ELSEVIER

Peptides in DNA delivery: current insights and future directions

Anita Mann, Garima Thakur, Vasundhara Shukla and Munia Ganguli

Institute of Genomics and Integrative Biology, Mall Road (near Jubilee Hall), Delhi 110007, India

Peptides are emerging as attractive alternatives to cationic polymers and lipids for nonviral DNA delivery. Their remarkable properties such as efficient condensation of DNA, translocation across the cellular membrane, pH-sensitive membrane disruption, and efficient targeting of attached cargoes to the nucleus make them lucrative for researchers to explore their application in DNA delivery. In this review article, we focus on how the chemical nature, structural features and DNA complexation strategies of different peptides have been utilized for efficient DNA delivery. We also discuss their potential problems hindering *in vivo* application.

Gene therapy using DNA-based therapeutic formulations attempts to revolutionize the conventional ways of treatment of genetic disorders [1]. The success of such formulations relies heavily on efficient intracellular delivery of exogenous DNA (e.g. oligonucleotides (ODNs) for blocking undesired gene expression or plasmid DNA containing a specific gene whose corresponding protein is insufficiently or aberrantly expressed). Naked DNA is unable to efficiently cross cellular barriers by passive diffusion because of its large size, strong negative charge, hydrophilicity and susceptibility to nuclease attack. Hence the major challenge for *in vitro* DNA delivery is designing suitable vectors which can protect DNA and efficiently deliver it to the intended sites in the cell. Successful application of such therapeutic vector-DNA formulations *in vivo* requires them to be stable during circulation in blood, resistant against rapid metabolic clearance and efficiently targeted to the appropriate tissue/cell. Viral vector-based DNA delivery systems are efficient but of limited use because of immunogenicity, possible random genomic integration and difficult preparative procedures. Nonviral vectors like liposomes and cationic polymers have been studied as possible alternatives [2]. However, lack of control during synthesis of cationic polymers often results in polymer–DNA formulations of nonuniform physico-chemical characteristics. Cellular toxicity and difficulties of selectively modifying the polymer with ligands for targeted delivery pose additional problems. Even though liposomes are commonly employed for *in vitro* DNA delivery, lipoplexes are unstable in the presence of serum and tend to change in size, surface charge and lipid composition in the presence of blood components, restricting their *in vivo* application. Low molecular weight (LMW) peptides present an alternative to polymer and lipid-based DNA delivery systems as they are relatively stable, easy to synthesize and functionalize, less toxic or immunogenic as compared to other nonviral and viral vectors and only weak activators of the complement system [3]. Different classes of peptides can overcome the major barriers faced in intracellular DNA delivery *in vitro* (Fig. 1). Depending upon their function, peptides involved in DNA delivery can be categorized as: (a) *DNA-condensing cationic peptides* (lysine and arginine-rich) which form complexes with DNA via electrostatic interaction; (b) *Cell-penetrating peptides* (*CPPs*) which efficiently translocate across the cell membrane along with the cargo; (c) *Membrane perturbing peptides* which primarily mediate DNA entry as a function of pH and (d) *Peptides containing specific Nuclear Localization Sequences* (*NLSs*) that target DNA to the nucleus for subsequent gene expression. DNA delivery formulations can be prepared by conjugating DNA to the peptide electrostatically (Figs 2 and 3a–d) or using covalent linkages (Fig. 3e–m). Such formulations can make use of different classes of peptides in a single formulation or designed multifunctional peptides having all the desired properties. In this review, we aim to highlight the key advancements as well as existing problems in the area of peptide-based DNA delivery.

Corresponding author: Ganguli, M. (mganguli@igib.res.in)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2007.11.008

