Expert Opinion

Nuclear gene delivery: the Trojan horse approach

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The nuclear envelope represents a formidable barrier to the transfer of plasmids to the cell nucleus, particularly in nondividing cells. The probability of intact plasmids arriving in the nucleus by a passive process is extremely low. There is substantial evidence in the literature that describes the transport of macromolecules, including plasmids, to the nucleus as a very inefficient process, and so far attempts to affect the active transport through the nuclear pores have achieved limited success. Several approaches have been attempted to improve nuclear transport of plasmids, including the condensation of plasmids to unimolecular complexes of minimal hydrodynamic diameter to favour passive transport through the nuclear pore complex, and the incorporation of nuclear localisation signals in the plasmid or in the delivery system to enhance the active transport of plasmids through the nuclear pores.

Keywords: *cis*-acting karyophilic sequences, gene therapy, nonviral gene delivery systems, nuclear localisation signals, nuclear pore complex, plasmid nuclear delivery, unimolecular complexes

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1. Introduction

Gene therapy can be viewed simplistically as a means to produce proteins *in vivo* using the patient's own cells as mini-bioreactors. The field of gene therapy has evolved since its inception from *ex vivo* methods of introducing genes to the direct administration of *in vivo* gene-based products. The clinical targets have also expanded from the treatment of genetic disorders such as cystic fibrosis, Duchenne muscular dystrophy and haemophilia, to the prevention and treatment of acquired diseases such as cancer, cardiovascular and neurological diseases. Gene therapy is also being explored for the production of innovative vaccines that can precisely modulate cellular and humoral immune responses, thus providing prophylactic or therapeutic treatments for several infectious diseases or cancers.

The rational design of plasmid-based therapeutics and vaccines requires a thorough understanding of the critical events that control both the location and functioning of the administered genes. Scientists have created and tested an array of novel delivery elements (and devices) to overcome some of the rate-limiting steps from the site of administration to the nucleus of the target cells. Those key steps have been described previously by the 'DART' approach to plasmid targeting [1-2] and include distribution (e.g., diffusion through injected tissues such as skeletal muscles or solid tumours), access (e.g., extravasation through sinusoids in the liver after intravenous administration), recognition (e.g., passive cell binding through ionic interactions; active targeting via cell ligands to affect receptor-mediated uptake) and trafficking (e.g., intracellular transfer of plasmids, including endosomal release, decomplexation if plasmids are associated with delivery carriers, plasmid cytoplasmic translocation and nuclear localisation) (Figure 1). So far, a limited number of first-generation delivery systems have been introduced into clinical trials (e.g., polymer- and lipid-based formulations) [3-4], and those primarily address the initial rate-limiting steps in gene

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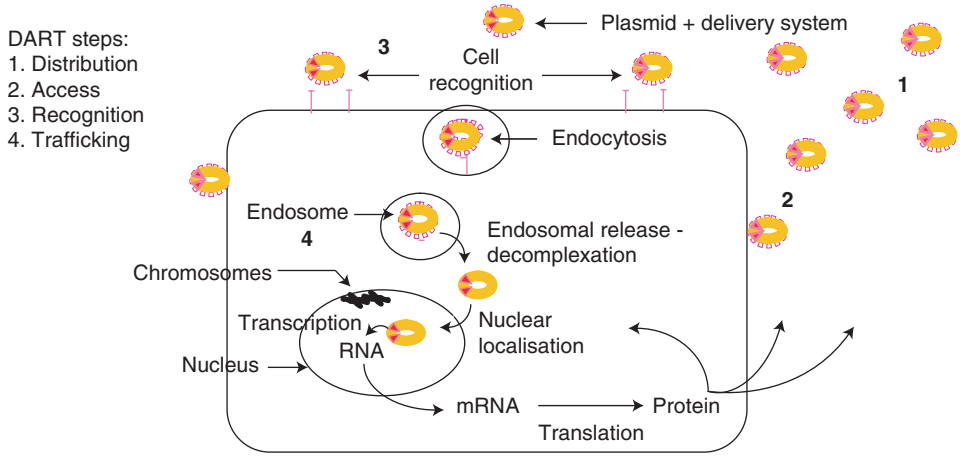

Figure 1. Rate-limiting steps in the delivery of plasmids from injection site to the nucleus of target cells.

delivery (e.g., plasmid diffusion in extracellular matrices, protection from nucleases and cell uptake via nonspecific mechanisms). More advanced delivery systems have been designed to address the different rate-limiting steps in DART, but so far their testing has remained limited to animal models. Major challenges and opportunities still remain in improving plasmid delivery to further enhance the potency of gene medicines and vaccines. Scientists must also continue to address the pharmaceutics of the resulting formulations, such as stability and scale-up, and develop analytical methods for quality control to ensure manufacturing process comparability and lot-to-lot consistency of plasmid-based therapeutics as "well-characterised biological products". In addition, once plasmids have reached the nucleus, following biodistribution and intracellular trafficking, the ability to control gene expression remains essential. This review focuses on the last step of gene transfer with plasmid-based systems, nuclear translocation, and evaluates some of the recent approaches that have been investigated to increase the number of intact plasmids gaining access to the cell nucleus.

