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Gene delivery by peptide-assisted transport

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Abstract

The diverse amino acid chemistries and secondary structures in peptides provide 'minimalist' mimics of motifs in proteins and offer many ideal properties for targeted delivery approaches. Several non-viral vectors (polymers and lipids) have been studied for their potential applications in gene delivery. However, non-specific uptake, lack of targeting, inability to escape endosomes, and inefficient nuclear delivery limit their application. Peptide-assisted trafficking of non-viral vectors can potentially overcome these biological barriers to improve gene delivery through targeted uptake using key cell-surface receptors (*e.g.*, integrins, growth factor receptors, and G-protein coupled receptors); membrane disruption for endosomal escape; and nuclear importation. Furthermore, the capacity of peptides to regulate spatio-temporal control over gene delivery opens multi-faceted avenues for effective gene delivery in a variety of complex applications. Rigorous on-going *in vitro* and *in vivo* studies utilizing peptides for targeted and microenvironment-sensitive gene delivery could promote their widespread clinical usage.

Keywords

Cell penetrating peptide; DNA; gene delivery; non-viral vectors; peptide

Introduction

Gene therapy is a revolutionary approach in medicine that has been heralded for its potential capacity to address the root causes underlying various diseases and not merely the symptoms [1]. The foundation of gene therapy is the modulation of gene expression through delivery of exogenous genetic materials like DNA and RNA [2]. Gene therapy was originally envisioned for treatment of inherited disorders, but it has risen in prominence due to its broader potential in a wide variety of both hereditary and acquired conditions ranging from cardiovascular and monogenic diseases to cancers. Recent gene therapy approvals by the US FDA are remarkable milestones in gene delivery technology. For example, in fall of 2017,

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Conflict of interest

The authors declare no conflict of interest.

two chimeric antigen receptor (CAR) T cell immunotherapies (Kymriah [3] and Yescarta [4]) were approved by the US FDA for the treatment of advanced stage hematological malignancies [5], representing the first clinical approvals for gene therapy products in the US. The CAR-T technology involves reconstruction of CAR receptors on T cells to enable antigen recognition and destruction of malignant B cells [6]. Patient-derived T cells are genetically modified *ex vivo* and subsequently injected back into the bloodstream of the patients [7]. Meanwhile, in December, 2017, Luxturna became the first US FDA approved *in vivo* gene therapy for treatment of inherited retinal disease. Luxturna corrects mutations in the RPE65 gene by using an adeno-associated viral (AAV2) vector to carry the functional gene into the affected eye tissue [8]. These new approaches represent promising steps forward in the advancement of gene therapy products that are ultimately targeted to a wider array of tissues and administered using a broader range of methods.

The last two decades have seen continual increases in the number of clinical trials involving gene delivery technologies [9]. Among the gene carriers used in these trials, viral vectors such as adenoviruses, AAVs, lentiviruses, and retroviruses have been the dominant carriers used in nearly all gene therapy applications. However, only a few products (Kymriah, Yescarta, and Luxturna [US]; Gendicine and Oncorine [China]; Rexin G [Philippines]; Neovasculgen [Russia]; and Glybera [Europe]) are available in the worldwide market, attributable in part to the inherent shortcomings in viral vectors: complex and costly processes for production, limited DNA packaging ability, cytotoxicity, broad tropism, immunogenicity, and tumorigenicity [10]. These limitations have spurred development and advancement of a range of non-viral gene carriers fabricated using innovative synthesis schemes [11]. Two major categories of non-viral vectors include: cationic lipids [*e.g.*, (N-[1-(2,3-dioleyloxy)propyl-]-N,N,N-trimethylammonium chloride (DOTMA); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); 1,2-Dioleyl-3-trimethylammonium propane (DOTAP)] and cationic polymers [*e.g.*, linear, branched, and dendritic polyethyleneimine (PEI); poly(2-dimethylaminoethyl methacrylate) (PDMAEMA); poly(beta-amino esters) (PBAEs)] [12,13]. Spontaneous electrostatic interactions between these non-viral carriers and nucleic acids lead to the formation of lipoplexes and polyplexes, respectively, which offer useful properties for gene delivery [14]. Cationic lipids offer efficient binding to negatively charged cell surface constituents to stimulate uptake, and cationic lipids can further interact with the endosomal membrane to facilitate cytoplasmic delivery [15]. Alternatively, cationic polymers, including branched, linear, and dendritic structures, offer flexible polymer chemistries that facilitate design and formulation of nanoscale polyplexes with tailorable properties, with facile polymer modification for use in plethora of biomedical applications [16].

Peptides for gene delivery

Non-viral vectors can encapsulate higher amounts of DNA and avoid detrimental immune responses associated with viral vectors. However, non-viral carriers exhibit gene expression levels that are typically several-fold lower than viral vectors. This inefficiency stems from the formidable extracellular and intracellular barriers precluding gene delivery using non-viral carriers (Figure 1). Recently, efforts have been focused on fine-tuning the functional components of non-viral vectors to enhance their biological efficacy.

Peptides are short amino acid chains of approximately 50 amino acids or fewer that can be used as unique tools in drug/gene delivery. Peptides are biodegradable and biocompatible, and their amino acid building blocks can be easily manipulated to accelerate several functions of barrier penetration and targeting of gene cargoes [17]. Peptides facilitate access to a wide array of chemistries through incorporation of both naturally-occurring amino acid monomers as well as non-natural amino acids (>30), resulting in a broad chemical modification repertoire. Furthermore, peptides can incorporate a variety of useful structural and functional motifs, including protein fragments with similar secondary structure features as their parent domains, and high-affinity/high-specificity cell-binding motifs selected through phage display or other high-throughput technologies. Additional advantages of peptides as compared to proteins include ease of production (in bacterial or mammalian cells, or by using solid phase peptide synthesis), and stability during storage and handling under ambient conditions [18,19]. Given their unique properties and facile production, peptides have been widely explored for their capacity to help overcome the barriers for gene delivery (Figure 2), including cell penetration, endosomal escape, and organelle targeting [19]. Different pathways through which a peptide can function as a barrier penetrating peptide are presented in Figure 2.