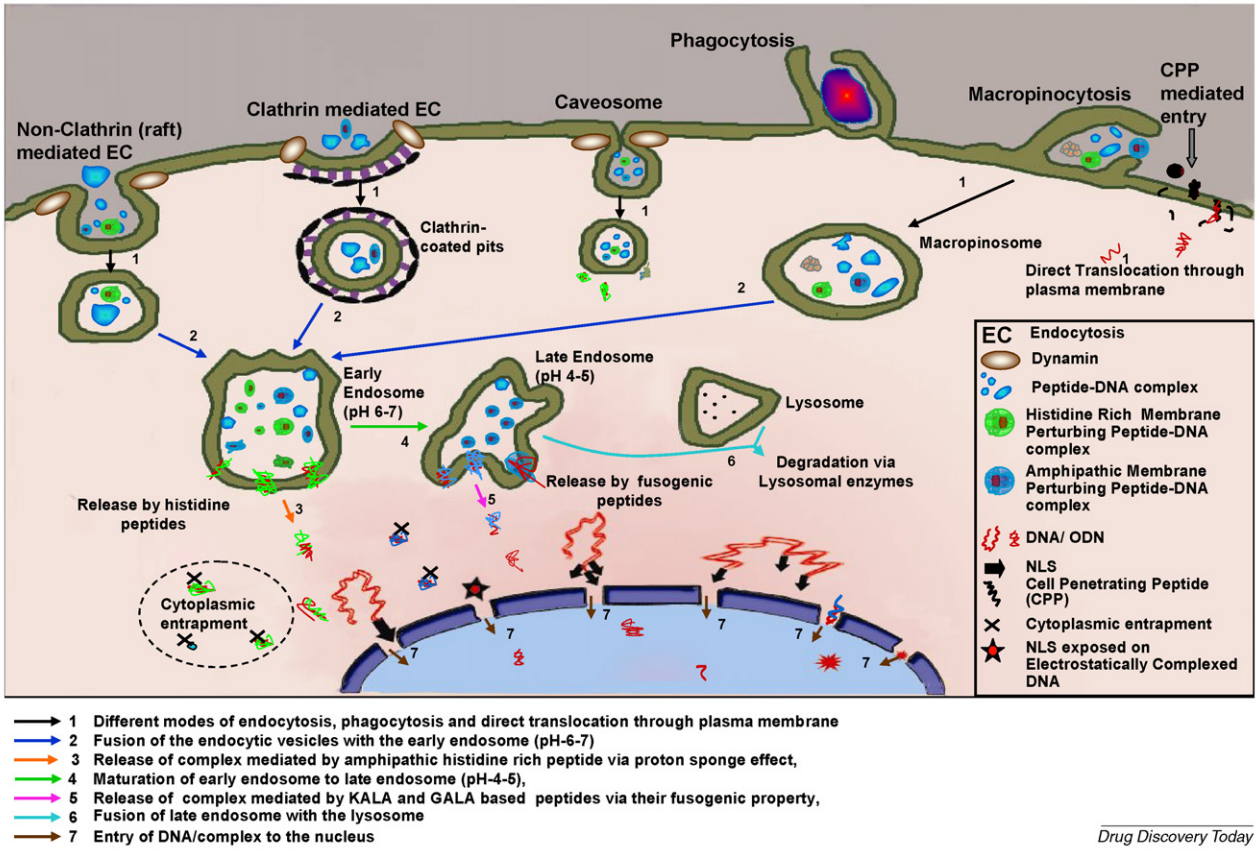

FIGURE 1

Pathways involved in intracellular entry of peptide–DNA complexes. The numbered arrows depict the different steps involved in intracellular delivery as described in the figure.

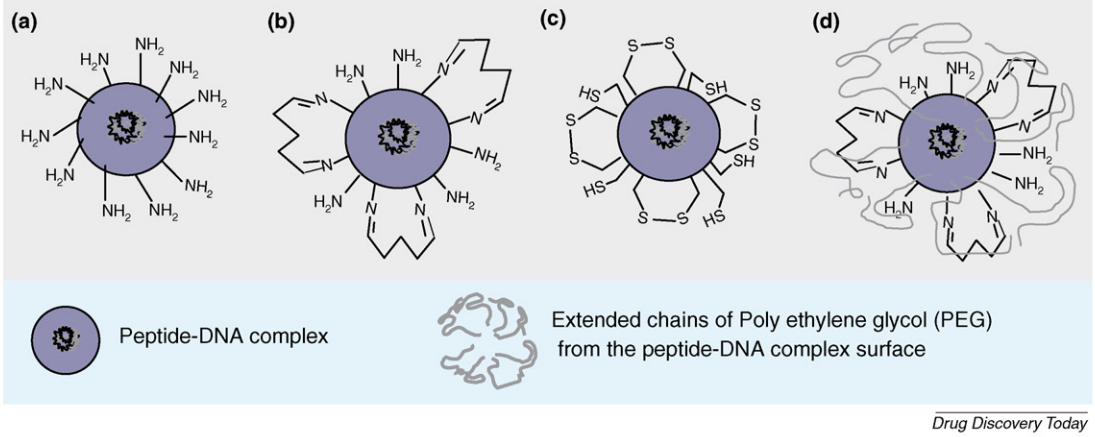

FIGURE 2

DNA condensation strategies used in preparation of peptide–DNA formulations: (a) simple electrostatic interaction between anionic DNA and cationic peptide; (b) crosslinking of exposed amine groups of peptides on the complex surface via glutaraldehyde; (c) crosslinking via oxidation of exposed cysteine groups of the peptide on the complex surface; (d) attachment of poly-ethylene glycol (PEG) chains to the peptides for preventing aggregation and increasing solubility.

