfection activity.

The mechanism underlying the enhanced transfection rates with pEI is unclear, although it is widely stated that its ability to enhance transgene expression involves an endosomal buffering capacity. It is proposed that multiple protonatable amine groups on the polymer act as a 'proton sponge' that quenches lysosomal acidification and prevents DNA degradation (Ref. 36). Consequently, increased osmolarity of the endosome might cause turgidity, rupture of the endosomal membrane and release of entrapped DNA complexes into the cytoplasm and promoting subsequent delivery to the nucleus. However, if pEI truly possesses endosomolytic properties, it is surprising that inclusion of other lysomotropic agents (e.g. chloroquine; see later) markedly increases gene expression (Ref. 37). One further possibility is that protonation of pEI leads to an expansion of the polymer structure, which produces physical swelling and endosomal disruption (Ref. 38).

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Determining the mechanisms by which the most successful polyplexes navigate the cellular barriers to transfection is crucial, and experiments using microinjection of complexes directly into the cytoplasm or nucleus separate the effects of internalisation, endosomal escape and nuclear import. Cytoplasmic microinjection of pEI–DNA complexes appears as efficient as simple polyplex transfection, implying internalisation is efficient and not the rate-limiting step. Cytoplasmic injection of DNA complexed with lipid, pLL or pEI results in 1%, 5% and up to 50% of cells exhibiting gene expression, respectively. Approximately 1% of plasmid molecules complexed with pEI and injected into the cytoplasm are calculated to reach the nucleus – greater than ten times more than the fraction for other polyplexes. Time to gene expression is also shorter, perhaps indicating cytoplasmic transport is enhanced. It has been proposed that the multiple positive charges on pEI mimic a nuclear import signal, which might also account for these findings (Ref. 39). In support of this, pEI complexes are less dependent on cell division to achieve gene transfection than either pLL- or lipid-based complexes (Ref. 40). Whereas nuclear injection of lipoplexes rarely results in gene expression, implying complex disassembly is not possible in the nucleus, polyplex nuclear injections do result in gene expression. Indeed, nuclear injection of pEI complexes or DNA alone result in similar rates of expression (~40–50%) (Ref. 39).

Similar to lipoplexes, polyplexes are cationic, rendering them prone to nonspecific interactions with plasma proteins. Complexes formed with pEI, rather than pLL, may be more susceptible to these effects (Refs 25, 26). Targeting ligands can be incorporated into the polymers to mediate entry into cells by binding cognate receptors (Refs 41, 42). This can lead to increased levels of uptake into receptor-positive cells, often with corresponding increases in transgene expression. Recent studies have shown that RNA can also be delivered as polyplexes (Ref. 43), provided the molecular weight of the cationic polymer is small. Peptides can also be incorporated into these systems to enhance selectivity and efficiency of expression (see later).

Polyplexes therefore represent a versatile strategy for gene delivery that is likely to fulfil several requirements. For a summary of the concept of targeted polyplex delivery see Figure 2.

Enhancement of transgene expression using electroporation

Local administration of plasmid DNA coupled with application of an alternating electrical field – electroporation – has been shown to yield high levels of transgene expression in the liver (Ref. 44), testes (Ref. 45), arteries (Ref. 46), skin (Ref. 47) and tumours (Ref. 48). The electrical pulse is thought to disrupt the cell membrane, forming transient pores through which DNA complexes can pass. However, high-frequency electrical fields applied for a long duration can cause local tissue damage and inflammation (Ref. 49). The most promising application of electroporation is to enhance plasmid-mediated gene expression in skeletal muscle, which might be useful for inherited muscular dystrophies or as an ectopic site from which a recombinant therapeutic protein can be secreted into the bloodstream. Using electroporation in a rat hind limb, erythropoietin