Cell penetrating peptides

Cell penetrating peptides (CPPs) are positively charged sequences 10-30 amino acids in length that can complex with nucleic acids via electrostatic interactions, or alternatively, be conjugated to lipidic or polymeric components in gene carriers. CPPs are broadly categorized as: cationic peptides [comprising arginine and lysine residues; *e.g.,* trans-activator of transcription (TAT) peptide, penetratin, and oligoarginines)]; amphipathic peptides (comprising both hydrophobic and hydrophilic segments; *e.g.,* buforin 2, crotamine, and azurin p18), and hydrophobic peptides [e.g., peptide derived from vascular epithelial cadherin (pVEC) and Pep-7] [26]. CPPs can internalize into the cells through three main pathways as presented in Figure 3a. The first pathway is through direct penetration, wherein positively charged CPPs interact with phosphate groups on the lipid bilayer of the cell membrane leading to the formation of cavities that facilitate direct access into the cytoplasm. The second pathway is through induction of a transitory structure within the lipid bilayer that allows penetration and uptake (*e.g.,* the inverted micelle structure formed by the penetratin dimer and negatively-charged phospholipids within the membrane). The third pathway is through endocytosis, an energy dependent pathway typically involving clathrin-mediated uptake, caveolar uptake, or macropinocytosis (detailed endocytic pathways are presented in Figure 3b) [23]. Both non-endocytic mechanisms involve CPP-mediated disturbances in the structural integrity of the cell membrane via non-covalent binding with membrane components, a process which allows the CPPs to translocate in an energy- and temperature-independent fashion. Meanwhile, CPP-triggered endocytosis is driven by CPP binding to membrane components, leading to energy- and temperature-dependent membrane invagination and uptake. For example, a study by Zhang et al. utilized HIV-1 TAT conjugated to PEI800 with 4’4-dithiodibutyric acid (DA) to develop CPP-labelled degradable gene carriers for efficient gene transfection (PEI-DA-TAT) [27]. These studies showed that the cationic HIV-1 TAT peptide interacted with the negatively charged cell

membrane and thereby stimulated endocytosis of the peptide along with the gene carrier system. The resulting transfection efficiency of PEI-DA-TAT was increased by 8% as compared to PEI-DA and PEI25K. Another interesting study by Jiao et al. investigated the concentration dependence of arginine-rich CPPs on import pathways [28]. At low concentrations (< 2 μM), the uptake was mediated by direct translocation across the plasma membrane, whereas at higher concentrations (> 2 μM), glycosaminoglycan-dependent endocytosis was induced. Recent applications of CPPs for enhanced gene delivery applications are illustrated in Table 1.

Peptides for targeting cells and organelles

Peptides have generated considerable interest both as cellular targeting ligands able to initiate transport across specific cellular membranes through interactions with cell surface receptors, and as subcellular targeting ligands able to navigate barriers within the intracellular membrane network. The design of targeting peptides for specific receptors requires comprehensive information about the natural ligand's binding site, secondary structure, and sequence to develop a biomimetic peptide. Alternatively, phage display can be used for screening a large library of peptides for desirable properties.

Peptides have been primarily explored against several major receptor classes including integrin receptors (*e.g.*, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$), growth factor receptors [*e.g.*, epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR)], and G-protein coupled receptors (*e.g.*, octreotide, exenatide, leuprolide, and abarelix). These receptor classes have generated enormous interest due to their broad relevance in cancers, cardiovascular conditions, tissue regeneration/repair, and other diseases (*e.g.*, diabetes mellitus, obesity, infectious, and inflammatory diseases).

Integrins are heterodimeric cell surface receptors involved in cell adhesion, cell differentiation, cell proliferation, cell migration, and cell survival. Integrin signaling through receptors such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrin is an important regulator of metastasis, and integrins also are involved in tumorigenesis through co-regulation of growth factor *(e.g.*, EGFR) activity in cancers. The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins bind to the RGD domain in vitronectin. $\alpha_5\beta_1$ integrin binds to the RGD domain in the 10th type III module (FNIII10) of fibronectin, as well as to the synergy site in the FNIII9 module, with the RGD motif forming, and the synergy site contacting only the head domain of the α subunit. Integrin-targeting approaches have especially benefitted from the design of integrin-targeting peptides that are able to mimic the presentation of native integrin ligands in extracellular matrix (ECM) and thereby garner increased receptor specificity. For example, the PR_b peptide (KSS**PHSRN**SGSGSGSGSG**RGDSP**) was designed to specifically target the $\alpha_5\beta_1$ integrin via spatially-constrained presentation of the RGD and synergy motifs using an SG spacer sequence. This peptide was tested for its capacity to enhance the selectivity of liposome-mediated gene transfection in DLD1 colorectal cancer cells [21]. In these analyses, integrin binding by the PR_b peptide triggered a combination of macropinocytosis and caveolar endocytosis of liposomes. The transfection efficiency of the PR_b-modified liposomes in DLD1 cells was enhanced by approximately 8-fold and 5-fold when compared

to PEI polyplexes and stealth liposomes, respectively, and targeting to the $\alpha_5\beta_1$ integrin was confirmed by evaluation of the binding affinity of PR_b peptide to $\alpha_5\beta_1$ integrin (76.3 ± 6.3 nM). In another example, a different peptide targeting the $\alpha_5\beta_1$ integrin receptor (CPIEDRPMC; *i.e.*, RPM) was used by Lee et al. for targeted delivery in a murine invasive colorectal cancer model [22]. RPM was conjugated to PEI-grafted PEG and incorporated into a polyplex system comprised of D-luciferin pDNA and PEI-grafted PEG (Figure 4) [22]. RPM stimulated $\alpha_5\beta_1$-mediated endocytosis, resulting in a 2-fold increase in transfection efficiency in HT29 colorectal cancer cells and enhanced tumor-accumulation in HT29 xenografts in mice. The proton sponge effect of PEI assisted in the endosomal escape process for gene delivery. Altogether, integrin targeting peptides can facilitate specificity, integrin-mediated internalization, and efficient transfection for potential treatment of diseased cells.