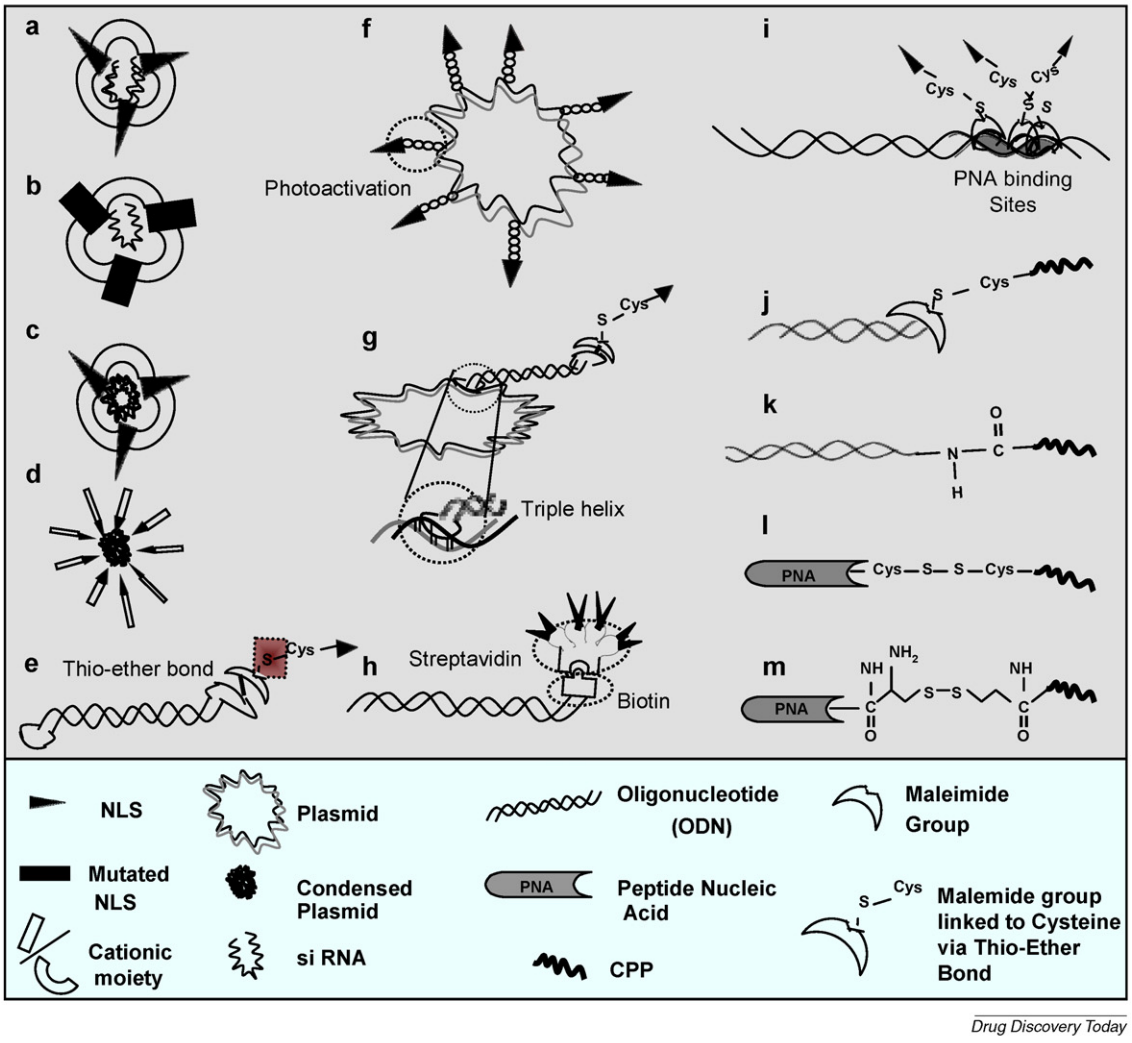

FIGURE 3

Linking strategies for nuclear localization sequence (NLS) and cell-penetrating peptides (CPP)- attachment to DNA (a–d: electrostatic interaction, e–m: covalent linkages): (a–d) oligonucleotide (ODN), plasmid or siRNA complexed with NLS-linked cationic peptide by electrostatic interaction; (e) single NLS attached to ODN via thiol-maleimide linkage; (f) multiple NLS peptides covalently attached to plasmid via photoactivation; (g) single NLS attached to padlock oligonucleotide which is attached to plasmid DNA through triple helix formation; (h) multiple NLS coupled at one end of ODN using biotin–streptavidin interaction; (i) NLS coupled to ODN via PNA by introducing PNA binding sites in the ODN; (j) CPP attachment to ODN via thiol-maleimide group; (k) CPP attachment to ODN via amide bond; (l) CPP attachment to PNA via thiol oxidation (disulfide linkage); (m) CPP attachment to PNA via thio-ester bond.

DNA-condensing cationic peptides

One of the important aspects in developing an efficient nonviral gene delivery formulation is to achieve a fine balance between complex formation through condensation of DNA and release of DNA from the complex when required for gene expression. Most of the cationic peptides used for DNA condensation are LMW oligolysines (Table 1), usually containing a minimum of eight consecutive lysine residues, which are less toxic than their high molecular weight counterparts [4]. For efficient DNA transfer, oligopeptides with ~18 lysine residues are required, which form small (<50 nm)-sized peptide–DNA complexes [5]. These are more stable in the presence of serum and salt which closely resemble the *in vivo* conditions than the peptide DNA complexes formed with 8-mer oligolysines [6]. For linear cationic oligolysines, number and position of the cationic residues in the peptide and complex size are critical determinants of DNA condensation and subsequent gene expression. For branched peptides, the number of residues and nature of the N-terminal sequence containing lysine, arginine or ornithine control DNA condensation. Arginine-based peptides show better condensation, while lysine-based ones are weaker activators of the complement system [7].

Stabilization of peptide–DNA complexes

The main drawback of these LMW peptide–DNA formulations (formed through electrostatic interaction between peptide and DNA, Fig. 2a) is their instability during *in vitro* and *in vivo* gene delivery because of the poor peptide–DNA binding leads to easy DNA release. To improve their applicability, several crosslinking strategies have been employed such that the complexes are more stable and can be 'tunably' disassembled in the intracellular environment.

TABLE 1

Common peptides used in DNA delivery

Peptide name	Sequence
DNA condensing peptides	
Linear lysine-based cationic peptides	Y K A K_n W K AlkC W K_n (n = 3 to 36) $(K)_n$ W C (s – s) C W $(K)_n$ CWK_{17} C (modification from CWK_{18})
Branched cationic oligopeptide	$(K K K)_2$ K G G C
Cell penetrating peptides (CPP)	
Penetratin	R Q I K I W F Q N R R M K W K K
Transportan Transportan-10	G W T L N S A G Y L L G K I N L K A L A A L A K I S I L A G Y L L G K I N L K A L A A L A K _ **K** I L
VP22	D A A T A T R G R S A A S R P T E R P R A P A R S A S R P R R P V D
HIV TAT	C G R K K R R Q R R R P P Q C
HIV-1 Rev	T R Q A R R N R R R R W R E R Q R
FHV coat	R R R R N R T R R N R R R V R
$(Arg)_n$, n=(7-16) Stearylated R8, Linear & Branched oligoarginine	$(R)_n$, Sterylated $(R)_8$ $(R)_n$-G-C
Haemagglutinin (HA-2) derived amphipathic peptides	
HA	G L F E A I A G F I E N G W E G M I D G
K5 E5 E5CA E5WYG H5WYG	G L F **K** A I A **K** F I **K G** G W **K** G **L** I **K** G G L F K A I A **E** F I **E** G G W **E** G L I **E** G G L F K A I A E F I E G G W E G L I E G **C A** G L F K A I A E F I E G G W E G L I E G **W Y G** G L F **H** A I A **H** F I **H** G G W **H** G L I **H** G W Y G
GALA KALA	W E A A L A E A L A E A L A E A L A E H L A E A L A E A L E A L A A W E A **K** L A **K** A L A **K** A L A **K H** L A **K A** L A **K** A L _ **K** A **C** E A
Cationic amphipathic histidine rich peptides	
LAH4 LAH4-L1 LAH6-L1 LAH6-L1-80	K K A L L A _ L A L H H L A H L A L H L A L A L _ _ _ K K A K K A L L A _ **H** A L H **L** L A **L** L A L H L A **H** A L _ _ _ K K A K K A L L A **L** H A L H **H** L A L L A **H** H L A H A L _ _ _ K K A K K **H** L L A _ H A L H **L** L A L L A **L** H L A H A L **A H L** K K A
JTS ppTG-1 ppTG-20	G L F E A L L E L L E S L W E L L L E A G L F **K** A L L **K** L L **K** S L **T K** L L L **K** A G L F **R** A L L **R** L L **R** S L T **A** L L L **R** A
Nuclear localisation sequences (NLS)	
SV40 large T Ag Ext SV40	P K **K*** K R K V E D P Y C S S D D E A T A D S Q H S T P P K **K*** K R K V E D P Y C
Mu	M R R A H H R R R R A S H R R M R G G
MPG	G A L F L G F L G A A G S T M G A W S Q P K S K R K V
HTLV	M P K T R R R P R R S Q R K R P P T W A H F P G F G Q G S L C
Mellitin	G I G A V L K V L T T G L P A L I S W I K R K R Q Q

Peptide sequences have been aligned in such a way that additions/modifications in the parent peptide leading to a novel peptide is clearly visible. Any change (addition/deletion/modification) in the sequence between the parent and modified peptide is marked in bold.