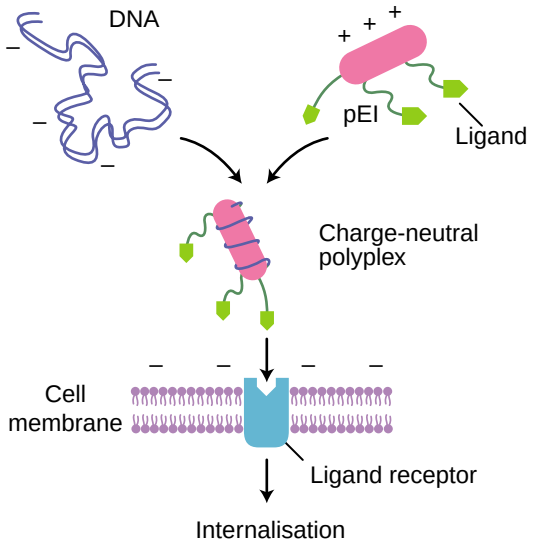

Figure 2. Receptor-mediated internalisation of ligand-labelled polyplexes. DNA can be complexed with cationic polymer (pEI; poly-ethylene-imine) that has been conjugated with ligands specific to a target cell type. These polyplexes can be used to target gene expression by docking with the cognate receptor **(fig002cns)**.

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(EPO) gene transfer was increased 100-fold, resulting in elevated serum erythropoietin (sEPO) levels for more than 3 months and increased haematocrit beyond 6 months, after a single administration. Although impressive, this was dependent on a high plasmid DNA concentration (50 μg kg^{-1}) to achieve physiologically active sEPO concentrations (pg l^{-1}). Most therapeutic molecules would require substantially greater physiological concentrations (ng l^{-1} to μg l^{-1}), necessitating multiple injection and electrode sites. Additionally, it is difficult to envisage how this technology could be applied to transfect internal organs.

Enhancement of transgene expression using ultrasound

Exposure to low-intensity ultrasound decreases the 'unstirred' layer adjacent to the membrane, temporarily increases cell membrane permeability and encourages the membrane-destabilising phase transition of the lipofection reagent DOPE (Refs 50, 51). These properties suggested that adjunctive ultrasound exposure (USE) could improve the efficiency of nonviral gene delivery (Ref. 52). Fechheimer et al. first demonstrated that murine fibroblasts could be transfected using sonication in 1987 (Ref. 53), and this was extended a decade later by the demonstration of a 2.4% transfection efficiency in primary chondrocytes using naked plasmid DNA combined with USE (Ref. 54). Up to tenfold enhancements of plasmid transfection in another poorly transfectable cell type, primary porcine vascular smooth muscle cells (PVSMCs), has since been demonstrated using USE (Ref. 55). These proof-of-concept results have been reproduced by other investigators in many other cell types (Ref. 52).

Although encouraging, these levels of enhancement are not of sufficient magnitude to be clinically relevant. In an attempt to increase efficiency, microbubbles have been added to the transfection medium prior to USE, on the basis that encouraging cavitation (the oscillation and/or collapse of microbubbles within an acoustic field) will lead to enhanced effects on transgene expression. USE during transfection of PVSMCs with naked plasmid was associated with >200-fold enhancements in reporter gene expression when the transfection medium was supplemented with microbubbles, compared with 16-fold enhancements using USE alone (Ref. 56). Transfection efficiencies of up to 43% have been reported using this approach (Ref. 57) and, furthermore, microbubble-enhanced ultrasound also facilitates polyplex transfection (Ref. 55).

Several studies have shown that USE also enhances nonviral gene delivery in vivo and appears to be nontoxic (Refs 58, 59, 60). Most recently, three papers have reported substantial effects of microbubble-enhanced ultrasound on the biological efficacy of therapeutic plasmid delivery to vascular and skeletal muscle (Refs 61, 62, 63). In combination with the flexibility, general safety and repeatability of ultrasound delivery in most organs and tissues, these early in vivo studies augur well for the potential use of ultrasound-assisted transfection in clinical gene therapy protocols.

