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Review

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Designer peptide delivery systems for gene therapy

Abstract: Gene therapy has long been hailed as a revolutionary approach for the treatment of genetic diseases. The enthusiasm that greeted the harnessing of viruses for therapeutic DNA delivery has been tempered by concerns over safety. These concerns led to the development of alternative strategies for nucleic acid delivery to cells. One such strategy is the utilization of cationic peptides for the condensation of therapeutic DNA for delivery to its target. However, success of DNA as a therapy relies on its delivery to the nucleus of target cells, a process that is complicated by the many hurdles encountered following systemic administration. Non-viral peptide gene delivery strategies have sought inspiration from viruses in order to retain DNA delivering potency, but limit virulence. This review summarizes the progression of peptide-based DNA delivery systems, from rudimentary beginnings to the recent development of sophisticated multi-functional vectors that comprise distinct motifs with dedicated barrier evasion functions. The most promising peptides that achieve cell membrane permeabilization, endosomal escape and nuclear delivery are discussed.

Keywords: biological barriers; DNA; gene therapy; non-viral; peptide.

Introduction

Since the inception of gene therapy in 1972, much progress has been made to underpin its present-day potential as a treatment for diseases of genetic origin (1). The capacity for foreign DNA to induce functional changes to the host's intracellular machinery is well established. However, DNA can only exert its therapeutic potential if delivered to the nucleus of the target cells. The major stumbling block in the development of gene therapies has been the dearth of efficacious delivery systems (2, 3).

Viruses are naturally adept at hijacking the host cells' machinery to enable their own proliferative agenda. Tailoring of known human viruses as potential gene delivery vectors therefore became the initial focus of research within the field (4). However, despite attempts to remove the pathogenic components of the viral apparatus, progress of these vectors has been thwarted due to safety concerns stemming from their use, and indeed patient perception (5). Non-viral based strategies manage to overcome some of the safety concerns associated with the use of viral vectors but have, as of yet, failed to match the efficacy of their viral counterparts (6). The use of naturally occurring and/or synthetic peptides whose designs are based upon viral sequences, therefore present an attractive alternative for nucleic acid delivery. Multi-functional peptide-based nanoparticles comprising distinct motifs with specific functionalities designed to overcome the extra- and intra-cellular barriers could, in theory, be used for safe and efficient gene delivery (7). Here we discuss the various hurdles that a gene delivery vector must overcome, functional peptides that are capable of facilitating their circumvention, and strategies to combine these peptides to develop effective bio-inspired gene delivery vectors.

Barriers to gene therapy

The ideal gene delivery system should be non-toxic, biodegradable, targeted, non-immunogenic and easily manufactured. In order to design such a peptide delivery system, numerous biological barriers must be understood. Following systemic injection, prospective peptides must firstly protect the therapeutic gene from the action of mononuclear phagocytes, complement and reticulo-endothelial

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systems, all of which results in rapid clearance from the body (8). Additionally the peptide must be able to extravasate from the circulation, pass through the fibrous extracellular matrix and reach the target tissue whilst ensuring any off-target effects are limited (9). Once the therapeutic reaches the target site, the peptide has to penetrate the cell membrane in order to deliver the genetic cargo. The mechanism of internalization has a consequential impact on its intercellular fate thereafter.

Peptides can be internalized by two main pathways: A) Endocytic or energy dependent pathways (clathrin-mediated, caveolae/lipid raft-mediated, clathrin and caveolae-independent endocytosis and macropinocytosis) and 2) direct penetration or energy independent pathways (e.g., inverted micelle model, pore formation, carpet model) (Figure 1) (10). If internalization is by endocytosis, as is predominantly the case, the objective is to escape the endosome, otherwise the genetic material will be degraded and expelled via a lysosome (11). If endosomal escape is successful then the DNA must be delivered to the nucleus where it can finally exact a sustained therapeutic effect (12). Each of these barriers must be overcome otherwise failure of the therapy is inevitable. Consequently, an in depth knowledge of these barriers is fundamental to effective peptide design.

Nucleic acid condensation

The capacity to effectively bind to and condense DNA into stable nanoparticles is essential in order to protect the cargo from enzymatic degradation in the systemic circulation and in the cytoplasm (13, 14). The use of condensing motifs can significantly enhance stability in vivo, protect DNA from the action of lytic enzymes, and ensure an appropriate nanoparticle size (<200 nm) to facilitate cellular uptake (15, 16).