Addition of glutaraldehyde (Fig. 2b) to preformed condensates for crosslinking generates metabolically stable particles but the slow reversal of the Schiff's bases results in limited gene expression [8]. The presence of cysteine residues in the peptide leads to interpeptide disulfide (S–S) bond formation (Fig. 2c) via oxidation of their thiol (–SH) groups. Complexes formed with such peptides can tunably release DNA in the intracellular reducing environment. The degree of stability can be controlled by changing the number of –SH groups: optimum stability and release is achieved in the presence of up to two cysteines [9]. For increased solubility and cell-specific targeting, PEG-conjugated peptides (Fig. 2d) and glycopeptides have been used, respectively [10]. Recent studies have also involved LMW peptides polymerized through oxidative polycondensation before their complexation with DNA giving rise to reducible polycations (RPCs) [11]. RPCs show further improvements in transgene expression by lateral stabilization of the complexes through addition of hydrophilic polymers [12]. In spite of the satisfactory stability-release balance of the crosslinked peptide–DNA complexes *in vitro*, their *in vivo* application is still challenging because of premature DNA release in strongly reducing environment in organs like liver, which results in rapid metabolism and low gene expression [10].

Cell-penetrating peptides

Transport of the peptide–DNA complex across the cellular membrane constitutes one of the major hurdles of DNA delivery. The recent discovery of peptides with inherent membrane translocation property has gained lot of attention because of their potential to enhance DNA delivery across the cell membrane [13]. Such CPPs include the naturally derived peptides (e.g. TAT and penetratin), chimeric peptides (e.g. transportan) and synthetic ones (e.g. oligoarginine) [14].

The chemical nature of the CPPs varies from highly basic to amphipathic and some are even totally hydrophobic [15]. Membrane translocation property of cationic CPPs seems to depend on the presence of arginine residues rather than the lysines [16]. At least seven arginines are required for translocation and 8-mer arginines are internalized best [16,17]. Translocation further improves with addition of spacers within heptaarginine [18]. Branched arginine-rich peptides translocate as effectively as linear ones, indicating that the number of arginines is a more crucial determinant of cellular entry than conformational flexibility and charge disposition [19]. The key structural feature of arginine needed for internalization is the guanidinium moiety and not the charge itself or the backbone structure [18,20]. It is conceivable that the molecular mechanism of internalization involves interaction between the guanidinium group and a hydrogen-bond acceptor moiety (proteoglycans – GAGs and heparan sulfate) present on the plasma membrane. This theory is supported by experiments showing that other peptoid structures based on guanidine moieties show similar translocation behavior [21]. In case of amphipathic CPPs, the most decisive structural requirement is the alpha-helical conformation of the peptide at neutral pH [22], which is considered to be necessary for membrane destabilization and subsequent internalization. However, in penetratin, which adopts a helical structure but also contains arginines, the positive charges rather than the helicity seem to control cellular translocation [23].

CPP-mediated delivery of nucleic acids

A number of CPPs have been utilized for efficient delivery of ODNs, peptide nucleic acids (PNA), morpholinos, plasmid DNA as well as siRNA [24–26]. It is important to choose an appropriate CPP–DNA conjugation strategy so that the strong cellular translocation property of CPP remains unhindered during delivery. For successful delivery of ODNs covalent bonding through thiol-maleimide (Fig. 3j), amide bond (Fig. 3k), disulfide (Fig. 3l) and thio-ester (Fig. 3m) linkages have been principally used, though electrostatic interaction has also been exploited [27]. Most of the CPP-PNA conjugates, however, need addition of endosome-disrupting agents for improvement in their delivery efficiency, for example, addition of photosensitizers which disrupt endosomes through production of reactive oxygen species [28]. Improved design, for example, the addition of arginine residues to penetratin, has a similar effect [29]. Similarly, conjugation to arginine-rich peptides significantly improves the cellular uptake of morpholino ODNs and their functional activity. The amino acid configurations (D or L isomer), incorporation of non-α amino acids, choice of crosslinkers and other external conditions control the metabolic stability of arginine-rich CPPs conjugated to morpholinos as well as the level of effectivity [30].

CPP-mediated delivery of plasmid DNA involves formation of peptide–DNA complexes primarily utilizing the electrostatic interactions between the anionic DNA and the cationic CPP. Oligoarginines are able to deliver plasmid DNA in a variety of mammalian cell lines [31] with 15 residues showing the best transfection efficiency. Unfortunately, this number does not necessarily correlate with the optimal number of arginine residues required for free peptide translocation. One of the major issues in using peptide–DNA complexes formed through electrostatic interaction is that the arginine moieties which impart efficient translocation ability are likely to be masked in the complex. Thus a careful control on the number and position of arginine residues in the peptide, peptide architecture, modifications on the peptide and charge ratios used in the formulation become important. Application of oligomers, dendrimers or macro-branched forms of CPPs can help in condensing DNA into stabilized nanoparticles keeping the membrane translocation property intact [32,33]. Similarly, N-terminal modification of 8-mer oligoarginine by hydrophobic moieties like stearyl also increases the transfection efficiency [34]. It is thought that the hydrophobic moieties exposed on the complexes might help membrane destabilization and permeability thus leading to high transfection efficiency. Optimizing the transfection efficiency by varying the lysine/arginine ratio of the condensing peptide sequence has also been analyzed [35]. Arginine substitution seems to improve compaction and stability in plasma, which is encouraging for *in vivo* studies. Cysteine modification and interspersing arginines with glycine and histidine residues achieve reversible plasmid condensation and efficient *in vivo* delivery [36].