Enhancement of gene transfer using biolistics

Sanford and colleagues first demonstrated particle bombardment, or biolistics, as a gene delivery system to overcome the inherent difficulty of transgene expression in plant cells (Ref. 64). This original method utilised a gunpowder acceleration system to propel DNA-coated tungsten particles at recipient cells. Penetration of the cell wall and membrane could result in the intracellular expression of reporter genes encoded by the exogenous DNA. Subsequently, helium-driven devices, such as the Helios™ gene gun (Bio-Rad Laboratories), have been developed, in which a helium pulse is used to accelerate DNA-coated gold microparticles through the cell membrane (Ref. 65). The integrity of large DNA constructs can be maintained on microparticles, allowing the delivery of complex genes. Conceivably, this system could have in vivo applications in humans and other animals, with genetic immunisation as a potential application.

Genetic immunisation uses nucleic acids that encode antigens capable of eliciting humoral and cellular immune responses. Biolistics can also be used to achieve effective immune responses in antigen-presenting cells, such as epidermal Langerhans cells and dermal dendritic cells. Intradermal rather than intramuscular delivery of nucleic acids is a more effective route for immunisation (Ref. 66) and has already shown success (Ref. 67).

Particle bombardment has several distinct advantages over other nonviral gene transfer techniques. Cationic complexing agents, lipids or dendrimers have varying degrees of cytotoxicity

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and these are avoided with biolistics. The gene gun approach has shown relatively high transfection efficiency in vitro compared with other nonviral systems, such as electroporation and lipofection, although this is dependent on the cell line used (Ref. 68).

Particle bombardment of mammalian tissues in vivo has not been investigated thoroughly, although currently there are many groups developing this technology. Skin conditions of genetic origin (genodermatoses) are favourable targets for gene therapy, as the skin is easily accessible (reviewed in Ref. 69). A variety of cell types have shown a significant level of reporter gene expression in vitro following particle bombardment (Ref. 68), whereas other studies, targeting brain neurons (Ref. 70), skeletal muscle (Ref. 71), liver (Ref. 72) and cutaneous and subcutaneous tissue (Refs 69, 72), have shown varying levels of reporter gene expression.

The challenge of systemic delivery

Systemic delivery represents one of the major hurdles for gene therapy, but if conquered will permit vectors to gain access to multiple targets, including metastatic tumour cells, haematological disorders and any of the internal organs. Mouse tail vein injections have been the most widely used experimental model in studies of systemic delivery. Early studies suggested that reporter gene expression following a single administration could provide intermediate-term expression (up to 3 months) in many tissues (Refs 73, 74, 75, 76). The lungs consistently demonstrated the highest levels of gene expression (10- to 1000-fold greater than other tissues), and the most prolonged expression, with the liver, spleen and heart showing lower levels (Refs 77, 78). Although most tissues examined demonstrated some evidence of gene expression, complex deposition was greatest in the lungs, possibly because this is the first capillary bed encountered, and trapped lipoplexes might act as a reservoir for sustained DNA release (Ref. 79). Alternatively, tantalising studies showing that lung transfection persists even when the lipids and DNA are administered separately (with the lipid administered first) might indicate that the lipid is involved in targeting the lipoplexes to the pulmonary endothelium, perhaps via an indirect action involving a plasma protein (Ref. 80).

It was assumed that the pulmonary expression was indicative of migration of the lipoplexes from the vasculature to the parenchyma. However, the fate of intravenously delivered lipoplexes has now been examined in more detail (Ref. 81). It appears that within minutes of the particles entering the circulation they aggregate with negatively charged plasma proteins, increasing the particle size to ~1 μm. Within 5 min, aggregates of particles are seen to adhere to the capillary endothelium, often sequestrating blood constituents including platelets, neutrophils and macrophages. These aggregates do not occlude the vessel and by 20 min the complexes begin a process of internalisation by endocytosis or phagocytosis. At 4 h the complexes are seen in the cytoplasm of endothelial cells, correlating well with gene expression. Thus, systemically delivered lipoplexes are internalised largely by endothelial cells, save for internalisation by macrophages in the spleen and Kupffer cells in the liver, and there is little evidence to indicate that they can reach the subendothelial tissues.