Gratton et al. (17) employed a technique known as particle replication in non-wetting templates (PRINT) to define the significance of particle size, shape and surface charge on non-specific cellular uptake. In the study a clear correlation is evidenced between particle size and the extent of cellular uptake. Particles with a size >1 micrometer exhibited significantly reduced internalization kinetics compared to those that occur within the nanometer scale. However, size, although significant, was not the only defining characteristic with regard to cellular uptake profiles of PRINT particles. Both surface charge and shape were also shown to be important determinants of internalization, with rod-shaped, high aspect-ratio PRINT particles carrying a positive zeta potential the most readily internalized. These, therefore, represent the fundamentals towards which scientists involved in the field of particle design should strive.

The common feature shared by all DNA condensing peptides is their cationic nature, and size-suitability in the condensed form (Table 1). One of the first polypeptides, Poly-L-Lysine (PLL) consisted of biodegradable repeated lysine residues that effectively condensed DNA; endosomal entrapment limited PLL's ability to successfully deliver genetic material (18). Subsequently numerous studies have examined the merits of using either lysine- or arginine-based peptides for gene delivery, with the more compelling evidence firmly supporting arginine. Arginine binds to the DNA in milliseconds (19), has a stronger affinity for the phospholipid phosphatidylserine (Ptd-Ser) on the inner leaflet of membranes (20), and is a superior internalizer to oligolysines (21). Furthermore, recent studies by Mann et al. (22) demonstrated that block distribution of arginine $R_5H_7R_4$ results in stronger condensation of DNA but that the addition of histidine residues either by R_9H_7 or $H_4R_9H_3$ were less effective at condensation but much better at releasing the genetic cargo giving a higher transfection. These studies support those of Hatefi's group, who suggested that vector architecture of the amino acid sequence is critical, and that clusters of lysine and histidine (KKKH-HHHKKK) were superior to interspersed (KHKHKHKHKK) sequences (23). As a cationic residue, histidine not only condenses nucleic acids to an extent but perhaps more importantly also facilitates the release of the genetic cargo from the endosome via the proton sponge effect (24).