Recently, CPPs have been used for siRNA delivery [37]. Although an in-depth discussion on this topic is beyond the scope of this review, it is worthwhile mentioning that both electrostatic interactions and covalent attachment strategies have been assessed. Although electrostatic interaction can result in positively charged particles with CPP moieties exposed outside, the challenge is to covalently link one CPP per siRNA without masking the CPP charge for improved delivery across the cell membrane.

Mode of cellular entry

The application of CPPs for DNA delivery is also complicated by technical difficulties associated with florescence microscopy and flow cytometry analysis because of fixation artifacts and residual binding of CPPs to proteoglycans on the cell membrane [14]. Initial fluorescence microscopy experiments on fixed cells (mainly with fluorophore-labeled TAT and penetratin) suggested a receptor and energy-independent entry process for CPPs [16,17]. These results were consistent with the theory of their direct translocation through the lipid bilayer. However, recent evidence shows that these results might have been misinterpreted. It is now evident that cellular entry of both CPPs or CPP–DNA complexes can occur either via energy-dependent endocytotic pathways or by direct translocation through the plasma membrane [38,39]. It seems unlikely that a single mechanism of entry exists for all CPPs and CPP–DNA complexes and multiple pathways could also exist for a single CPP. The particular mechanism used will depend on the CPP itself (e.g. octaarginine enters via macropinocytosis whereas penetratin does not [40]), whether the CPP is bare or conjugated [14], the cell line used [41] and other factors. Correct elucidation of the internalization mechanism along with intelligent peptide design should go a long way in developing more effective CPPs for DNA delivery.

Membrane perturbing peptides

Upon entering the cell via endocytosis, peptide–DNA complexes have to escape from the endosome before endosome–lysosome fusion in order to avoid degradation by lysosomal enzymes. This endosomal escape property has been utilized for DNA delivery and

the design of membrane fusogenic peptides that can mediate escape of peptide–DNA complexes at endosomal pH. Another way of endosomal escape is employed by the 'endosomolytic' histidine rich peptides through a 'proton sponge effect' wherein the buffering nature of the histidine amine groups (pK_a 6.5) leads to additional pumping of protons into the endosome. This is accompanied by influx of chloride ions to maintain charge neutrality; thereby increasing the ionic strength. The resultant osmotic swelling of the endosome and its rupture allows release of the DNA–pepetide complex into the cytosol [42].

Fusogenic peptides

Most fusogenic peptides adopt an amphipathic alpha-helical structure at endosomal pH that can interact with the endosomal membrane to cause membrane disruption/pore formation. Some fusogenic peptides are components of viral proteins (e.g. HA-2, the N-terminal end of influenza virus hemagglutinin), their derivatives (GALA, KALA, etc.) or synthetically designed (JTS) (Table 1) [42]. To use these for DNA delivery, positive charges have to be introduced into the peptide by substituting negatively charged glutamic acids with lysine and arginine. Peptides like GALA and JTS-1 thereby give rise to KALA and ppTG1, ppTG20 peptides, respectively [43]. The latter has shown *in vivo* activity [44]. CPPs like penetratin (nonamphipathic) and transportan (amphipathic) also have endosome-disrupting property [45].

Fusogenic peptides are commonly used as auxillary agents to improve the efficacy of known DNA delivery formulations. For example to improve delivery efficiency, HA-2 was conjugated to a cationic moiety poly-L-lysine and then coated on to transferrin-poly-L-lysine condensates (Fig. 4a) [46]. HA-2 has also been used by covalent conjugation as well as surface coating of preformed complexes (Fig. 4b) to enhance transfection in dividing, nondividing and postmitotic cells [47]. KALA has been used in different methods of complexation with PEI and poly-L-lysine. It seems that both the complexation strategy of KALA and the content of free KALA in the formulation determine its working pH range as free KALA can mediate its action over a pH range of 4–8.5 in association with liposomes like POPC/POPG [46].

Endosomolytic peptides

Histidine-rich peptides are usually endosomolytic in nature and assist in both early and late endosomal release of DNA cargoes. H5WYG peptide, a histidine-rich variant of HA-2, shows improved transfection ability because of early endosome (pH < 7) disruption as compared to the glutamic-acid-substituted anionic peptide E5WYG, which is effective only at the late endosome (pH < 5.5). H5WYG is active even in the presence of serum which makes it a good choice for *in vivo* delivery [48].

Linear oligolysines have also been modified with different amounts of histidine for improved cytosolic delivery as well as nuclear accumulation of ODNs [49]. Several branched lysine–histidine peptides show efficient transfection in the presence of liposomes [50]. Two modified branched peptides have been prepared by addition of a histidine-rich tail (H2K4bT and H3K4bT) such that the buffering capacity can be increased further. The former shows improved transfection efficiency *in vivo* compared to the un-modified peptide while the latter, with higher amount of histidine, does not show any improvement presumably because of the lower lysine content, which hindered effective condensation [51]. High molecular weight multi-block copolypeptides consisting of composite series of histidine-rich peptide and SV40 NLS peptide linked together with disulfide bond have been shown to be effective. The reducible nature of the polycation ensured controlled DNA binding and efficient release intracellularly, also resulting in lowered cellular toxicity [52].