Growth factor receptors such as EGFR, PDGFR, and VEGFR are glycoproteins with an extracellular ligand-binding domain, a hydrophobic transmembrane region, and intracellular sequences containing regulatory and catalytic (kinase) domains that are overexpressed in many malignant gliomas. The versatility of peptide chemistries has proven especially useful in GFR targeting, with several examples demonstrating the benefits of phage display for selection of high affinity/high specificity GFR binders. For example, an EGFR-binding peptide (an 11-amino acid oligopeptide termed GE11) with high EGFR specificity and a K_D of 22 nM was identified based upon phage display. GE11 was used for EGFR targeting within self-assembling peptide nanovesicles (SPVs) that were assembled from GE11-glycidyl hexadecyl dimethylammonium chloride (GE11-GHDC)/cholesterol/DOPE using thin-layer evaporation and reverse-phase evaporation methods [34]. The delivery of doxorubicin and the acetylcholinesterase gene using EGFR-targeted SPV resulted in excellent *in vitro* and *in vivo* drug/gene delivery with a significant growth-suppressing effect on liver cancer xenografts. Tumor weights in treated mice were 4-fold lower than tumor weights in animals treated with non-EGFR targeted SPV. Moreover, EGFR-specific nanovesicle targeting was clearly demonstrated based upon hyperspectral confocal fluorescence microscopy and *in vivo* tumor distribution in the xenograft mouse model, framing the benefits of peptides for enabling cell-targeted therapy.

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors and their overexpression is associated with promotion of cancer aggressiveness. A total of 118 peptide-binding GPCRs have been identified and hence peptides mimicking their natural agonists or antagonists can be used for targeting the orthosteric binding site of the receptor for cargo delivery. For example, a group of researchers designed a hybrid adeno-associated virus and phage (AAVP) vector displaying biologically active octreotide (Oct) for ligand-directed gene delivery of tumor necrosis factor (TNF) [35]. Oct can emulate the specific binding to somatostatin receptor 2 (SSTR2) in pancreatic neuroendocrine tumor (NET) cells to enhance uptake. A 5-fold increase in binding of Oct-AAVP-TNF to the NET cells was observed as compared to the untargeted control AAVP-TNF. The enhanced accumulation of Oct-AAVP-TNF in insulinomas of a human multiple endocrine neoplasia syndrome type 1 (MEN1) mouse model, and the subsequent TNF expression in tumors, provided clear evidence for improved targeted gene delivery by Oct. Taken together, the utilization of various natural ligand-mimetic peptides for GPCRs can enhance targeted cargo

delivery of gene or small drug molecules to diseased cells for improvement in therapeutic efficacy.

Following endocytosis, many gene carriers become entrapped within endosomes, which can fuse with lysosomes or recycle their contents back to the cell surface. To avoid these fates, gene carriers must either escape endosomes or traffic within the cell through alternative trafficking strategies involving non-acidifying/non-recycling vesicles [36,37]. Several groups have taken advantage of the sophisticated chemical/physical properties of peptides to design peptide nanostructures with elegant membrane-disruptive capacity inside the acidic endosome. The mechanisms of action by such peptides include pore formation in the endosomal membrane [*e.g.*, listeriolysin O, pneumococcal pneumolysin, virus inspired polymer for endosomal release (VIPER)] [38,39]; the proton sponge effect (*e.g.*, histidine-rich peptides) [40]; or conformational changes in peptide secondary structure that assist membrane insertion and endosomal escape (*e.g.*, hemagglutin derived peptide HA2, INF7, GALA) [41]. For example, a recent study by Asseline et al. demonstrated the cytosolic targeting ability of the histidine-rich peptide H5WYG (GLFHAIAHFIHGGWHGLIHGWYG) derived from the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin following its conjugation to the 5’-end of the RNase H-incompetent antisense 2’-O-methyl-phosphodiester oligonucleotide (2’-Ome RNA705) [42]. (Note: the oligonucleotide targets an aberrant splicing of luciferase pre-mRNA in HeLa pLuc705 cells, such that successful delivery induces the increased expression of active luciferase.) The H5WYG peptide undergoes a conformational change between pH 7.0 and 6.0 after imidazole protonation, resulting in permeabilization of the cell membrane in a weakly acidic medium; accordingly, H5WYG is an excellent candidate for pH-mediated delivery of oligonucleotides to the cytosol from early endosomes. Confocal imaging demonstrated an approximately 5-fold higher level of cytosolic accumulation of H5WYG-2’-Ome-RNA705 as compared to 2’-Ome-RNA705; moreover, H5WYG-2’-Ome-RNA705 exhibited significant levels of nuclear accumulation due to the ability of cytosolic oligonucleotides to pass through the nuclear pores without the requirement of an active process. A 2660- and 400-fold increase in luminescence for H5WYG-2’-Ome-RNA705/lipofectamine treated HeLa cells was observed as compared to untreated control and 2’-Ome-RNA705/Lipofectamine treatments, respectively. This result is attributable to the successful entry, endosomal escape, and nuclear delivery of H5WYG-2’-Ome-RNA705 that directed the splicing machinery toward complete intron 2 removal by masking the cryptic splice site to induce luciferase expression.