2. Effect of cell division on transfection efficiency

Transfection of dividing cells is generally effective, as the nuclear membrane disintegration during mitosis allows plasmids to enter the nucleus before the membrane reforms. There is experimental evidence to prove that gene transfer occurs most readily in dividing cells [5-7]. However, transfection of postmitotic cells is typically ineffective (Figure 2). A few exceptions to the transfection of differentiated cells have been found *in vivo* after intramuscular administration of plasmids to skeletal (and cardiac) muscle, and the use of hydrodynamic (bolus injection of a high volume) gene transfer to the liver and muscle [8-11]. The reason for those exceptions is not very well understood, but it is possible that in addition to the transient cell membrane permeability created by the high volume of injection, a transient increase in nuclear membrane permeability may also occur. *In vitro* transfection experiments have shown that unprotected plasmids can be rapidly degraded in the cytoplasm, and typically < 1% of plasmids accessing the cytoplasm will translocate to the nucleus [12-13]. Nuclear entry of unformulated plasmids in postmitotic cells has been shown by cellular microinjection studies to be extremely low, with expression being ~ 1000-fold lower after intracytoplasmic injection compared with injection into the nucleus [14]. In addition, nuclear microinjection of cationic lipid/DNA complexes has been shown to inhibit expression, suggesting that plasmids need to be released from complexes (pre- or post-entry in the nucleus) to allow gene expression [15]. However, cationic polymers such as poly-L-lysine (PLL) and polyethylenimine (PEI) [16] have been suggested to mediate nuclear targeting (e.g., PLL is reminiscent of the nuclear localising signal Pro-Lys-Lys-Lys-Arg-Lys-Val).

3. Nuclear transfer

3.1 Passive transfer

The nuclear membrane prevents the passive transfer of most macromolecules > ~ 50 kDa into the nucleus, unless they can interact with the nuclear pore complex (NPC) for active transport. The nuclear envelope is composed of two lipid membrane bilayers creating a formidable barrier between the cytoplasmic and nuclear compartments. The inner and outer membranes are connected at NPCs throughout the envelope. It is estimated that there are 2000 – 4000 nuclear pores/cell. The NPC has a reported diameter of 10 – 25 nm and does not allow the direct transfer of plasmids across the nuclear membrane due to the intrinsic colloidal characteristics of plasmids (persistence length or average linearity of ≥ 50 nm) and their net negative surface charge. However, the NPC permits the passive transfer of gold particles < 9 – 10 nm in diameter and

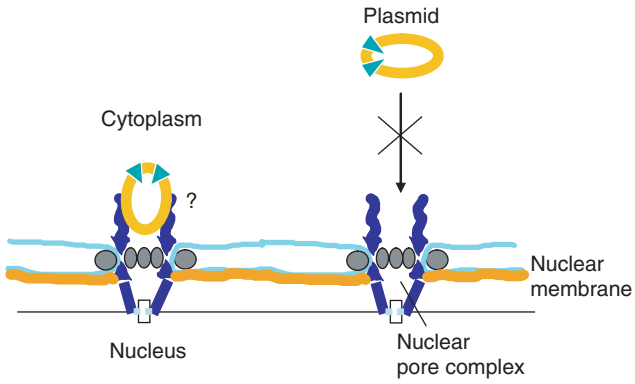

Figure 2. Passive nuclear transfer of unformulated plasmid is typically inefficient in postmitotic cells.

linear DNA fragments ≤ ~ 300 bp [17]. Plasmids are too large to cross the nuclear membrane efficiently and most delivery systems condense DNA to multimolecular complexes with diameters in the hundreds of nanometer to micron range that are also incompatible with passive nuclear entry (assuming no prior cytoplasmic decomplexation). One approach that has been evaluated to improve the passive transfer of plasmid molecules through the NPC is the condensation of plasmids into unimolecular complexes. Each of these complexes contains one molecule of DNA and has the minimum possible compacted size based on computation.