Intravenous application of polyplexes in vivo faces the same limitations as systemic delivery of lipoplexes, with most formulations showing poor solubility in physiological salts, significant nonspecific interaction with plasma proteins, membranes and cells, as well as rapid elimination from the circulation. A few studies have shown that the persistence of polyplexes in the circulation can be influenced by the physical properties of the polyplexes themselves, although these long-circulating forms might be bound to erythrocytes (Ref. 82). Recently, there have been attempts to alter the systemic pharmacokinetics of polyplexes by surface modification with hydrophilic polymers, to produce 'stealth' vectors. However, initial studies using monovalent poly(ethylene glycol) (pEG) to modify the surface of the vector have been disappointing, with little evidence for improved systemic circulation. Indeed, such modifications might render polyplexes more prone to destabilisation after interactions with positively charged molecules in the circulation. One interesting modification to this has been the use of an amino-crosslinking reagent to stabilise the surface of pEG-modified complexes, which leads to extended circulation in the bloodstream (Ref. 83). Further work has evaluated the benefit of surface modification of polyplexes with hydrophilic polymers that bear more than one reactive group, to stabilise the surface of the vector by introducing a level of 'lateral stabilisation'. Polyplexes stabilised in this

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manner with multivalent polymers based on poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA) show substantially increased stability to biological challenge, which is manifest by substantial improvements in systemic circulation time (Refs 84, 85).

Another desirable property for systemically delivered polyplexes is cell-specific targeting. Condensed pEI–DNA complexes carry a net positive charge rendering them prone to unwanted cellular interactions. This can be countered in vitro by conjugating ligands to pEI. In one study, DNA was condensed to neutrality with pEI bearing 5% galactose and delivered to two different hepatocyte cell lines containing an asialoglycoprotein receptor. This conferred a specificity of 10^4–10^5 greater in cells expressing the cognate receptor than those not and achieved reporter gene expression in up to 50% of cells (Ref. 42). There is little evidence to suggest this targeting strategy will be useful in vivo. However, one tail vein injection study, which conjugated an anti-PECAM antibody to pEI, demonstrated a 20-fold increase in reporter gene expression in the lungs, resulting from an increase in DNA retention from 20% to 32%. Only a third of delivered DNA was retained in the lungs; there was a nonspecific increase in the heart and there was persistently high hepatic gene expression (Ref. 86).

Technologies therefore exist for the targeted delivery of both lipid and polycation-based vectors to selected cells in vivo. Currently, a major challenge is to achieve efficient transgene expression following arrival of the targeted vector within the endosome, or cytoplasm, of the target cell. This challenge is common to all nonviral gene delivery systems, and intracellular factors limiting the efficiency of transgene expression are considered in detail below.

Intracellular barriers to transgene expression efficiency

Escape from the endosome

Many macromolecular complexes enter cells by endocytosis, so the first major challenge to successful transgene expression is endosomal escape. As discussed, lipoplexes are thought to achieve this by mixing neutral lipids with the endosome bilayer and releasing the nucleic acid into the cytoplasm (Ref. 87). By contrast, polyplexes, with the exception of pEI-based complexes, are generally not intrinsically fusogenic. An agent that is widely used in vitro to enhance transgene expression using polyplexes is chloroquine, although its mechanism of action is not fully delineated (Refs 16, 37). Chloroquine is a weak base that leads to increased osmolarity of the endosome and might induce lysis; however, it is also capable of inhibiting nuclease activity, which might be important in maintaining integrity of the DNA in both the endosome and the cytoplasm. In addition, chloroquine binds to DNA under acid conditions and might lead to release of some of the cationic polymers, perhaps increasing their interaction with the endosome membrane as the pH falls. Although its precise action is unclear, chloroquine is widely thought to promote entry of the DNA into the cytoplasm.