Larger DNA cargoes typically require more active strategies to be trafficked into the nucleus, as most gene carriers are too large in size to passively diffuse through the nuclear pore membrane [43]. Peptides offer a potential solution to this issue through their ability to mimic the nuclear localization sequences (NLSs) found in proteins, viruses, and other native structures, particularly when NLS targeting is coupled to strategies that shuttle gene carriers into the perinuclear cytosol to access the nuclear uptake machinery. In one study, the simian virus 40 (SV40) large T-antigen derived NLS was conjugated to the LAHA4-L1 peptide, a pH-responsive (cationic amphipathic) peptide that promotes endosomal escape of nanocarriers. The coupled SV40 NLS-LAHA4-L1 peptide was assembled with luciferase DNA to form complexes that were ~130-165 nm in diameter, whereas LAHA4-L1/DNA

complexes lacking the SV40 sequence formed ~227 nm complexes. DNA delivery efficiency was significantly improved in slow-dividing cells and dendritic cells, with up to a 10-fold increase in gene expression using NLS-linked LAH4-L1 as compared to LAHA4-L1 alone in slow-dividing epithelial cancer cells (Calu-3), macrophages (RAW264.7), dendritic cells (JAWSII), and thymidine-induced growth-arrested cells [25].

An alternative approach is the design of peptides that can shuttle gene carriers to the nucleus by using retrograde transport or other alternative trafficking patterns employed by some viruses and toxins. Ross, Munsell, and coworkers demonstrated the ability of histone tail motifs [histone 3 (H3)-derived peptides] to traffic DNA polyplexes into the nucleus by enhancing caveolar uptake and increasing polyplex transport through the Golgi/ER en route to the nucleus [36]. H3/PEI/DNA polyplexes trafficked through the Golgi/ER via a non-acidifying vesicular route that overlapped with H3K4 methyltransferase (H3K4MT)-linked pathways and also was regulated in part by Rab6 (a marker of retrograde transport from the Golgi to the ER). The H3/PEI/DNA polyplexes ultimately reached the nucleus through post-mitotic ER membrane redistribution in a process that was facilitated by the H3 NLS and importin-4 proteins [36,44]. Recently, the benefits of the H3-targeting approach were demonstrated in mesenchymal stem cell (MSC)-targeted gene delivery for bone repair applications. H3-targeted polyplexes induced a 4-fold enhancement in osteogenic bone morphogenetic protein-2 (BMP-2) expression by MSCs as compared to gene carriers lacking H3 [37]. Moreover, 100-fold more recombinant BMP-2 protein was needed to achieve similar levels of BMP-2-mediated chondrogenic gene and protein expression as H3-targeted BMP-2 gene transfer, demonstrating clear translational impacts.

Peptides for spatiotemporally-controlled gene delivery

Techniques that enable spatiotemporal control over which cells receive and express gene cargoes are of great interest to minimize off-target effects following gene transfer. The chemical versatility in peptides has been exploited for this purpose, often by designing peptides whose properties depend upon the application of an exogenous or endogenous stimulus. For example, an interesting photoswitchable CPP design offered a route to spatiotemporally control peptide activation and uptake in cancer cells [45]. The CPP was comprised of cell-penetrating oligoarginine (positively charged) linked to an inhibitory oligoglutamate (negatively charged) via an azobenzene (AB) moiety within the peptide backbone. The turn-like structure of the *cis*-form AB allowed efficient pairing of the oppositely charged peptide sequences, whereas the extended *trans*-form disrupted this pairing to release oligoarginine for cellular uptake. The light-responsive nature of AB allowed the structure and behavior of the peptide to be directly controlled by the application of light, with the *cis*-AB induced by application of certain wavelengths of light ($\lambda \approx 360$ nm), and the *trans*-AB induced by distinct wavelengths ($\lambda \approx 440$ nm). A 4- to 10-fold increase in cellular uptake of the *trans*-form vs. the *cis*-form occurred in cultured HeLa cells. This unique structural modification could potentially be further utilized to control uptake of gene carriers and other cargoes.

Studies by Urello et al. demonstrated an alternative approach to use ECM-inspired peptides to enable endogenous (proteolytic) control over the release/expression of gene cargoes from

within collagen matrices. Their work employed collagen mimetic peptides (CMPs) to attach DNA polyplexes to collagen scaffolds such that the polyplexes were only released during matrix metalloproteinase (MMP)-mediated collagen remodeling and endocytic collagen uptake by fibroblasts [46-48]. CMPs are peptides comprised of GXY repeats such as the GPO triplet, and these peptides can strand invade into collagen to form a collagen triple helix incorporating one strand of the peptide. Urello and coworkers synthesized CMP-PEI conjugates, and they tuned CMP-PEI composition in DNA polyplexes to enable stable binding of CMP-polyplexes within the collagen gel matrix. Their approaches showed significant benefits for spatiotemporally-controlled platelet derived growth factor (PDGF) gene delivery in response to high levels of MMP expression, such as those present in the chronic wound bed. MMP activity mediated polyplex release through collagen degradation, and polyplex release could be modulated based upon the CMP conjugation ratio (0% to 50% CMP-PEI) to extend release from 20 days (for 0% CMP-PEI) to 35 days (for 50% CMP-PEI) [46]. *In vitro* wound closure with PDGF polyplexes was improved by 2-fold and 2.5-fold with 20% CMP-PEI polyplexes and mixed polyplexes (20% CMP-PEI and 50% CMP-PEI mixtures), respectively, as compared to non-CMP-modified polyplexes [47]. On-demand polyplex release and gene expression delivery was also were demonstrated *in vivo*, wherein gene expression could be tailored to peak at 6-20 days depending on the levels of CMP modification [48].

Spacing and arrangement of peptides on non-viral vehicles: Strategies and implications for gene delivery

Nanocarrier modification with peptides not only requires careful peptide design, but also careful consideration of the number and arrangement of ligands required for cell binding, uptake, and/or trafficking. Ligand density is of critical importance, as it defines the binding avidity of the ligand and therefore strongly affects cellular responses. Furthermore, peptides can be arranged to mimic the specific presentation of clustered surface motifs in viruses and other naturally-occurring structures. For example, the effect of clustered RGD ligands on the transfection efficiency in DNA/PEI polyplexes was studied by covalent conjugation of RGD peptide-modified gold nanoparticles on the surfaces of polyplexes [49]. The clustering mimicked the physical structure of adenovirus type 2, which possesses five penton-base proteins located at each of the 12 vertices of the virus surface, with protruding RGD peptide sequences located 5.7 nm apart for viral cell entry (Figure 5). The presence of RGD nanoclusters on the DNA/PEI polyplex resulted in a 5.4-fold or 35-fold increase in gene transfer efficiency over unmodified polyplexes in HeLa cells with low- or high-integrin surface density, respectively.