According to Zuber *et al.* [18], the volume of a 5.5-kbp DNA is 11,800 nm^3 if hexagonal packing is assumed (based on the model that DNA helices form a hexagonal array with counterions in the interstices between the helices, resulting in a stable three-dimensional phase with high structural order as reported by Shellmann and Parthasarathy [19]). With a calculated 2700 cationic lipid molecules to neutralise the DNA negative charges, and with an assumption of the dimensions of a hydrated lipid molecule of 0.8 nm^2 × 4 nm, the resulting volume of the complex is 20,440 nm^3, which translates to a sphere of 34 nm in diameter. Plasmid condensation into unimolecular complexes also alters the surface properties of the DNA (e.g., by providing a more hydrophobic surface with less exposed negative surface charges), which may also facilitate penetration via the NPC.

3.2 Active transport

The NPC enables the active transport of endogenous nucleoproteins (e.g., histones and transcription factors) into the nucleus and the export of RNA to the cytoplasm. The nuclear transfer of those macromolecules involves energy-independent recognition of the nuclear localisation sequences (NLS)-containing protein by the NLS receptor (a heterodimeric complex composed of the NLS-binding importin-α and the NPC docking importin-β protein), docking of the substrate at the NPC, energy-dependent translocation of the complex through the NPC and release within the nucleus [20]. Nucleoproteins contain NLS, typically a cluster of four to seven basic amino acids (e.g., Pro-Lys- Lys- Lys-Arg-Lys-Val), as found in the Simian virus (SV)40 large T antigen, for example, which interact with karyopherins or importins that act as shuttle proteins to the nucleus. Many nucleoproteins have clusters of the cationic sequences (e.g., bipartite) separated by a spacer of 10 – 12 residues. Covalent coupling of NLS to proteins such as bovine serum albumin, ferritin and IgM [21] has been shown to enhance their nuclear localisation. Many viral proteins also contain NLS and are assumed to reach the nucleus by active transport:

- viral DNA enters the nucleus by way of one or more nucleoprotein(s) coupled to its DNA (e.g., adenovirus having a copy of the terminal protein coupled to each 5′-terminus of the viral duplex DNA);
- histone-like proteins in the viral core mediate the uptake of viral DNA; these cationic proteins, noncovalently bound within the viral core, may form complexes of sufficient stability to contribute to the uptake of the DNA (e.g., adenoviral core proteins V and VII may work that way);
- viral DNA has sequences that bind host cell nucleoproteins after the viral capsid has dissociated, resulting in active uptake of the viral DNA (e.g., SV40 sequence).

In a similar manner, it is conceivable that NLS could be used to improve the nuclear transport of plasmids.

In an attempt to increase the active transport of plasmids to the nucleus, approaches that mimic the transport of nucleoproteins and viral DNA have been investigated and are reviewed below. NLS have been associated with plasmids by various means (direct linkage of NLS to DNA; noncovalent association with NLS-bearing synthetic polymers; association of DNA with virus-derived proteins/peptides, nuclear proteins such as high mobility group [HMG] proteins and histones). DNA *cis*-element recognised by a nuclear protein have also been examined.

4. Unimolecular complexes for enhanced passive nuclear transfer

Improvement in the nuclear transfer of plasmids could be achieved by improving the passive diffusion rate through the NPC by plasmid condensation to unimolecular complexes of a size amenable for entry via the NPC (≤ 25 nm) (Figure 3). Experiments reported by Liu *et al.* [22] show the effect of unimolecular compaction of a plasmid (~ 5 kbp) by 30-mer lysine polymers (PLL conjugated with poly(ethylene glycol) PEG_{10K}) on transfection efficiency of terminally differentiated human neuroblastoma SY5Y cells (by exposing the cells to all-*trans*-retinoic acid for 6 days) or growth-arrested human hepatoma HuH-7 cells after intranuclear or -cytoplasmic microinjection. There was no difference in timing, level and extent of gene expression after intranuclear injection of either

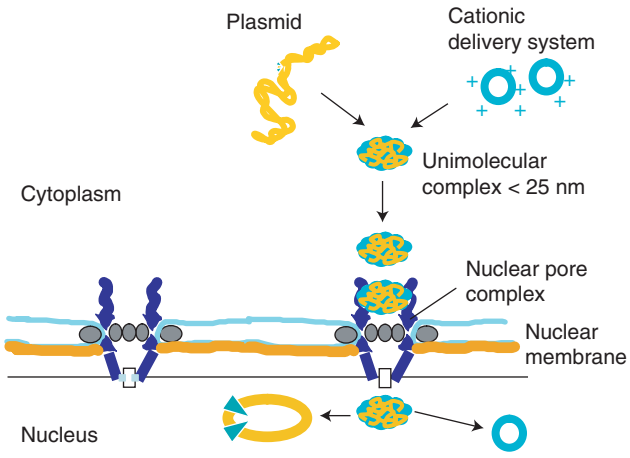

Figure 3. Formation of unimolecular complexes with a hydrodynamic diameter of ~ < 25 nm enhances the passive transfer of plasmids to the nucleus.