Other arginine-rich sequences exist in nature such as protamine, which plays a key role during spermatogenesis by replacing histones, thus ensuring tight condensation of the DNA (25). The Mu peptide (MRRAHHRRRRASHR-RMRGG) is a 19 mer, arginine-rich peptide first identified and isolated from the adenovirus core complex in 1976 (26). The Mu peptide has been shown to consistently bind DNA into small, stable nanoparticles, which has led to its application in a number of peptide-based delivery systems (27, 28). Nevertheless peptides such as protamine and Mu remain uni-functional and so frequently they are imported into multi-functional systems to take advantage of their DNA binding characteristics. For example protamine has been utilized as the core DNA binding component of a multifunctional envelope type nano device (MEND) incorporating the fusogenic peptide GALA and the nuclear localization signal (NLS) maltotriose which boosted transfection efficiency 15.8-fold higher than that of the commercially available in vivo-jet-PEI™-Gal (29).
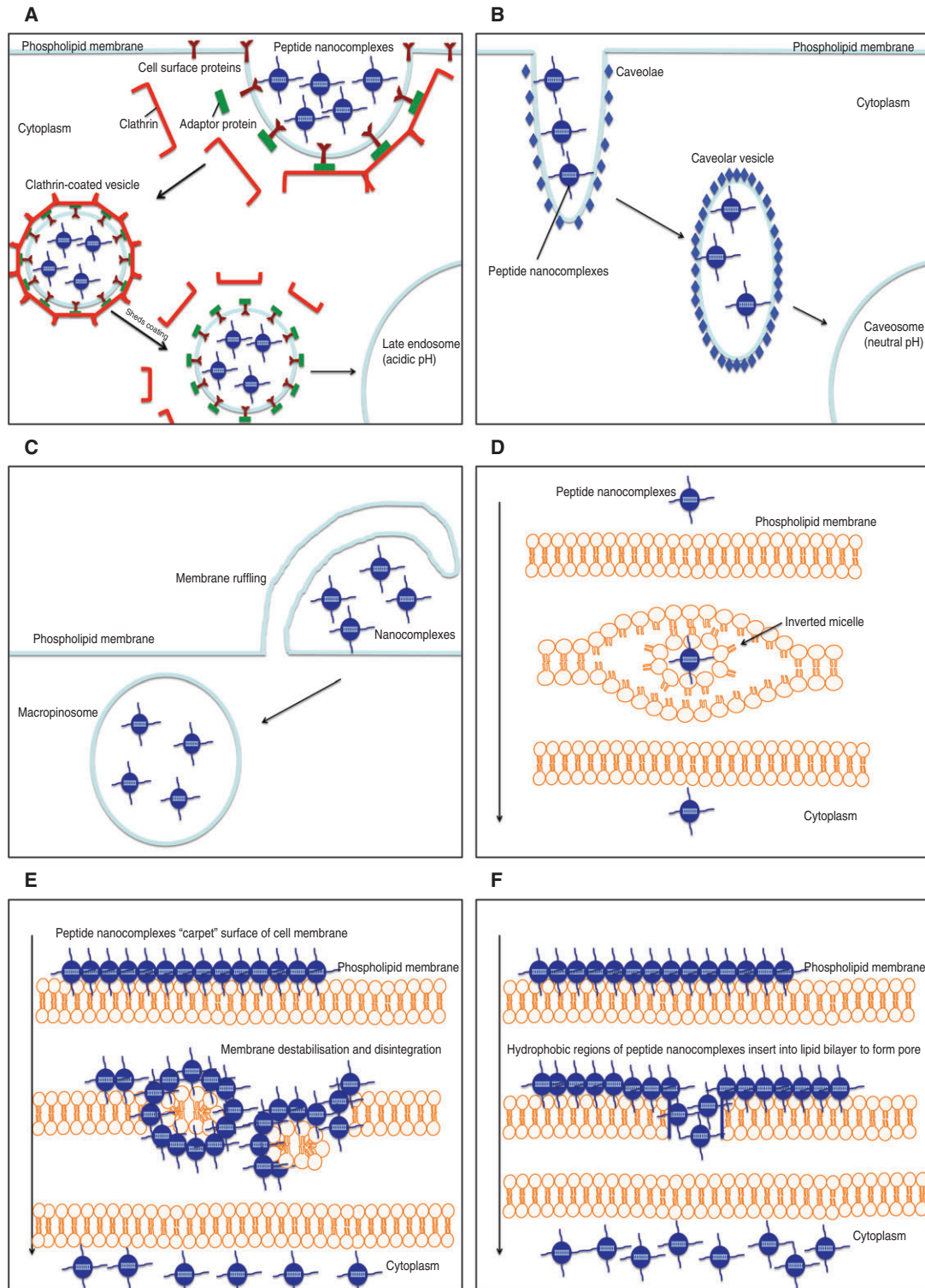
Figure 1: Schematic representation of endocytic (A, B and C) and direct penetration (E, F and G) mechanisms of internalization. (A) Clathrin-mediated endocytosis: Peptides are engulfed in clathrin-coated vesicles. Clathrin coating is shed prior to fusion with acidic, late endosomes. (B) Caveolea-mediated endocytosis: Peptides are engulfed in caveolae-coated vesicles and transported via microtubules to pH neutral caveosomes. (C) Macropinocytosis: Protrusive flaps of the cellular membrane non-selectively engulf extracellular material. (D) Inverted micelle model: Inverted micelles are generated upon interaction of peptides with negatively charged phospholipids. Peptides remain in a hydrophilic environment as they transverse the hydrophobic core of the lipid bilayer. (E) Carpet model: Peptides "carpet" the surface of the membrane imposing curvature strain and membrane collapse. (F) Pore formation model: Hydrophobic residues insert into the phospholipid core resulting in transient pore formation.

Table 1: DNA condensing motifs.

Peptide	Sequence	Origin	Reference
Poly-L-lysine (PLL)	(L)n	Synthetic	(18)
$R_5H_7R_4$	RRRRRHHHHHHHRRRR	Synthetic	(22)
$K_3H_4K_3$	KKKHHHHKKK	Synthetic	(23)
Protamine sulfate	RSSSRPVRRRRRPRVSRRRRRRGGRRRR	Salmon sperm	(25)
Mu (μ)	MRRAHHRRRRASHRRMRGG	Adenovirus core complex	(26)
Tat (48–60)	GRKKRRQRRRPPQ	HIV-1 transactivator	(48)
Oligoarginine	(R)n	Synthetic	(56)

Karjoo et al. (30) reported transfection efficiencies (>95%) in ovarian cancer SKOV3 cells with a viral mimetic nanoparticle system designated THG/Mu-PEG5K. The THG biopolymer, which consists of a targeting peptide (T), four repeating units of histone (H) and the fusogenic peptide GALA (G), was mixed at a ratio of 8:8 with the covalently bonded Mu-PEG5K to form stable nanoparticles with pEGFP-N1 (30).