The multifunctional LAH series of peptides has been designed to improve plasmid DNA delivery. These peptides are rich in alanine and leucine and have been modified with lysine and histidine to introduce DNA compaction and endosomal release properties. LAH peptides adopt a transmembrane orientation at neutral pH. When the pH is lowered the histidines become protonated and the peptide flips into an in-plane orientation at the membrane surface

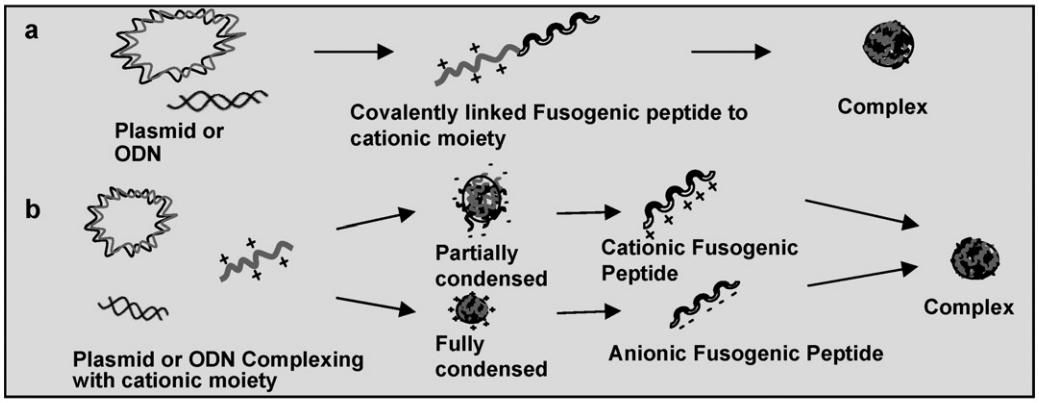

FIGURE 4

Different formulation strategies using fusogenic peptides (cationic and anionic): (a) Fusogenic peptide can be covalently conjugated to a cationic moiety for complexation of plasmid DNA or oligonucleotide (ODN). (b) Plasmid DNA/ODN first forms either a partially or fully condensed structure with the cationic moiety. Cationic or anionic fusogenic peptide can be subsequently added to form the final formulation.

thereby destabilizing anionic lipids of the membrane. The position and number of histidine residues (4–5 at the core) is crucial in obtaining the best transfection efficiency. Further modifications like increase in the number of histidines have led to improved serum stability of the complexes and strong interaction with anionic lipids like phosphotidylserine often expressed on tumor cell surface, thereby making them suitable for *in vivo* applications [53,54].

Peptides with nuclear localization sequence

Transport of DNA across the nuclear membrane through the nuclear pore complex (NPC) constitutes another major barrier in gene delivery. Only small molecules (40–60 kDa) can passively diffuse because the pore size of the NPC is about 9 nm. The entry of large DNA or DNA complexes can be mediated by attachment to certain classes of peptides containing specific nuclear localization sequences (NLSs) [55]. The classical NLS sequences used in DNA delivery have stretches of highly basic amino acids: either one cluster (monopartite) or two clusters of basic residues separated by 10–12 neutral residues (bipartite).

Coupling of DNA to NLS

Conjugation of DNA (plasmid, linearized plasmids and oligonucloetides) to NLS moieties has been achieved using both electrostatic and covalent methods. Positively charged NLS peptides can interact electrostatically (Fig. 3a–d) with DNA, forming complexes with multiple NLS moieties exposed on the surface. However, easy disassembly of these complexes in the cytoplasm can lead to loss of the NLS signature and thereby prevent efficient nuclear entry. Even when the NLS peptide is attached to another cationic peptide (e.g. NLS-mu peptide), there is little control over whether the NLS moieties will be exposed on the surface (for nuclear targeting) because both NLS and cationic peptide can interact with the DNA electrostatically [56]. However, covalent method of NLS–DNA conjugation allows greater control on the peptide–DNA formulation. NLS peptides can be covalently coupled to the DNA backbone through first, chemical modification using spacers/thiol-reactive maleimide group (Fig. 3e)/streptavidin–biotin (Fig. 3h) conjugates [57,58]); second, covalent triple helix formation via Psoralen-based reactions [59] and ODN padlock (Fig. 3g) [60]; third, PNA attachment (Fig. 3i) [61]; fourth, photoactive linkages (Fig. 3f) [62]. The attachment of NLS to DNA can differ in number and position from one NLS [57,60], to a bunch of NLS at one end [58] and also NLSs randomly spread across the DNA [59].

The number of NLS sequences required for efficient nuclear entry depends on the conformation, size and length of the DNA used. For example, covalent attachment of a single NLS to linear DNA through thiol-maleimide linkage results in high transfection efficiency. It has been suggested that there is an inverse correlation between the number of NLS and the efficiency of nuclear transport of large linear DNA as the molecule would attempt to enter through multiple pores [57]. However, in the case of supercoiled plasmid DNA, covalent attachment of a single NLS does not increase transfection efficiency [60]. Linear DNA shows a size dependency on the nuclear accumulation in digitonin-permeabilized cells: nuclear accumulation of DNA of 310 bp onwards increases significantly with covalent attachment of NLS [58]. The length of NLS and the overall charge are important. An extended form of NLS SV40 with reduced positive charge, which is covalently attached to DNA, has been more effective than the highly charged one possibly because of reduced electrostatic interaction between the NLS peptide and the DNA [58].

Problems and future prospects

Although peptides have the potential to develop new paradigms in cellular gene delivery, application of peptides for *in vivo* delivery of therapeutic DNA is still in its infancy and various areas of concern need to be critically addressed before significant solutions emerge. Some of the important issues are addressed below.

CPP entry with and without cargo: gray areas

As mentioned earlier, one of the main problems of using CPPs for DNA delivery is the difficulty to assess entry and cellular distribution quantitatively. Suitable assays need to be developed for checking the metabolic stability of the CPPs, the rate of internalization, degradation and the biological activity of the cargoes transported for accurate quantitative comparison of the uptake of different CPPs and CPP–DNA conjugates. It would be important to quantitate the fraction of CPPs entering through endocytosis versus the fraction that is not. Moreover, the correlation between cargo size, its nature and the pathway of entry also needs to be looked into. This would help us design tailor-made CPPs with improved cellular uptake. Endosomal trapping of CPP-ODN conjugates is an additional area of concern. Application of endosomolytic peptides in the formulation might help in overcoming this problem.