~ 500 copies/cell of naked DNA or PLL-formulated plasmid, suggesting a rapid plasmid decomplexation from PLL in the nucleus. After intracytoplasmic injection, however, there was a ~ 10-fold increase in the extent of gene expression with compacted DNA compared with naked DNA, but the expression was about fivefold lower than following the intranuclear injection of compacted DNA. The effect of plasmid compaction on transfection efficiency after intracytoplasmic injection was abolished by coinjection of wheat germ agglutinin, a compound that is known to block importin-mediated transfer. The effect of the particle size on transfer through the NPC was investigated by the microinjection of condensed plasmids of different sizes (3 – 28 kbp) in the cytoplasm or nucleus. Nuclear microinjection of naked and condensed plasmids resulted in comparable gene expression, indicating that transcriptional efficiency did not decrease as a function of plasmid size increase. However, gene expression was inversely proportional to particle size after intracytoplasmic injection. There was a significant decrease in expression above an approximate threshold of 25 nm, approximating the physical limitation of the NPC size for passive transfer to the nucleus.

In another study [20,23], fluorescently labelled PLL/plasmid complexes of different charge ratios were evaluated for their translocation to the cell nucleus *in vitro*. PLL by itself did not localise to the cell nucleus. With an increased amount of PLL in the complex (from a charge ratio (+/-) of 0.4 – 4), there was a significant increase in plasmid accumulation in the nucleus. Electron and atomic force microscopy revealed that as the amount of PLL increased in the complex their size changed from 60 nm (charge ratio 0.4, +/-) to > 150 nm (charge ratio 2, +/-) to 15 – 20 nm (charge ratio 4, +/-). At a charge ratio of 4, the coupling of a NLS to PLL did not increase plasmid nuclear transfer because the plasmid/PLL complex is sufficiently condensed to enable the complex to diffuse through the NPC.

Other delivery systems have recently been developed for creating small, unimolecular complexes [18]. The principle is based on plasmid condensation with cationic detergents (containing a cysteine residue) at an appropriate concentration to form unimolecular complexes (detergents are used rather than lipids due to higher solubility), followed by oxidative dimerisation of the detergent into a lipid structure with increased stability.

Although very limited success has been reported in the literature so far, condensation of plasmids into unimolecular complexes that have the ability to cross the nuclear membrane passively is an attractive concept to increase plasmid translocation to the cell nucleus. However, significant hurdles remain in controlling the compaction of plasmids to such discrete and homogeneous complexes, as well as in their scale-up and quality control. Even if such unimolecular complexes can be manufactured at sufficient scale and display adequate shelf-life stability, it will be extremely challenging to demonstrate *in vivo* that such complexes retain their physicochemical properties (in particular a hydrodynamic diameter of ~ 25 nm with a narrow size distribution) when placed in contact with biological milieu such as body fluids, extracellular matrices and intracellular proteins.

5. Approaches for active plasmid transport to the nucleus

Improvement in nuclear transfer of unformulated or formulated plasmids could also be achieved by improving the active transport of plasmids through the NPC. NLS have the potential to be incorporated in either the gene delivery system or in the plasmid sequence to facilitate the active transport of plasmids to the nucleus through the NPC (Figure 4).

5.1 Nuclear localisation proteins and peptides

5.1.1 Viral and nuclear proteins

Several viral proteins have been used to improve nuclear transport of plasmids (e.g., adenovirus hexon protein linked to PEI [24]; C-terminal domain of HIV type 1 viral protein R [25]), resulting in increased *in vitro* gene expression. As the use of microorganism proteins may lead to immunogenicity/safety issues, endogenous nuclear proteins (HMG proteins, histones) have instead been investigated for the active transport of plasmids. HMG group 1 (HMG-1) non-histone chromosomal protein was associated with plasmid-loaded liposomes and resulted in a shorter onset of expression, but with no significant impact on expression levels [26].