When selecting a peptide sequence it must be noted that a fine balance is required between protecting the DNA from extracellular degradation, achieving favorable pharmacokinetics and ensuring effective intracellular release. Indeed the very characteristics that make cationic peptides powerful condensers of nucleic acids may also detrimentally affect nanoparticle biodistribution in vivo and cytoplasmic release of DNA. Whilst condensation with cationic peptides can dramatically reduce interaction of DNA with enzymatic elements in vivo, peptides too, particularly highly cationic, arginine-rich peptides, are susceptible to rapid clearance from the body and degradation by proteolytic plasma enzymes (31). Such problems may be overcome by functionalization with polyethylene glycol (PEG) (32). However, modification in this way has been shown to adversely affect intracellular kinetics (33). This problem may, in turn, be overcome by the use of sheddable PEG coatings. Zhu et al. (34) reported improved tumor accumulation and tumor-specific cleavage of a self-assembly block copolymer (PEG-pp-PEI-PE) due to the use of an MMP2 labile linker for PEG.

Therefore, with regard to the method of nucleic acid condensation, there is much to consider. And this is only the first step in a complex process.

Membrane destabilization

Traversing the cellular membrane is the next critical step for successful gene delivery. Cell-penetrating peptides (CPPs) are a class of peptides that can facilitate the permeabilzation of biological membranes. Endocytosis is the predominant mechanism of membrane translocation by CPPs with subsequent entrapment in the endosome, acidification and degradation of the genetic cargo unless escape to the cytosol can occur (35). Alternatively if CPPs enter the cell via direct membrane translocation, the endosome is by-passed and the need for an escape mechanism in the design of the peptide is circumvented. Elucidation of the mechanisms by which specific peptides are internalized is therefore crucial to effective vector design.

In the literature, CPPs have been categorized either according to their origin as protein-derived, chimeric or synthetic; or, according to their amphipathic profile as primary amphipathic, secondary amphipathic or non-amphipathic (Table 2). Their designation as such depends largely on the specific amino acid engineering and physiochemical properties of the peptide in question (10). Unsurprisingly arginine abundance is a common feature of CPPs, characterized by the presence of the guanidine head group, thus facilitating formation of strong bidendate hydrogen linkages with anionic components of the cell membrane (36–38). There is to date no consensus on the precise mechanism of cellular internalization of arginine-rich CPPs. However the degree and manner of initial interaction with the cell surface membrane ultimately dictates the eventual pathway of internalization and is generally recognized as being the first step of the CPP internalization process.

In a landmark study, cell surface activity and internalization was examined using Penetratin (RQIKI WFQNR RMKWK K-amide), a 16 amino acid peptide derived from the third helix of the Antennapedia homeodomain, PenArg (RQIR IWFQ NRRM RWRR-amide) and PenLys (KQIK IWFQ NKKM KWKK-amide) (39). Studies revealed that the levels of internalization with PenArg were 10 times higher than those seen with PenLys, indicating that arginine interacted more strongly with phospholipid membranes than lysine. Åmand et al. (39) then went on to quantify the relationship between strength of CPP interaction with cell

Table 2: Cell-penetrating peptides.

	Peptide	Sequence	Origin	Amphipathic designation	Reference
Protein derived	Penetratin	RQIKIWFQNRRMKWKK-amide	Antennapedia homeodomain	Secondary-amphipathic	(39)
	PenArg	RQIRIWFQNRRMRWRR-amide	Antennapedia homeodomain	Secondary-amphipathic	(39)
	PenLys	KQIKIWFQNKKMKWKK-amide	Antennapedia homeodomain	Secondary-amphipathic	(39)
	Tat (48–60)	GRKKRRQRRRPPQ	HIV-1 transactivator	Non-amphipathic	(48)
Chimeric	TP 10	AGYLLGKINLKALAALAKKIL	Galanin + Mastoparan	Primary-amphipathic	(52)
Synthetic	C6	RLLRLLLRLWRRLLRLLR	Synthetic	Secondary-amphipathic	(47)
	RWmix	RWRRWRRWRRWR	Synthetic	Secondary-amphipathic	(49)
	RWR	RRRRWWWWRRRR	Synthetic	Non-amphipathic	(49)
	Olioarginine	(R)n	Synthetic	Non-amphipathic	(56)
	Oligolysine	(R)n	Synthetic	Non-amphipathic	(56)