Another means to enhance endosome-to-cytoplasm transfer is the use of membrane-active peptides, such as those based on the influenza virus haemagglutinin protein (Ref. 88). These N-terminal peptides are pH-dependent and adopt amphipathic configurations at low pH that are thought to insert into the endosome membrane and lead to permeabilisation. One major advantage of these peptides over chloroquine is that they can be used in vivo, at least in principle, since the endosomolytic agent can be covalently linked to the nucleic acid delivery vector and targeted to specific cells.

Nuclear import and transcriptional instability

Most present-day DNA delivery systems can achieve appreciable levels of transgene expression only in proliferating cells. This is a major limitation to the usefulness of such vectors, since the majority of target cells in vivo are postmitotic. The main reason for this lack of efficiency in nonproliferating cells, similar to the problems faced by retrovirus, is thought to be the barrier of the intact nuclear membrane. In proliferating cells, the nuclear membrane disintegrates during the G2–M stage of the cell cycle, facilitating vector access to the transcriptional machinery; this does not occur in nonproliferating cells. Entry into the nucleus of noncycling, or postmitotic, cells is restricted to passage through the nuclear pore complexes (Ref. 89), and several research groups are presently trying to devise means to achieve this efficiently. Most groups are focused on exploiting cellular pathways for nuclear import of nuclear proteins, involving basic strands of amino acids known as nuclear localisation sequences (NLSs) that bind cytoplasmic importins

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and mediate efficient import of the protein into the nucleus. In principle, NLSs can be linked to cationic polymers, or even directly to the DNA. An extensive review of this field is beyond the scope of this article, but for more information see Ref. 90. In short, nuclear entry is a major problem for most DNA delivery systems and, presently, attempts to overcome the problem using NLSs appear more successful for oligonucleotides than plasmid delivery, implying plasmid DNA might be an intrinsically 'non-permissive' polynucleotide form (Ref. 91).

Gene expression following nonviral transfection is often transient, falling rapidly within the first few days and disappearing altogether within one week. This mirrors a reduction in plasmid DNA that can be recovered from transfected tissues soon after transfection. Three reasons for this reduction might be the removal of cells that have been lethally damaged during transfection, an immune-mediated response to cells expressing the transgene, and removal of plasmid DNA that has reached the nucleus but has subsequently been recognised as foreign by the cell.

Although substantially more immune-inert than viruses, plasmid DNA synthesised by bacteria differs from vertebrate chromosomal DNA in that it contains many unmethylated CpG motifs. These are recognised by the immune system and induce an inflammatory response, involving tumour necrosis factor α, interferon γ, interleukin 6 (IL-6) and IL-12, which suppresses transgene expression. To counter this phenomenon, partially CpG-depleted plasmid vectors (with motifs reduced from 526 to 256 sites per plasmid molecule) have been constructed, which reduce cytokine levels by 43–81% and increase expression twofold after tail vein injection. The enhancement is modest, and only in part due to a reduced immune response, but suggests plasmid DNA design is important (Ref. 92).

Further modifications to the plasmid can markedly affect in vivo transcription and expression. Optimisation of tissue-specific promoters, intron sequences and polyadenylation sequences were shown to increase reporter gene expression in the liver of mice after tail vein injections by 69-fold. Expression during the first few days, but more particularly thereafter, falls disproportionate with plasmid DNA loss and is due to transcriptional silencing of strong viral promoters. Tissue-specific promoters produce lower levels of gene expression initially, but transcription and thus expression is maintained for longer (Ref. 93). Modified constructs with a liver specific α1-antitrypsin promoter have demonstrated sustained expression of human factor IX beyond 18 months (Ref. 6). These results are particularly impressive since plasmid DNA remained episomal – that is, it was not integrated into the genome. Stable integration with a nonviral system could avoid the mechanisms hampering episomal gene transfer, and recent studies have exploited transposon-mediated integration to this end. One transposase, called 'Sleeping Beauty' (SB), is able to control integration into mammalian (including human) DNA of a cotransfected gene flanked by SB-specific terminal repeats (Refs 94, 95). Using mouse tail vein injections to treat a murine model of a metabolic recessive disorder (tyrossinaemia), hepatocyte integration rates of 0.1–4.0% were observed, with more than 95% of these being single-gene SB-transposase-specific insertions (cf. retroviruses) (Ref. 96).