The spacer length for conjugated peptides is another important parameter that must be optimized to enable incorporation of bioactive peptide sequences without compromising the non-viral gene transfer activity. An interesting study by Stefanick et al. analyzed the effect of peptide linker length on cellular uptake of liposomes in breast cancer cells [50]. As compared with PEG2000 (~45 repeat units of ethylene glycol), the use of short linkers (EG12, with 12 repeats of ethylene glycol) for HER-2-targeting peptide conjugation significantly improved the uptake of liposomes by ~9-fold and ~ 100-fold, respectively, in

breast cancer cells and multiple myeloma cells. These authors studied the effects of different linker lengths (EG6, EG12, EG18, EG24, EG30, EG36, EG45, and EG72) to determine that minimal uptake with the EG6 linker was either attributable to the inability of such a short linker to present the HER-2 peptide in a sufficiently extended fashion beyond the liposomal PEG coating, or to the insufficient linker length for allowing the peptide to reach the binding pocket on the receptor. Enhanced liposomal uptake was observed with EG12 and EG18 linkers, but gradually declined with EG24 and completely disappeared with EG45 and EG72. Shorter EG linkers (EG12, EG18) can adopt a more linear conformation than longer linkers (EG24, EG30, EG36, EG45, and EG72), which form globular mushroom-like structures with reduced capacity for peptide interactions.

Peptide-ligand combinations for synergistic enhancement in gene delivery

Gene delivery is affected by several intracellular barriers in sequence, including the cell membrane, endosomal membrane, and nuclear membrane. Accordingly, employing peptide-ligand combinations that can overcome different gene transfer barriers in series can provide synergistic improvements in gene transfection efficiency and reduced off-target effects. For example, use of a peptide for endosomal escape alone may not help in nuclear delivery of pDNA in nondividing cells because pDNA primarily enters the nucleus in conjuction with nuclear membrane breakdown during mitosis. This issue was addressed in a study exploring the synergistic effects of anchoring the fusogenic peptide GALA and the NLS maltotriose onto lipid nanocarriers [51]. The modification with fusogenic peptide GALA alone (for endosomal escape) enhanced transfection efficiency in liver of mice (following injection via tail vein) by 1 and 2 orders of magnitude for lipid nanocarriers prepared with DOTAP and DOTMA, respectively. Addition of maltotriose (for nuclear targeting) resulted in a further enhancement in transfection activity by 1 order of magnitude to reach the higher level obtained for a conventional lipoplex but with less hepatic toxicity. These results were attributable to the individual effects of GALA and maltotriose on endosomal escape and nuclear transfer to potentiate enhancement in transfection.

Future prospects

Recent gene therapy approvals by the FDA have encouraged a substantial investment in advanced gene delivery technologies. The chemical versatility and unique biochemical properties in peptides can be exploited to improve targeting specificity and enhance transfection in non-viral gene carriers. Furthermore, the stimuli-responsive properties in peptides offer new opportunities for spatiotemporal control in gene delivery and open avenues for multi-faceted, peptide-based systems in treatment of different diseases such as cancer, chronic wounds, and hereditary diseases. Appropriate chemical modifications of peptides that can sense changes in the disease microenvironment would be highly promising for effective treatment. Furthermore, peptides that are responsive to external stimuli such as light may be useful for switching the active/non-active forms of peptides to enable efficient uptake and transfection only in desired sites of interest.

Several PEI- and lipid-based nanoparticles for gene delivery have been developed for clinical trials, mostly focused on the treatment of different cancers [52]. However, peptide-

mediated gene delivery is still in its infancy for successful application in the clinic. Rigorous *in vitro* and *in vivo* studies using peptide-mediated gene delivery are necessary to uncover the immunogenic properties, off-target effects, and efficacy of peptides in specific applications. The diverse amino acid chemistries and secondary structures will ultimately offer rich opportunities for targeted gene transfer in a variety of applications.

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Abbreviations

AAVP	adeno-associated virus and phage
AB	azobenzene
Api	Arginine rich peptides containing unnatural amino acid 4-aminopiperidine-4-carboxylic acid
BMP-2	bone morphogenetic protein-2
CAR	chimeric antigen receptor
CMP	collagen mimetic peptide
CPP	cell penetrating peptide
DA	4’4-dithiodibutyric acid
DMBT1	deleted in malignant brain tumor 1
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleyl-3-trimethylammonium propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl-]-N,N,N-trimethylammonium chloride
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
Get	Lysine with a guanidinylethyl
GHDC	glycidyl hexadecyl dimethylammonium chloride
GPCR	G protein-coupled receptor
MEN1	multiple endocrine neoplasia syndrome type 1
MMP	matrix metalloproteinase

MSC	mesenchymal stem cell
NET	neuroendocrine tumor
NLS	nuclear localization sequence
Oct	octreotide
PBAE	poly(beta-amino ester)
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDMAEMA	poly(2-dimethylaminoethyl methacrylate)
PEG	polyethylene glycol
PEI	polyethyleneimine
Pro^{Gu}	proline derivative with guanidine in the side chain
pVEC	peptide derived from vascular epithelial cadherin
RISC	RNA-induced silencing complex
SCO	splice correcting oligonucleotide
SSTR2	somatostatin receptor 2
SV40	simian virus 40
TAT	trans-activator of transcription
TNF	tumor necrosis factor
VEGFR	vascular endothelial growth factor receptor
VIPER	virus inspired polymer for endosomal release.