membrane and degree of cellular internalization, demonstrating conclusively an almost linear correlation between the two factors and verifying the previously unsubstantiated supposition that strength of interaction with the cell membrane is the crucial first consideration in deciphering CPP mechanism of internalization. Self-stimulated macropinocytosis was also shown to be the primary mechanism of membrane translocation for the PenArg CPP. Yet the cellular uptake of a chimera hybrid consisting only of D- and L-arginine isomers has been shown to be via direct membrane translocation following inhibition of endocytic pathways by both physical and pharmacological means (40). Direct translocation was further confirmed when the transmembrane potential was eliminated resulting in a drastic reduction in chimeric oligoarginine in the cytosol. Transmembrane potential is therefore another critical factor to consider in the translocation of guanidium-rich peptides (41).

The differences in transmembrane activity exhibited by arginine-rich CPPs can also be related to the amphipathic profile of the peptide (42, 43). For example the presence of two tryptophan (W) residues in the Penetratin backbone has led to its designation as a secondary amphipathic peptide, described as such due to the distribution of hydrophobic and hydrophilic charges on its secondary structure following interaction with phospholipid membranes (44). The presence, size and hydrophobic character of W has been shown to functionally enhance the internalization activity of CPPs primarily through improved anchoring of peptides to cell membranes (45). To what degree this amphipathic quality dictates the route of internalization is not clear. However, complete loss of function of Penetratin was observed following substitution of tryptophan (W6) for phenylalanine (46). It was then postulated that Penetratin followed a two-step model for internalization that involved initial electrostatic interaction followed by tryptophan-dependent membrane destabilization. The evidence is mounting that tryptophan therefore has a key role to play in the design of secondary amphipathic peptides. Indeed studies by Jafari et al. (47) demonstrated that replacing three leucine residues in the 18 mer C6 peptide with tryptophan to give C6M1 not only increased peptide solubility and secondary helical structure but also reduced cytotoxicity and increased intracellular uptake. Incorporation of tryptophan and leucine residues into modified Tat 48–60 markedly enhanced leakage from plasma membrane vesicles compared to those lacking a hydrophobic component (48).

Rydberg et al. (49) took the studies with CPPs one step further by examining the effect of arginine and tryptophan positioning within the peptide. Results indicated that positioning 1–4 tryptophans at the N-terminus significantly impaired efficacy; while cellular uptake was highest for the RWmix (RWRRWRRWRRWR), attributable to greater secondary amphipathicity afforded by equal spacing of the residues, cytotoxicity was lower in a RWR (RRRRWWWWRRRR) sequence that achieved greater accumulation in the cytoplasm and nucleus, aided by the non-endocytic uptake route (49). The positioning of the amino acid residues then becomes a critical factor with only the RWmix entering via endocytosis. Taken together it becomes apparent that when designing a peptide for nucleic acid therapeutics, the distribution of the selected residues can have a profound influence on the mechanism of uptake.

The effects of cargo and cell membrane composition on internalization are also key considerations that cannot be overlooked. The size and type of cargo, as well as the manner of binding to the CPP, can influence CPP translocation characteristics (50–52). Much information to this regard has been gleaned from the implementation of unilamellar vesicles as model membranes to analyze the

interaction of CPPs with lipid membranes. A recent publication by Vasconcelos et al. (53) used large unilamellar vesicles (LUVs) to delineate the relationship between peptide hydrophobicity and membrane perturbation characteristics of sterylated analogues of Transportan 10. They demonstrate that the interaction between the peptide and its given cargo can have an important influence on CPP secondary structure and therefore internalization profiles (53). However, the use of liposomal models as a tool to elucidate CPP mechanism of action is discouraged by some, who claim they do not adequately represent the environmental complexity of live cells (48).

Cell membrane glycosiaminoglycan (GAG) content has also been cited as a crucial mediator of internalization (54, 55). Naik et al. (56) examined the effect of surface-bound and free GAGs on the permeabilization characteristics of R_{16} and K_{16} homo-peptides in live cells. Results found that DNA complexed with the R_{16} peptide enter cells via non-endocytic and endocytic pathways, but that both are GAG independent. Complexes of DNA and the K_{16} peptide enter primarily via an endocytotic pathway, and is dependent on GAG presence (56, 57). Subrizi et al. (48) further challenged the role played by GAGs in cellular uptake, producing evidence that they actually inhibit movement of the Tat peptide across biological membranes in live cells. Indeed many of the methods used to analyze CPP behavior exhibit a high degree of analytical variability that only fuels the debate surrounding the mechanisms of cellular uptake (36).