5.1.2 NLS peptides

The simple addition of NLS peptides to plasmids has also been tested [27-30] and has resulted in an enhanced rate and amount of plasmid taken up by nuclei. A 4.4 kDa tetramer of NLS from the SV40 large T antigen was synthesised and tested for interaction and compaction of plasmids, as well as

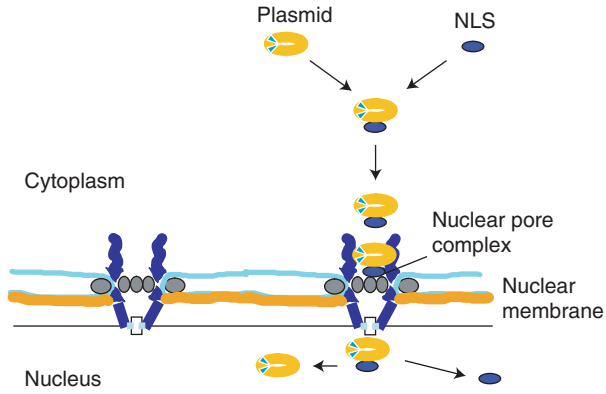

Figure 4. Active transport of plasmids through the nuclear pore complex with NLS (NLS can be added to or incorporated in the plasmid by means described in Section 5).

NLS: Nuclear localisation sequence.

transfection efficiency [31]. Positively charged nanoparticles were obtained by interaction of the tetramer peptide with plasmids, resulting in some protection *in vitro* against DNase I degradation. Rapid accumulation of the complexed plasmids in the nucleus of the transfected cells *in vitro* was observed by fluorescence *in situ* hybridisation, and a concomitant early onset of expression was evidenced to be less cell cycle-dependent than with other non-NLS carriers such as PEI or fractured dendrimers. Interestingly, when the tetrameric NLS peptide was combined with a PEI carrier to facilitate cellular uptake, a further increase in gene expression was observed *in vitro*. Due to its similarity with the NLS of SV40 large T antigen, PLL has been investigated for gene delivery. However, it has been shown that PLL by itself does not function as a NLS [23].

Other polycationic peptides have been designed to facilitate the transfer of plasmids to cell nuclei [32]. The arginine-rich TAT peptide from HIV-1 has been shown to be able to transport proteins, nanoparticles and liposomes by a mechanism apparently different from endocytosis, and it also acts as a nuclear translocation domain. Oligomers of the TAT peptide were able to compact plasmids to nanoparticles and protect against nuclease degradation. *In vitro* transfection experiments showed an increase in accumulation of plasmids in the cell nucleus using dimer and trimer forms of the TAT-peptide repeats with intervening glycine residues, leading to a six- to eightfold increased expression compared with poly-L-arginine-mediated delivery. By precompacting plasmids with TAT oligomers then adding PEI, a 1 – 2 Log increase in gene expression was observed compared with PEI–plasmid complexes. When the order of addition of the compacting delivery systems was reversed, no benefit of the TAT oligomers on transfection efficiency was observed. Intratracheal instillation of TAT dimer/PEI/plasmid complexes in mice resulted in a marginal increase of gene expression in the lungs compared with PEI–plasmid complexes. In a similar approach, branched peptides derived from one to eight identical fragments of the HIV-TAT protein were synthesised [33], but *in vitro* results were disappointing. The plasmid formulation with the eight TAT moiety peptide resulted in the highest transfection efficiency in dividing cells compared with the other TAT peptides, although transfection was similar to lipofectamine-mediated delivery. In nondividing endothelial cells, the eight TAT moiety peptide did not increase gene expression significantly above the naked DNA control and expression was ~ 100-fold lower than lipofectamine-mediated transfection.

In another study, plasmids were condensed with NLS peptides, either the adenoviral core peptide mu or the SV40-derived peptide, and formulated with a cationic lipid system, 3(*N*-*N*′,*N*′-dimethylaminoethanecarbamoyl)cholesterol/dioleoyl-phosphatidyl-ethanolamine [34]. It was observed *in vitro* that, although the two peptides were able to translocate rapidly to the cell nucleus on their own, when formulated with plasmids and the cationic lipid system, a rapid intracellular dissociation of plasmids from the condensing, nuclear targeting peptides occurred. As a consequence, with such a delivery system there was no clear benefit of the incorporation of either NLS peptides on plasmid translocation to the nucleus in growth-arrested cells.