Single and composite multifunctional peptides

Each class of peptides discussed in this article can overcome at least one of the barriers to intracellular delivery (e.g. cell penetration by CPPs, endosomal release by histidine-rich peptides). For a synergistic effect on the delivery efficiency, however, peptides need to be engineered in such a way that various functionalities of DNA condensation, cellular translocation, endosome disruption and nuclear targeting can be brought together in an optimal fashion. The LAH peptide series mentioned earlier is an interesting example of a modification on a single peptide to generate the required delivery characteristics [53,54]. Composite systems like MPG peptide vector based on fusion peptide domain of HIV-1 gp41 protein (for cell membrane entry in receptor-independent pathway) and NLS of SV40 large T antigen (for nuclear localization) also show improved delivery [63]. Such multifunctional peptides that can efficiently condense DNA generating nontoxic formulations stable in serum and are likely to be future candidates for successful peptide-mediated DNA delivery.

In vivo *efficacy and physico-chemical properties of peptide–DNA complexes*

In addition to choosing the right peptide/combination, it is important to improve the stability and understand the physico-chemical properties of the formulations to enhance their *in vitro* and *in vivo* efficacy. Several strategies (e.g. crosslinking, different peptide–DNA conjugation methods) have been used for a good condensation-release balance of the peptide–DNA complexes, a critical parameter in complex stability. Even then, the physico-chemical parameters of the complex (e.g. peptide–DNA interaction, complex size, surface charge and colloidal stability) and their role in delivery are still not well-understood. Generation of formulations with uniform particle

size and controlled surface charge is still a challenge. Future work also needs to focus on the peptide–DNA assembly mechanism in relation to DNA delivery. *In vivo* application of formulations well characterized *in vitro* would entail overcoming additional barriers like stability in circulation, stimulation of the immune system, loss because of metabolism as well as targeted tissue/cell-specific delivery. For this purpose, formulations with controlled release properties which are stable in physiological fluids would have to be prepared along with attachment of cell-specific ligands for receptor targeting. Intrinsic physico-chemical properties of the DNA–peptide complex are also known to control the biodistribution. For targeted gene delivery (e.g. in case of cancerous tissue), these intrinsic properties might need to be fine-tuned further such that they least interfere with the specific targeting, thus controlling the biodistribution for effective *in vivo* DNA delivery.

Conclusion

Establishing peptides as DNA delivery agents is an interdisciplinary area of research which is of importance for the pharmaceutical industry. Further advancements in this area would require interdisciplinary approaches to understand the role of peptide chemistry, peptide–DNA coupling strategies and physico-chemical properties of the DNA–peptide complex in relation to DNA delivery. Novel formulations are likely to emerge only when such structure–function correlations are established.

Acknowledgements

The authors would like to thank CSIR, India for research support (Task Force project on Nanomaterials and Nanodevices: application in health and disease) and Vinod Yadav for assistance in preparing figures.

References

1 Edelstein, M.L. *et al.* (2007) Gene therapy clinical trials worldwide to 2007-an update. J. Gene Med. 10.1002/jgm.1100
2 Luo, D. and Saltzman, W.M. (2000) Synthetic DNA delivery systems. *Nat. Biotechnol.* 18, 33–37
3 Fabre, J.W. and Collins, L. (2006) Synthetic peptides as non-viral DNA vectors. *Curr. Gene Ther.* 6, 459–480
4 Gottschalk, S. *et al.* (1996) A novel DNA–peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* 3, 48–57
5 McKenzie, D.L. *et al.* (1999) Comparative gene transfer efficiency of low molecular weight polylysine DNA condensing peptides. *J. Peptide Res.* 54, 311–318
6 Adami, R.C. *et al.* (1998) Stability of the peptide-condensed plasmid DNA formulations. *J. Pharm. Sci.* 87, 678–683
7 Plank, C. *et al.* (1999) Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. *Hum. Gene Ther.* 10, 319–332
8 Adami, R.C. and Rice, K.G. (1999) Metabolic stability of glutaraldehyde cross-linked peptide-DNA condensates. *J. Pharm. Sci.* 88, 739–746
9 Mckenzie, D.L. *et al.* (2000) Low molecular weight disulphide cross-linking peptides as non-viral gene delivery carriers. *Bioconjugate Chem.* 11, 901–909
10 Kwok, K.Y. *et al.* (2003) In vivo gene transfer using sulfhydryl cross-linked PEG-peptide/glycopeptide DNA co-condensates. *J. Pharm. Sci.* 92, 1174–1183
11 Read, M.L. *et al.* (2003) Vectors based on reducible polycations facilitate intracellular release of nucleic acids. *J. Gene Med.* 5, 232–245
12 Oupicky, D. *et al.* (2002) Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of the DNA delivery vectors. *J. Am. Chem. Soc.* 124, 8–9
13 Schwarze, S.R. and Dowdy, S.F. (2000) In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol. Sci.* 21, 45–48
14 Trehin, R. and Merkle, H.P. (2004) Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur. J. Pharmaceut. Biopharmaceut.* 58, 209–223
15 Futaki, S. (2006) Oligoarginine vectors for intracellular delivery: design and cellular uptake mechanisms. *Biopolymers* 84, 241–249
16 Futaki, S. *et al.* (2001) Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* 276, 5836–5840
17 Suzuki, T. *et al.* (2002) Possible existence of common internalization mechanisms among arginine-rich peptides. *J. Biol. Chem.* 277, 2437–2443
18 Rothbard, J.B. *et al.* (2002) Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J. Med. Chem.* 45, 3612–3618
19 Futaki, S. *et al.* (2002) Translocation of branched chain arginine peptides through cell membranes: flexibility in the disposition of positive charges in membrane permeable peptides. *Biochemistry* 41, 7925–7930
20 Mitchell, D.J. *et al.* (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Peptide Res.* 56, 318–325
21 Wender, P.A. *et al.* (2000) The design, synthesis and evaluation of molecules that enable or enhance peptoid molecular transporters. *Proc. Natl. Acad. Sci.* 97, 13003–13008
22 Oehlke, J. *et al.* (1998) Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytotically. *Biochim. Biophys. Acta* 1414, 127–139
23 Scheller, A. *et al.* (1999) Structural requirements for cellular uptake of alpha-helical amphipathic peptides. *J. Peptide Sci.* 5, 185–194
24 Gait, M.J. *et al.* (2003) Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues. *Cell. Mol. Life Sci.* 60, 844–853
25 Koppelhus, U. *et al.* (2003) Cellular delivery of peptide nucleic acids. *Adv. Drug Deliv. Rev.* 55, 267–280
26 Eguchi, *et al.* (2001) Protein transduction domain of HIV TAT protein promotes efficient delivery of DNA into mammalian cells. *J. Biol. Chem.* 276, 26204–26210
27 Zorko, M. and Langel, U. (2005) Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv. Drug Deliv. Rev.* 57, 529–545
28 Shiraishi, T. *et al.* (2006) Enhanced delivery of cell penetrating peptide-peptide nucleic acid conjugates by endosomal disruption. *Nat. Protoc.* 1, 633–636
29 Abes, S. *et al.* (2007) Efficient splicing correction by PNA conjugation to a R_6-penetratin delivery peptide. *Nucl. Acids Res.* 35, 4495–4502
30 Wu, R.P. *et al.* (2007) Cell penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. *Nucl. Acids Res.* 35, 5182–5191
31 Kim, H.H. *et al.* (2006) Characterization of gene delivery in vitro and in vivo by the arginine peptide system. *Intl. J. Pharmaceut.* 335, 70–78
32 Rudolph, C. *et al.* (2003) Oligomers of the arginine-rich motif of the HIV-TAT protein are capable of transferring plasmid DNA into cells. *J. Biol. Chem.* 278, 11411–11418
33 Liu, Z. *et al.* (2005) Macro-branched cell-penetrating peptide design for gene delivery. *J. Control. Rel.* 102, 699–710
34 Futaki, S. *et al.* (2001) Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjugate Chem.* 12, 1005–1011
35 van Rossenberg, S.M.W. *et al.* (2004) Stable polyplexes based on arginine-containing oligopeptides for in vivo gene delivery. *Gene Ther.* 11, 457–464
36 Siprashvili, Z. *et al.* (2003) Gene transfer via reversible plasmid condensation with cysteine flanked, internally spaced arginine-rich peptides. *Hum. Gene Therapy* 14, 1225–1233
37 Meade, B.R. *et al.* (2007) Exogenous si-RNA delivery using peptide transduction domains/cell penetrating peptides. *Adv. Drug Deliv. Rev.* 59, 134–140
38 Richard, J.P. *et al.* (2003) Cell-penetrating peptides. A re-evaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 278, 585–590
39 Kaplan, I.M. *et al.* (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Control. Rel.* 102, 247–253
40 Nakase, I. *et al.* (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther.* 10, 1011–1022
41 Patel, *et al.* (2007) Cell penetrating peptide: Intracellular pathways and pharmaceutical perspectives. *Pharm. Res.* 24, 1977–1992
42 Wagner, E. (1999) Application of membrane-active peptides for non-viral gene delivery. *Adv. Drug Deliv. Rev.* 38, 279–289
43 Wyman, T.B. *et al.* (1997) Design, synthesis and characterization of a cationic peptide that binds to nucleic acids and permeates bilayers. *Biochemistry* 36, 3008–3017