Therefore until standardized methods are agreed amongst the field for evaluating the CPP phenomenon, correlations between peptide composition and cellular uptake will be difficult to elucidate. What is commonly accepted is that CPP permeabilzation occurs via two or more pathways and the propensity of any given CPP toward a particular pathway is highly variable and depends on a number of factors. These factors include CPP size, distribution of charge, hydrophobicity and peptide conformation, as well as considerations of cell membrane composition and cargo. These variables need to be accurately accounted for in experimental design to ensure reproducibility and consistency of results. Once this is achieved peptides can be tailored to ensure maximal accumulation within the desired intracellular location.

Endosomal escape

Endocytosis is the primary mechanism for movement of extracellular material across biological membranes (11).

Once endocytosed, the transported material is engulfed within an endosome. Lysosomes fuse with endosomes resulting in the acid degradation of the endosomal contents. This presents a significant barrier to gene delivery, one that must be overcome in order for DNA to reach its site of action, namely the nucleus. Fusogenic peptides are a class of amphipathic peptides derived from the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin (58). The HA2 peptide (GLFGAIAGFIENG-WEGMIDG) forms an α-helix under acidic conditions and fuses with the endosomal membrane, enabling cargo delivery into the cytosol (59). At physiological pH, the lytic activity of the HA2 peptide is negligible, which confers a level of targeting for endosome of the target cell. This then renders HA2 peptide and subsequent derivatives suitable for systemic administration.

One of the first derivatives of the HA2 sequence was the 30 mer designer peptide GALA (WEAALAEALAEAL-AEHLAEALAEALEALAA), characterized by a glutamic acid-alanine-leucine-alanine repeat (60). The maximum α–helical conformation of GALA occurred at a pH 5 which gives rise to a hydrophobic face on one side of the peptide and a subsequent interaction with the endosomal membrane. This results in pore formation and endosomal escape of the cargo to the cytosol. The repeating glutamic residues in GALA render it anionic and therefore ineffective for condensing and protecting nucleic acids. Nevertheless GALA has been utilized in several multi-functional systems as a discrete endosomal-disrupting motif. For example, the GALA peptide was incorporated into a biomimetic vector that also had four repeats of histone proteins to condense DNA, a targeting motif for HER2 and a cathepsin substrate that acts as an intracellular cleavage site (61). Studies demonstrated that positioning GALA on the N-terminus of the multifunctional vector ensured fusogenic amphipathic activity. GALA has also been utilized in the R8-MEND system to significantly improve gene expression in the liver (618-fold) in nanoparticles with a pDNA/PEI negative core (62).

In a bid to increase the functionality of GALA, Wyman et al. (63) substituted the negatively charged glutamic acid residues of GALA with positively charged lysine to produce the cationic peptide KALA (WEAK-LAKALAKALAKHLAKALAKALKACEA), which not only retains its fusogenic activity but can also condense negatively charged nucleic acids thanks to the positive charge conferred by the lysine. KALA has been utilized as an independent transfection agent alone and also to improve the activity of other delivery vehicles. KALA coating of PEG-g-PLL not only increased transfection efficiency but also displayed negligible toxicity compared to PEG-g-PLL alone (64). KALA has also been used to coat magnetic

mesoporous silica nanoparticles capped with PEI to deliver VEGF siRNA (M-MSN-siRNA@PEI-KALA) to not only reduce cytotoxicity but also significantly delay tumor growth in A549 lung tumors in vivo (65).

Given the superiority of arginine over lysine as previously discussed, McCarthy et al. (66) went on to create another cationic peptide termed RALA (WEAR-LARALARALARHLARALARALRACEA) to deliver DNA. Studies demonstrated that the fusogenic activity of RALA remained pH-dependent, toxicity was reduced in vitro compared to a commercial agent and that cellular entry was via caveolin- and clathrin-mediated endocytosis. Furthermore the RALA/pDNA nanoparticles retained activity following lyophilization with trehalose giving a suitable isotonic formulation for in vivo administration. Following systemic administration, gene expression was maximally observed in the lungs and liver (66).