An importin β-binding peptide (31 amino acid sequence) from the amino terminus of the human T-cell leukaemia virus type 1 (HTLV) Rex post-transcriptional regulatory protein was also used to condense plasmids [35]. The HTLV peptide was found to bind the intracellular transport receptor importin-β_1 *in vitro* using an enzyme-linked immunosorbent assay-based assay, but also unexpectedly to importin-α_1 and transportin 1. The HTLV peptide was shown to condense plasmids into discrete particles at nitrogen:phosphate (N:P) ratios of 2 – 3. At a higher N:P ratio of 10 the diameter of the plasmid/HTLV peptide complexes was measured to < 90 nm by photon correlation spectroscopy. Significant transfection was observed in PC-3 human prostatic carcinoma cells with HTLV peptide-condensed plasmids. The HTLV peptide mediated levels of expression ~ 15- to 32-fold higher than PLL and K18, respectively.

5.1.3 NLS peptides linked to templates

An alternative approach to ensure the colocalisation of plasmids and NLS in the target cells has been to link the NLS to templates (e.g., peptide nucleic acid [PNA] clamp, cationic peptides) that bind DNA [36-37]. Some of these approaches have also resulted in enhanced *in vitro* transfection efficiency. The SV40 large T antigen NLS that binds to importin-α_1 was attached to plasmids at specific locations using PNA clamp [35]. A very modest increase in gene expression (twofold) was observed *in vitro* when circular plasmids were used, whereas a sevenfold increase was achieved with linearised plasmid compared with unmodified DNA. A 38-mer peptide (M9) derived from the heterogeneous nuclear ribonucleoprotein A1 was investigated *in vitro* to enhance nuclear transport of plasmids

in a variety of cell types [38-39]. Heterogeneous nuclear ribonucleoprotein is a mRNA-binding protein complex that shuttles RNA between the nucleus and the cytoplasm via the NPC. The M9 peptide binds to the nucleoporin via karyopherin-β_2 (transportin-1) to permit nuclear transport through the nuclear pores. The karyopherin–M9 complex then dissociates in the nucleus in response to Ran-GTP present at high concentration in the nucleus. The M9 peptide was coupled to a scrambled version of the SV40 large T antigen to enhance the ability of the peptide to bind plasmids via ionic interactions. In combination with low concentrations of lipofectamine to facilitate cell entry *in vitro*, the modified M9 peptide was shown to enhance nuclear trafficking of plasmids in oesophageal mucosal cells and fibroblasts *in vitro* and transfection efficiency. An NLS peptide derived from the SV40 large T antigen was also linked with high affinity to plasmids by fusion with the tetracycline repressor protein TetR that can bind to a short DNA sequence, the tetracycline operator sequence *tetO*, included in the plasmid sequence [40]. *In vitro* studies demonstrated that the TetR–NLS fusion protein enhanced transfection efficiency by ~ 10-fold in cell cycle-arrested cells. Increased transfection was observed in a number of cell lines, including primary mesenchymal stem cells, and the enhancement was higher with a shorter linearised fragment than for the corresponding plasmid. In another study, a SV40 large T antigen NLS peptide was bound to plasmids in an irreversible way without affecting the plasmid physicochemical properties: the NLS peptide was linked to plasmids via triplex-forming oligonucleotides [41]. A 63-mer triplex-forming oligonucleotide was designed to bind a specific plasmid sequence in the phage f1 replication origin using a heating step and that oligonucleotide was then ligated using a T4 DNA ligase to a 32-mer hairpin oligonucleotide that had been conjugated to the NLS. This resulted in a circular 95-mer oligonucleotide/NLS with a dumbbell-like structure catenated to a supercoiled plasmid. Using either a cationic lipid- or PEI-based formulation, those modified plasmids with a single NLS peptide did not lead to increased expression levels *in vitro* compared with unmodified plasmids. The authors hypothesised that supercoiled plasmids may require more than one NLS peptide per DNA molecule or different NLS peptides.