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Highlights

- Peptides possess diverse amino acid chemistries and secondary structures suitable for targeted nucleic acid delivery.
- Peptides can assist in nanocarrier trafficking by triggering cellular uptake, facilitating endosomal escape, and/or enabling nuclear targeting.
- Peptides can promote spatio-temporal control over gene delivery.
- Modified peptides responsive to external stimuli (*e.g.*, light) can switch into active/non-active forms to assist uptake and transfection in target cells.

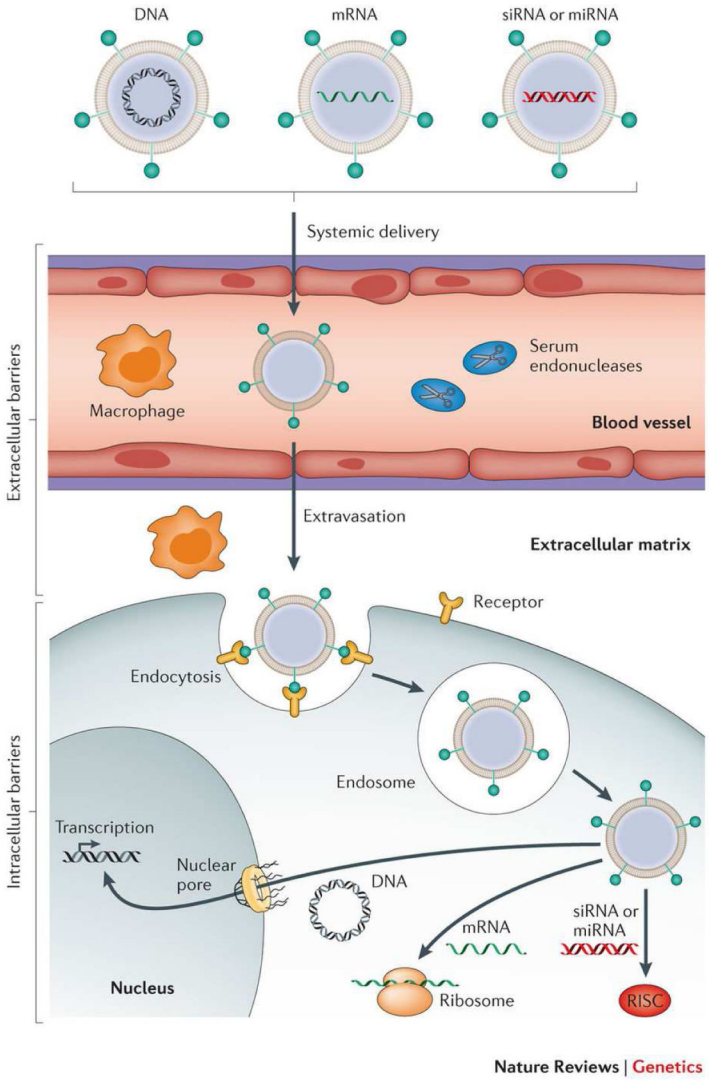

Figure 1:

Barriers to successful *in vivo* delivery of nucleic acids using non-viral carriers. Various non-viral carriers can be used to deliver DNA, mRNA and short double-stranded RNA [*e.g.,* small interfering RNA (siRNA) and microRNA (miRNA)]. If administered systemically, these carriers must help their cargoes avoid serum nuclease-mediated degradation, evade immune detection, and prevent nonspecific interactions with serum proteins. Moreover, for non-hepatic applications, these vectors need to avoid renal clearance, extravasate into desirable target tissues, and mediate both cell entry and appropriate subcellular trafficking. siRNA and miRNA ultimately must be loaded into the RNA-induced silencing complex (RISC), whereas mRNA must bind to the translational machinery. DNA must be further transported into the nucleus to exert its activity (Reproduced from reference [10], with permission from Springer Nature).

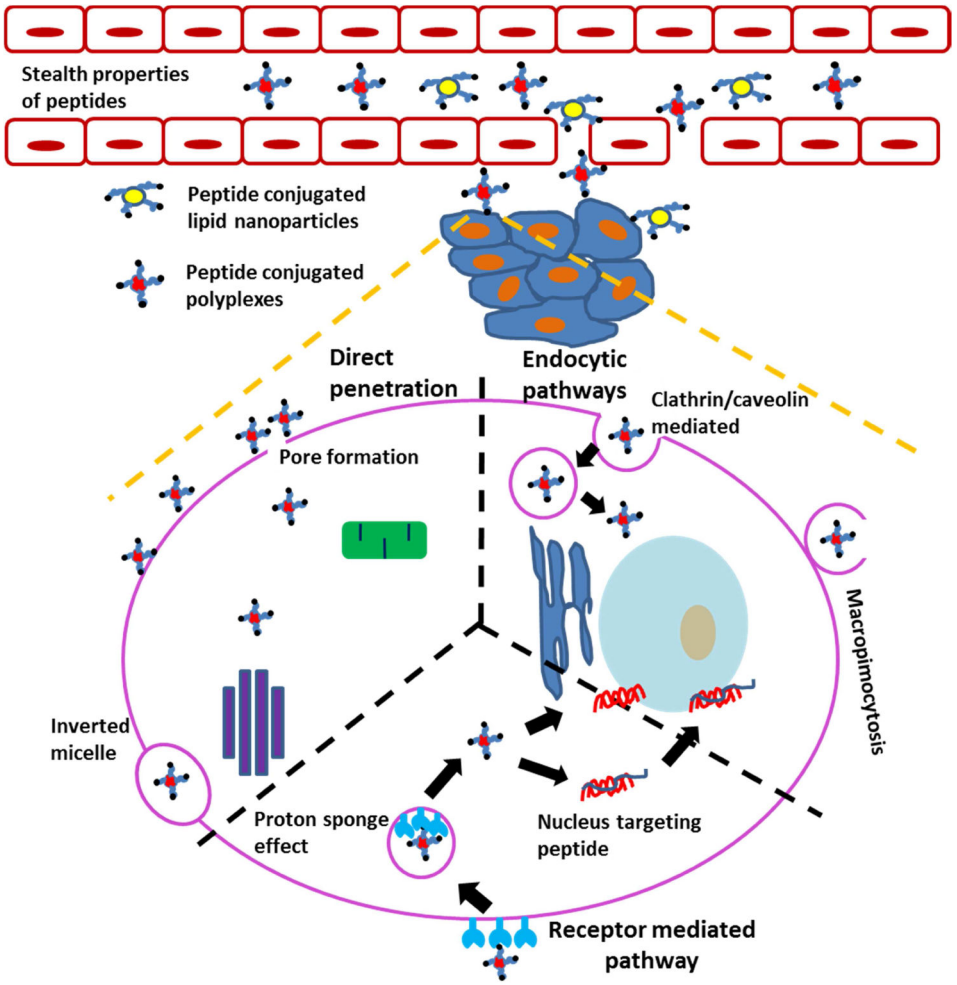

Figure 2:

Schematic representation of pathways for peptide-mediated penetration of biological barriers to address key issues in non-viral gene carriers. Peptides can provide stealth properties to prevent opsonization while in circulation [20]. Furthermore, peptides can facilitate penetration of non-viral carriers across various cellular and sub-cellular barriers. Peptide-mediated transport mechanisms across the plasma membrane include uptake through endocytosis or macropinocytosis (right) [21]; active uptake through receptor-mediated endocytosis (bottom) [22]; and pore formation (left) [23]. Some peptides can mediate transcytosis through sequential endocytic uptake, subcellular transport, and exocytosis [24]. Within cells, peptides can facilitate endosomal escape [22] or nuclear targeting [25], leading to increased RNA delivery or gene transfection.

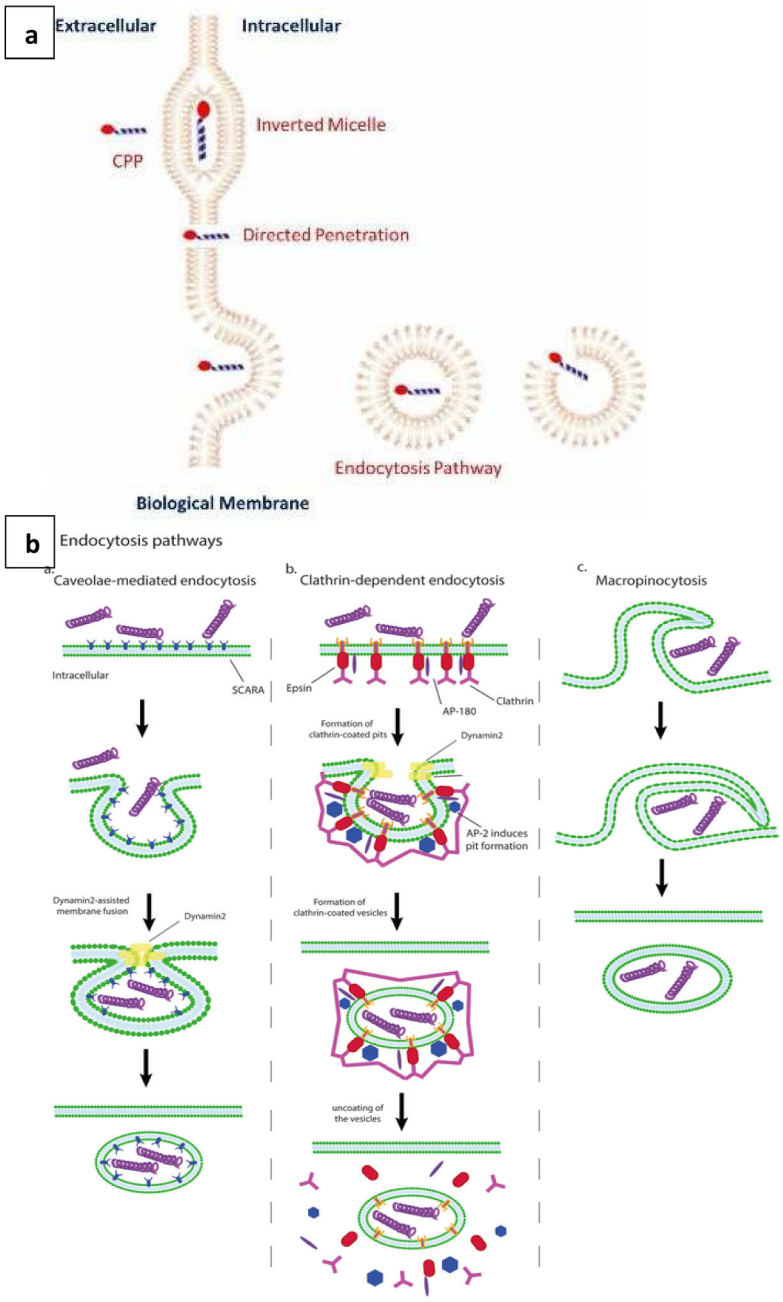

Figure 3:

(a) Translocation mechanisms of CPPs into the cells through direct penetration, formation of inverted micelles, and endocytic mechanisms (Reproduced from reference [23], with permission from). **(b)** Endocytic pathways of CPPs through clathrin and caveolin mediated endocytosis, and macropinocytosis (Reproduced from reference [24], with permission from Springer Nature).