44 Rittner, K. *et al.* (2002) New basic membrane-destabilizing peptides for plasmid based gene delivery in vitro and in vivo. *Mol. Ther.* 5, 104–114
45 Lindgren, M.E. *et al.* (2004) Passage of cell-penetrating peptides across a human epithelial cell layer in vitro. *Biochem. J.* 377, 69–76
46 Wagner, E. *et al.* (1992) Influenza virus hemagglutinin HA-2N terminal fusogenic peptides augment gene transfer by transferrin-polylysine–DNA complexes: toward a synthetic virus-like gene transfer vehicle. *Proc. Natl. Acad. Sci.* 89, 7934–7938
47 Parker, A.L. *et al.* (2007) (Lys)16-based reducible polycations provide stable polyplexes with anionic fusogenic peptides and efficient gene delivery to post-mitotic cells. *Biochim. Biophys. Acta* 1770, 1331–1337
48 Midoux, P. *et al.* (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chem.* 9, 260–267
49 Pichon, C. *et al.* (2000) Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. *Nucl. Acids Res.* 28, 504–512
50 Chen, Q.R. *et al.* (2001) Branched co-polymers of histidine and lysine are efficient carriers of plasmid. *Nucl. Acids Res.* 29, 1334–1340
51 Leng, Q. *et al.* (2006) A branched histidine/lysine peptide, H2K4b, in complex with plasmids encoding antitumor proteins inhibits tumor xenografts. *J. Gene Med.* 8, 1407–1415
52 Manickam, D.S. *et al.* (2006) Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery. *Bioconjugate Chem.* 17, 1395–1403
53 Kichler, A. *et al.* (2003) Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc. Natl. Acad. Sci.* 100, 1564–1568
54 Mason, A.J. *et al.* (2007) Optimising histidine-rich peptides for efficient DNA delivery in the presence of serum. *J. Control. Rel.* 118, 95–104
55 Escriou, V. *et al.* (2003) NLS bioconjugates for targeting therapeutic genes to the nucleus. *Adv. Drug Deliv. Rev.* 55, 295–306
56 Akita, H. *et al.* (2006) Evaluation of the nuclear delivery and intra-nuclear transcription of plasmid DNA condensed with mu and NLS-mu by cytoplasmic and nuclear microinjection: a comparative study with poly-L-lysine. *J. Gene Med.* 8, 198–206
57 Zanta, M.A. *et al.* (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci.* 86, 91–96
58 Ludtke, J.J. *et al.* (1999) A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *J. Cell Sci.* 112, 2033–2041
59 Neves, C. *et al.* (1999) Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. *FEBS Lett.* 453, 41–45
60 Roulon, T. *et al.* (2002) Coupling of a targeting peptide to plasmid DNA using a new type of padlock oligonucleotide. *Bioconjugate Chem.* 13, 1134–1139
61 Bremmer, K.H. *et al.* (2004) Factors influencing the ability of nuclear localization sequence peptides to enhance non-viral gene delivery. *Bioconjugate Chem.* 15, 152–161
62 Ciolina, C. *et al.* (1999) Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin-alpha. *Bioconjugate Chem.* 10, 49–55
63 Morris, M.C. *et al.* (1997) A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucl. Acids Res.* 25, 2730–2736