5.1.4 *Covalently bound NLS peptides*

The direct covalent linkage of NLS to DNA has been achieved via chemical and photochemical reactions. Although covalent coupling of increased number of NLS to DNA has been shown to result in stronger binding to importins, a concomitant increase in expression was not observed [42], suggesting that the location and number of NLS per plasmid may be important for nuclear import of plasmids. Using a capped 3.3-kbp linearised cytomegalovirus (CMV)-luciferase plasmid linked to a single NLS (PKKKRKVEDPYC) using a synthesised oligonucleotide peptide, a 10- to 1000-fold increase in transfection efficiency was achieved *in vitro*. The enhancement in transfection was independent of the cell type and cationic formulation used [43]. A single mutation in the NLS (a lysine to threonine replacement of the third amino acid) completely abolished the NLS effect. In another recent study, Van der Aa *et al.* [44] tested the effect of covalently linking NLS peptide to linear DNA on transfection efficiency when formulating the modified DNA with cationic polymers. To avoid premature degradation of linear DNA from the 5′- and 3′-sides, linearised plasmid DNA was capped at both ends with a hairpin, and a NLS peptide derived from SV40 large T antigen was conjugated to one of the hairpins. When formulated with various cationic polymers, the linear DNA–peptide conjugate did not lead to increased *in vitro* transfection and no nuclear localisation was observed. The reason for the failure of that specific study was hypothesised to be partly related to the absence of a spacer linking the NLS peptide to DNA, making ionic interactions between the cationic NLS peptide and the linear DNA more likely, thus potentially masking the peptide efficiency. Plasmid–NLS peptide conjugates were also prepared by covalent binding via diazocoupling using a short (C_5 alkyl chain) or a long spacer (PEG 3400 Da) [45]. Covalent modification of plasmids with the SV40 large T antigen NLS sequence resulted in binding to the nuclear transport proteins, importin-α and -β, as a function of the number of NLS sequences and spacing with the DNA molecule. Similarly, there seemed to be a trend in increased transfection efficiency with a higher number of NLS sequences and the longer spacer. However, after intracytoplasmic microinjection there was no clear evidence of nuclear translocation of the covalently modified plasmids.

The association of plasmids with nuclear proteins has presented limited success so far and safety concerns remain with such approaches, in particular the potential for immune responses to proteins derived from microorganisms. The use of shorter synthetic peptides for plasmid translocation to cell nucleus has shown mixed results *in vitro* and some of the proposed approaches to bind NLS peptides to plasmids still suffer from a lack of characterisation and potential for scale-up. In addition, there is a possibility that some of the noncovalently bound NLS peptides may dissociate prematurely from plasmids *in vivo* and, therefore, not improve plasmid transfer to the nucleus. Although a few approaches with covalent binding of NLS to intact or linearised plasmids have shown some promising results *in vitro*, it is unlikely that such strategies will be economically and technically viable in the near future.

5.2 *Cis*-elements for nuclear localisation

The introduction of a minimal sequence from the SV40 enhancer/early promoter to the plasmid sequence has been shown in *in vitro* microinjection experiments [46-47] to promote nuclear import (in contrast to other viral promoter/enhancer sequences from CMV or Rous Sarcoma Virus) in mammalian cells from mouse, rat, monkey and human in a variety of cell types, including smooth and striated muscle cells, fibroblasts, endothelial and epithelial cells. In contrast to all of the above approaches that have been tested *in vitro* only, the effect of such a *cis*-element recognised by a nuclear

protein has also been evaluated in animal models. In a study of transfection of rat vasculature using electroporation, Young *et al.* [48] compared green fluorescent protein and luciferase plasmids driven by the human cytomegalovirus immediate-early promoter to constructs containing the SV40 enhancer downstream of the reporter genes. A 10- to 40-fold increase in gene expression was observed with the plasmids containing the SV40 sequence with a concomitant nuclear localisation as shown by *in situ* hybridisation. In another *in vivo* study, the comparison of plasmids driven by a CMV enhancer/promoter with or without the SV40 enhancer at either the 5′-end of the CMV promoter/enhancer or at the 3′-end of the polyadenylation site resulted in a ≤ 20-fold increase in gene expression in mouse skeletal muscle [49]. The minimum size of the enhancer was found to be a single copy of a 72-bp element of the SV40 enhancer. The addition of the SV40 sequence in forward and reverse orientation to plasmids driven by other promoters (e.g., skeletal actin) resulted in significant enhanced gene expression from mouse muscle, also suggesting that the 72-bp sequence (that does not contain the origin of replication of SV40) was as effective as the full-length SV40 sequence (245 bp).

Similarly, *cis*-acting nucleotide sequences for NFκB (inducible transcription factor transported to the nucleus by the nuclear import machinery) were added to a luciferase plasmid and shown to increase nuclear localisation by ~ 10-fold with an associated increase in gene expression [50]. This approach would be dependent on NFκB activation, as in the absence of stimulus the proteins would predominantly be cytoplasmic.