A recent study carried out by Nouri et al. (67) compared the fusogenic activity of GALA, KALA and a number of other synthetic HA2-derived fusogenic peptides, INF7 (GLFEAIEGFIENGWEGMIDGWYG) (68), H5WYG (GLFHAI-AHFIHGGWHGLIHGWYG) (69) and RALA, each coupled with four histone repeats and a targeting motif. Although GALA outperformed the other peptides in terms of both the percentage of cells transfected and the levels of green fluorescent protein expressed, H5WYG performed best in the hemolytic assay, suggesting that of the five fusogenic peptides investigated, H5WYG is superior at disrupting endosomal membranes. This result was not unexpected as the presence of an imidazole ring in H5WYG acts as a proton sponge, thus enhancing the pH-buffering capacity of the peptide. H5WYG has a pKa value of approximately 6.0 and will be protonated at around pH 6. This property therefore facilitates early escape from weakly acidic endosomes whilst remaining in an inactive conformation at physiological pH (68). It should be noted, however, that the contribution offered by improved buffering is currently debated, with some finding that improved buffering of polymer complexes at low pH may not always enhance endosomal escape (70).

Histidines have also been employed to improve TAT as a gene delivery vehicle. Although TAT is excellent at condensing DNA and traversing the cell membrane, it cannot escape the endosome, rendering it ineffective for gene delivery. However, Lo et al. covalently added histidine residues to the C-terminus of TAT and found that the addition of 10 residues resulted in a 7000-fold increase in gene expression (71). Further modifications included the addition of two cysteine residues to improve stabilization and an equal distribution of histidine to give C-5H-Tat-5H-C which improved transfection a further 1000 fold (71).

It is important to note however, that the fusogenic activity of these peptides is a consequence of a pH-dependent shift in their conformational status, occurring in the late endosome or upon fusion with a lysosome following clathrin-mediated endocytosis. Endocytosis by non-acidic pathways such as caveolae-mediated endocytosis and macropinocytosis will nullify the membrane lytic activity of fusogenic peptides, in which case entrapment within the endosome remains a major problem. Therefore, before employing a fusogenic peptide in any delivery system, due consideration must first be given to the mechanism of cellular entry.

Nuclear import

Of all the barriers to gene delivery, nuclear import is by far the most challenging to overcome. The nucleus is enveloped by a highly impermeable double lipid bilayer known as the nuclear membrane (72). Movement across this membrane is regulated by highly restrictive nuclear pore complexes, interacting protein domains that form aqueous channels between the cell cytoplasm and the nucleoplasm. At their narrowest point these channels are a diameter of around 10 nm, allowing passive diffusion of ions and small proteins (<10 nm) into the nucleus. Movement of larger molecules into the nucleus relies on nuclear localization signals (NLS), small peptide sequences that interact with components of the importin super family of proteins, which mediate macromolecular movement into the nucleus. The genetic cargo must be within close proximity to the nuclear membrane to enable binding to nuclear transport factors, a process that can be facilitated by components of the cell cytoskeleton, which coordinate movement of molecules through the "cytoplasmic sieve" (12). The challenge is therefore to identify a NLS that can: 1) Retain functionality by not binding to the genetic cargo that is to be delivered, 2) interact sufficiently with elements of the cytoskeleton to mediate accumulation of the genetic cargo around the outer membrane of the nuclear envelope and 3) bind specifically with importin adaptor proteins that arbitrate nuclear uptake.

Over the past number of decades many peptide NLSs have been identified for the purposes of active shuttling of DNA to the nucleus and their ability to enhance nuclear accumulation of cargo. However, despite numerous attempts to improve transfection with non-viral vectors through the use of NLSs, nuclear accumulation in quiescent cells remains a significant barrier to successful gene delivery (73). When selecting a NLS in peptide

design, the key factors that need to be considered are 1) NLS characteristics, 2) cellular characteristics and 3) cargo characteristics.

NLS characteristics

Classical NLSs, such as the REV peptide derived from HIV (RQARRNRRNRRRRWR) and the large tumor antigen of the simian virus 40 (SV40) (PKKKRKV) are short, basic peptides that interact with importin-α adaptor proteins to mediate transport to the nucleus. Kim et al. (74) recently demonstrated enhanced transfection when a cysteine-enriched SV40 derivative (GYGPKKKRKVGGC) was complexed with pLuc DNA before cationic liposome encapsulation. Several variants of the SV40 were tested, but only the C terminal disulfide homodimer resulted in improved efficiency and DNA release (74). The human mRNA-binding protein hnRNP M9 (GNYNNQSSNF-GPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY) is an example of a non-classical NLS, which binds directly to importin-β without binding to importin-α adaptor proteins (75). The unordered M9 peptide is distinctly useful in multifunctional peptide systems because the lack of basic residues reduces interaction with the DNA cargo and therefore the NLS functionality remains intact. Canine et al. (76) utilized the M9 NLS in a multifunctional biopolymer termed FP-DCE-NLS-TM where FP is a fusogenic peptide, DCE a DNA condensing and endosomolytic sequence and the TM a targeting motif. A truncated version FP-DCE-TM evoked negligible gene expression thus proving that the M9 NLS remained functional in the biopolymer (76).