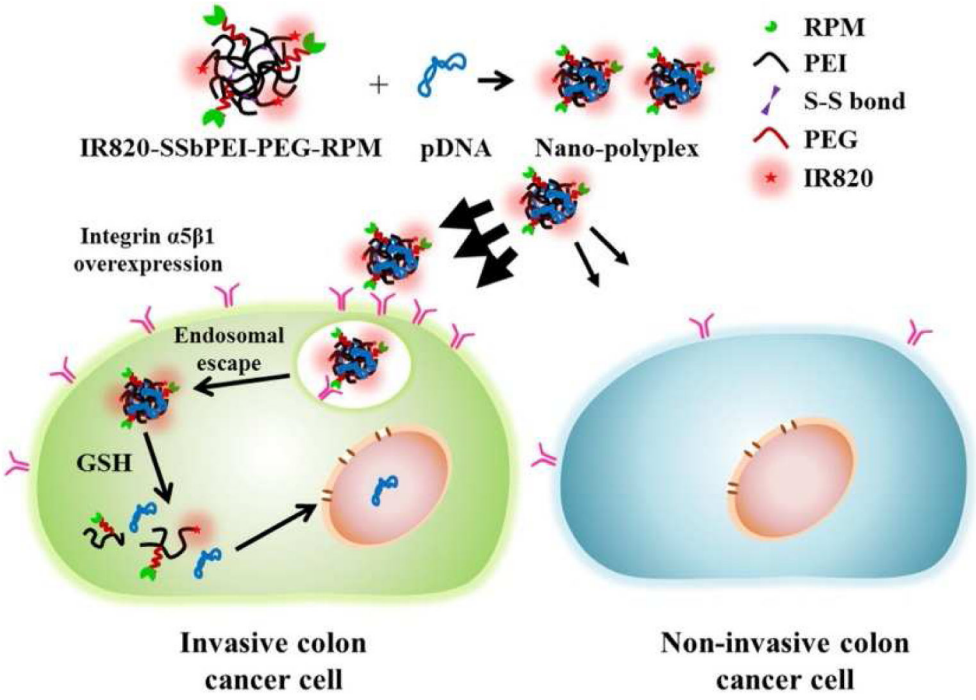

Figure 4:

Schematic diagram of gene delivery using RPM-conjugated bioreducible gene carriers (SSbPEI-PEG-RPM) (Reproduced from reference [22], with permission from Elsevier). In this work, $\alpha_5\beta_1$ integrin receptor-overexpressing invasive colon cancer cells (HT29) interacted with RPM peptides to enhance receptor-mediated endocytosis of the PEGylated polyplexes. Enhanced uptake was not observed in non-invasive colon cancer cells (HCT116). Following endosomal escape through the proton sponge effect of PEI, released pDNA was successfully transfected into the nucleus. IR820 was used for infrared imaging in transfected cells.

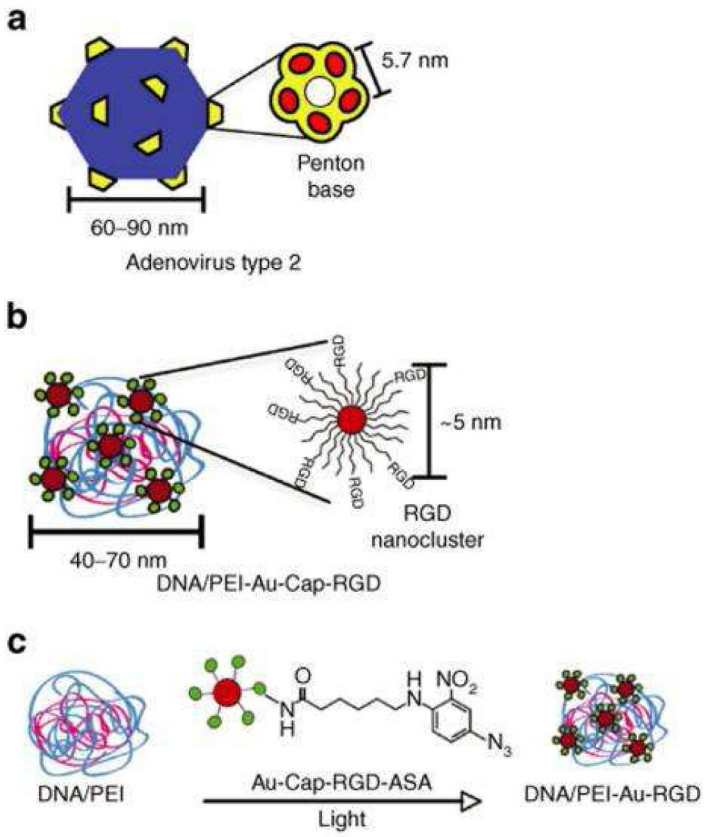

Figure 5:

Schematic comparing adenovirus type 2 and DNA/PEI-Au-Cap-RGD-ASA polyplex (Au-Cap-RGD-ASA: azidosalicylic acid-RGD-modified gold nanoparticles). **(a)** Type 2 adenovirus with penton-base proteins. **(b)** DNA/PEI-Au-Cap-RGD-ASA polyplex with RGD nanoclusters. **(c)** Au-Cap-RGD-ASA nanoparticles are conjugated to preformed DNA/PEI polyplexes when exposed to the UV light (Reproduced with permission from reference [49]).

Table 1:

CPPs for gene delivery applications.

Delivery carrier	CPPs	Purpose	Delivery system	Mechanism of uptake	Ref
Peptide (CPP)	Arginine rich peptides containing unnatural amino acid 4-aminopiperidine-4-carboxylic acid (Api) or proline derivative with guanidine in the side chain (Pro^{Gu})	pDNA delivery	Peptide/pDNA complex	Endocytosis (pathway not specified)	[29]
Peptide (CPP)	Lysine with a guanidinylethyl (GEt) amine structure in the side chain [Lys(GEt)-peptide]	pDNA delivery	Lys(GEt)-peptide/pDNA complex	Endocytosis (pathway not specified)	[30]
Nanoparticle [magnetic nanoparticles (MNPs)]	PF14 (Stearyl-AGYLLGKLLOOL AAAALOOLL-NH_2)	Splice correcting oligonucleotide (SCO) delivery	PF14-SCO-MNPs	Receptor-mediated endocytosis	[31]
Lipid [pH-sensitive cationic lipid (YSK05)]	Octaarginine (R8)	pDNA delivery	Lipid nanoparticles	Macropinocytosis	[32]
Peptide (CPP)	Human protein DMBT1 (deleted in malignant brain tumor 1)-derived CPP	siRNA delivery	DMBT1 peptide/siRNA complex	Direct membrane translocation and endocytosis	[33]