The Epstein-Barr virus (EBV) *oriP* sequence has also been incorporated into plasmids to increase nuclear import and gene expression (levels and duration) in the presence of the EBV nuclear antigen 1 (EBNA1) [51-52]. For nuclear transport of plasmids containing the EBV *oriP*, EBNA1 is required as it contains the DNA binding domain and the nuclear localisation signal for transport through the nuclear pore. The *oriP* is ~ 2 kbp, but the family of repeats *FR* comprising 20 copies of a 30-bp binding site motif for EBNA1 may be sufficient (deletion of the dyad symmetry element *DS*, the site for initiation of replication, should not affect nuclear localisation). The presence of the EBNA1 protein in cells at the time of transfection seemed to be critical to enhance nuclear transport (shown by cotransfection of EBNA1 versus EBNA1 stably expressing cells). In the presence of EBNA1, the *oriP*-containing plasmid increased *in vitro* gene expression as a result of transcriptional or post-transcriptional activation (as shown by intranuclear injection) and increased nuclear transport (as shown by intracytoplasmic injection). The increase in *oriP*-EBNA1-dependent gene expression was not restricted to a CMV promoter as replacement by other promoters resulted in the same effect. Safety concerns, however, may be raised with that approach if EBNA1 was used in conjunction with the *oriP* as EBNA1 has been shown to be tumorigenic in mice [53].

The introduction of *cis*-acting sequences in plasmids that favour binding to karyophilic proteins has shown promise, increasing nuclear translocation and gene expression both *in vitro* and *in vivo*, but some of those approaches continue to raise some safety concerns.

6. Expert opinion

Efficient gene delivery to the nucleus of target cells *in vivo* still remains a major limitation to effective therapy. Many barriers to gene delivery have been addressed through the rational design of delivery systems that tackle some of the rate-limiting steps from the site of administration to the cell nucleus. Although first-generation plasmid formulations designed to primarily address the early steps in gene delivery (i.e., passive distribution, diffusion in solid tissues and cellular uptake) have reached human clinical status, improvement of plasmid intracellular trafficking still remains one of the major challenges to significantly improve transfection efficiency.

Although plasmids can access the cell nucleus during mitosis and be retained in the nucleus following cell division [54], in nondividing cells strategies will continue to need investigation to facilitate either the passive or active transfer of plasmids to the cell nucleus [55]. Many approaches to improve on the penultimate step in gene transfer, nuclear translocation, designed primarily to enhance the number of plasmid copies that can reach the nucleus in order to improve transfection efficiency in both dividing and nondividing cells, have been described in this review. Condensation of plasmids with cationic carriers to form unimolecular complexes of minimal hydrodynamic size have shown some promise in enhancing the passive nuclear diffusion of plasmids. A product candidate based on the concept of condensation of single plasmid molecules with PLL is currently being tested in cystic fibrosis patients [101], but few data have been reported on the effect of such a system on enhanced plasmid translocation to the nucleus and gene expression. Significant technical hurdles seem to remain to develop stable, unimolecular complexes at a large scale and to ensure their stability once exposed to biological milieu. The use of one or more NLS peptides or proteins linked to plasmids either covalently or noncovalently has shown some promising results primarily *in vitro* in helping active transport of plasmids to the cell nucleus, resulting in enhanced gene expression. However, challenges still remain associated with controlling the rate of dissociation of NLS from plasmids, preventing potential immune responses to NLS, needing in some instances linearisation of plasmids and overcoming technical challenges in scale-up and characterisation of such systems. The introduction of *cis*-acting sequences in plasmids to bind nuclear transport proteins seems to be the most straightforward approach to enhance nuclear translocation and gene expression. Although promising results have been observed *in vivo* with plasmids containing NLS elements (e.g., the SV40 enhancer sequence), in some instances the need to coexpress or introduce karyophilic proteins has raised concerns as they may impact cell function and require controlled expression *in vivo*.

The combination of unimolecular complexes and NLS for nuclear transport, although untested so far, could represent a more effective way to enhance the transport of plasmids to the cell nucleus. Alternatively, cytoplasmic transgene expression remains a possible option that may bypass the need for nuclear localisation, thus potentially providing a solution to enhance expression and/or biological responses [56-61]. Synthetic gene delivery is still at an early stage of discovery and there are many lessons that can be learnt from the intracellular trafficking of viruses that have evolved over millions of years. The development of better models and techniques to evaluate nuclear localisation both *in vitro* and *in vivo*, rational design of effective and safe NLS and formulations that can remain stable in a biological environment will continue to be critical to advance more efficient second-generation gene delivery systems to human clinical testing. Gene therapy remains a promising biotechnological breakthrough that is likely to revolutionise the medicines of this new century.

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