An interesting alternative, circumventing the need for nuclear entry, is to employ cytoplasmic transgene expression systems. Some authors have demonstrated cytoplasmic transcription in mammalian cells using plasmids encoding transgenes under the control of the phage T7 polymerase (Refs 97, 98). Encoding the T7 polymerase itself as an 'autogene' achieves earlier expression in the cytoplasm and might be effective in postmitotic cells. While cytoplasmic expression systems present a tantalising approach, the selectivity of expression that can be conferred by using carefully chosen promoters or other transcriptional regulatory elements is essentially lost.

The discussion so far has concentrated on DNA-based strategies to introduce potentially therapeutic genes. However, RNA interference (RNAi), an RNA-based technology, promises to provide an effective strategy to achieve gene knockdown. Double-stranded RNA molecules that are similar to a target gene transcript can reduce mRNA levels >90% (Ref. 99). The RNA molecules are short (21–23 nt in length) with 3' overhangs, and therefore do not induce an IFN-γ response like longer RNA molecules do. Because they are double-stranded the molecules are more stable, but potentially small enough not to be encumbered by the problems of plasmid

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transfection. This technology serves as a useful biological tool and might also prove valuable in the field of gene therapy (Ref. 100).

Implications for molecular medicine

Nonviral gene delivery vectors, designed from well-characterised components, are likely to exhibit improved safety profiles compared with present viral systems. Therefore, they should be more acceptable for the treatment of nonmalignant disease. Nonviral systems can already be employed effectively in some settings, although the efficiency of transgene expression is typically several orders of magnitude lower than the most efficient viral vectors. Inefficient transfection should not represent a particular barrier to clinical gene vaccination, and the modest immune response mounted against plasmid DNA might in fact prove helpful in this context.

In the future, substantially more-efficient nonviral gene delivery could be used in several settings. One example is as a one-off delivery simultaneous with a clinical intervention, such as to prevent post-angioplasty restenosis or saphenous vein graft failure (SVGF). Both of these disorders are typified by a vasculoproliferative response to vessel-wall injury leading to an occlusive lesion, and a number of well-characterised genes have been implicated in this effect. Theoretically, transfection of only a proportion of the cells in the vessel wall will be sufficient to inhibit the development of a completely occlusive lesion. SVGF is a particularly attractive target for gene therapy because after harvesting the veins from the lower limb there is a window of opportunity to deliver the genes more efficiently ex vivo prior to coronary implantation. Another example of clinical application is in life-threatening conditions such as cancer. It is possible that local toxicity and inflammation induced by the DNA, carrier molecules or mechanical modes of augmentation might have a synergistic effect.

Nonviral gene delivery might also prove valuable in the treatment of certain inherited recessive disorders. The adverse clinical sequelae of disorders such as haemophilia or metabolic recessive disorders can be prevented by recovery of less than 5% or normal protein/enzyme function. This implies suboptimal transfection would be sufficient, provided stable expression was achieved, to produce a cure. Additionally, it should be possible to stably transfect cells, such as progenitor cells, ex vivo and re-implant a small population of them afterwards.

Hence, the rational design of vectors for specific purposes is evolving and is likely to exploit a combination of technologies that have been alluded to in order to address the particular barriers encountered. Combining several such modifications into multicomponent vectors is an ongoing challenge but should prove possible. The present dominance of viral vectors in the field of gene therapy reflects a 'top down' approach where professional pathogens are being harnessed and used for therapeutic purposes; nonviral vectors represent a 'bottom up' approach that should eventually yield superior technology.

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Further reading, resources and contacts

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Features associated with this article

Figures

Figure 1. Lipoplex-mediated transfection and endocytosis (fig001cns).
Figure 2. Receptor-mediated internalisation of ligand-labelled polyplexes (fig002cns).

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