Cellular characteristics

The binding affinities of nuclear transport proteins to particular NLS is cell-type dependent. Gu et al. (77) characterized the nuclear import characteristics of the HIV-1 derived Rev peptide across different cell lines, with results suggesting that HeLa, U937 and THP-1 cell lines employed transportin as the major transport receptor to rev, whereas in 293T, Jurkat or CEM cell lines, importin-β was the primary mediator of nuclear uptake. Intranuclear transport characteristics are also known to alter once cells become cancerous (78, 79). A truncated form of importin-α has been found to lack a NLS binding domain in ZR-75-1 breast cancer cells, which would restrict the efficacy of any NLS operating on that pathway, e.g., SV40 (80).

Cargo characteristics

Direct conjugation of the NLS to the DNA cargo has largely failed to significantly enhance gene expression (12, 72). In its uncondensed form, DNA is susceptible to degradation by cytoplasmic enzymes and movement through the densely packed cytosol is impeded due to the unordered state and size of uncondensed pDNA (81, 82). Use of cationic condensing agents such as the core protein Vll of adenovirus type 2 or histones that contain inherent NLS have been shown to help overcome such issues (83, 84). However, more success has been derived from the conjugation of NLS to polycation binding proteins, thus reducing interference from cargo. Yi et al. (85) reported a 200-fold enhancement in transfection efficiency of Tat conjugated to the NLS PKKKRKV-NH$_2$ (PV) compared to Tat/DNA complexes alone. Furthermore, complexes formed by non-specific electrostatic interaction (Tat/PV/DNA) showed no significant enhancement in transfection. Therefore with respect to peptide design it is better to covalently attach the NLS to the peptide and also ensure availability of the NLS to the importin proteins within the cytosol.

Conclusion

The phrase "from needle to nucleus" is one that has been coined as the ideal in vector development for gene therapy. In reality however, the process is a stepwise one, with numerous hurdles that must first be overcome before the nucleus of target cells is reached. The purpose of this article is to identify peptides that can be utilized to help advance this agenda.

One criticism of peptide-based gene therapy might be the failure, thus far, to identify a single peptide sequence independently capable of highly efficient gene delivery. Progress therefore, relies on a multifaceted approach to nanoparticle design; one that involves collaboration across various non-viral disciplines and one that is based on systematically addressing the biological barriers faced. Such a philosophy has led to the development of a variety of peptide-enhanced, multifunctional nanoparticle systems, some of which have been referred to herein. Examples include the amelioration of polymers, lipids, micelles, chitosan and MENDs with peptides for improved gene delivery. For a comprehensive analysis of multifunctional non-viral vectors in gene therapy the reader is referred to a recent review by Wang et al. (86).

Therefore, whether required for targeting, nucleic acid condensation, membrane destabilization, endosomal

escape or nuclear localization, peptides offer a wealth of promise when incorporated into multifunctional system designs such as these.

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Bionotes

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Stephen Loughran was awarded a Masters in Pharmacy (1st Class) by Queens University Belfast in 2011. He is currently in his second year of a PhD research project which focuses on the design of multifunctional peptide vectors capable of delivering microRNA to treat metastatic prostate cancer.

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Cian McCrudden was awarded a BSc (Hons) degree in Biomedical Sciences (which included a year's research placement in the Pharmacology Department in the University of Nevada's Medical School) and a Masters in Research by the University of Ulster, and received his PhD in peptide pharmacology from Queen's University, Belfast. Since 2007, Dr McCrudden has gained extensive postdoctoral experience in cancer pharmacology, and since 2012 has been characterizing the potential of a DNA delivery peptide for the inducible nitric oxide synthase gene therapy treatment of metastatic breast and prostate cancer.

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Graphical abstract

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**Designer peptide delivery
systems for gene therapy**

Original Research Article: From
'needle to nucleus': The journey of
self-assembling, multifunctional
peptide nanoparticles, the extra- and
intra-cellular barriers they face and
the mechanisms by which these
barriers are overcome.

Keywords: biological barriers; DNA;
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