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Review article

Drug delivery of oligonucleotides by peptides

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Abstract

Oligonucleotides are promising tools for in vitro studies where specific downregulation of proteins is required. In addition, antisense oligonucleotides have been studied in vivo and have entered clinical trials as new chemical entities with various therapeutic targets such as antiviral drugs or for tumour treatments. The formulation of these substances were widely studied in the past. With this review we will focus on peptides used as drug delivery vehicles for oligonucleotides. Different strategies are summarised. Cationically charged peptides from different origins were used e.g. as cellular penetration enhancers or nuclear localisation tool. Examples are given for Poly-L-lysine alone or in combination with receptor specific targeting ligands such as asialoglycoprotein, galactose, growth factors or transferrin. Another large group of peptides are those with membrane translocating properties. Fusogenic peptides rich in lysine or arginine are reviewed. They have been used for DNA complexation and condensation to form transport vehicles. Some of them, additionally, have so called nuclear localisation properties. Here, DNA sequences, which facilitate intracellular trafficking of macromolecules to the nucleus were explored. Summarizing the present literature, peptides are interesting pharmaceutical excipients and it seems to be feasible to combine the specific properties of peptides to improve drug delivery devices for oligonucleotides in the future.

Keywords: Oligonucleotides; Drug delivery; Fusogenic peptides; Poly-L-lysine; Protamine; NLS

1. Introduction

Antisense oligonucleotides (ON) have been used for many years to down regulate specific proteins in cells. The principle of antisense technology, invented in 1978 [1–3], was the sequence specific binding of an antisense oligonucleotide to target mRNA preventing gene translation. Further antisense methods like triplex forming ON targeting DNA, aptamers [4,5] and small interfering RNA (siRNA) [6] were studied for years.

The most described problems in oligonucleotide delivery are degradation by nucleases, insufficient affinity for the target, lack of specificity and most important, the poor bioavailability [7]. To overcome these problems, many chemical modifications were introduced [8]. Another approach is the development of drug delivery systems. Different carrier systems for antisense ON have been described in the past years. These systems utilise synthetic polymers like polyethyleneimine (PEI), cationic liposomes (e.g. DOTAP, Lipofectine, etc.), dendrimers and various peptides. Such peptides consisting of less than 10 amino acids are called oligo-peptides, if they have more than 10 and up to 100 or more amino acids these are poly-peptides. However, there is no clear definition for the transition of a peptide to a protein [9].

Cationic peptides, for example poly-L-lysine (PLL) can complex ON, protect them against nuclease digestion and enhance the cellular uptake via non-specific endocytosis. To get more specificity in the targeting of cells, various ligands like folic acid, steroids, transferrin, mannose, growth factors were conjugated to PLL to improve the uptake of the oligonucleotide–peptide complexes or conjugates via receptor-mediated endocytosis. Another functionality of peptides is the endosomolytic potential. Arginine- and histidine-rich peptides mediate an endosomal escape of the ON into the cytosol. Furthermore, special amino acid sequence motifs have the ability to transfer oligonucleotides to the cell nucleus, if they include a nuclear localisation sequence (NLS-peptides).

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There are many excellent reviews dealing with the different strategies of oligonucleotide delivery and gene therapy, for examples see [10–18]. In this present work we will focus especially on oligonucleotide (ON) delivery using peptides.

2. Poly-L-lysine

PLL attracted much interest for a variety of (bio)medical applications like gene carriers and drug delivery systems for oligonucleotides. PLL could be synthesised by various synthesis-strategies with different molecular weight (typical 1000 up to >300,000 Da), in linear conformation or as dendritic polypeptides [19,20]. A disadvantage is the significant cytotoxicity of the polypeptides depending on the size. By increasing the molecular weight PLL was less toxic [21]. The cytotoxicity could be further reduced by adding polyanionic maromolecules like ON. Such oligonucleotides were conjugated covalently to PLL or formed complexes with oligonucleotides via electrostatic interaction. Both strategies enhanced the cellular uptake via a non-specific adsorptive endocytosis [13]. An overview of in vitro and in vivo studies are given in Table 1.

2.1. PLL-oligonucleotide conjugates and complexes without specific ligands

A conjugation of oligonucleotides (ON) with PLL via the oxidation of the 3′-terminal cytidine of the oligonucleotide and afterwards covalent binding of these ON to a primary amino group of the PLL was performed. An example was demonstrated for an ON sequence which was complementary to the vesicular stomatitis virus (VSV) N protein mRNA initiation site. These conjugates promoted a specific and efficient antiviral activity in a concentration of more than 100 nM [22]. Higher concentration up to 1 μM of these ON–PLL conjugates resulted in more than 90% inhibition of the VSV replication in a fibroblast-like cell line (L929). Without PLL the oligonucleotide showed no effect up to a concentration of 50 μM [23]. Beside this effect the internalisation pathway of the conjugates were studied. PLL greatly increased the uptake of fluorescent tagged oligonucleotide following a classical endocytic pathway. It was also shown that the ON has to be cleaved from the PLL inside the cell to exhibit an antiviral effect [24].

Human T lymphocyte cells were protected against the succumbing of the cytotoxic effects of an infection of the human immunodeficiency virus type 1 (HIV-1) by administration of PLL–ON conjugates including an oligonucleotide sequence complementary to the HIV-1 tat-protein splice donor site [25]. Additionally, studies were carried out to observe the specificity of the PLL–ON conjugates against the tat-protein. Very significant data about the reduction of the antiviral activity of the conjugates were collected using two mismatches in the oligonucleotide sequence. Furthermore, an EC_{50} of approximately 1.5 μM of the conjugates were found in a HIV-1 acute infection cell assay with a cytopathic-effect readout, which represented a strong reduction in concentration as compared to

Table 1
Poly-L-lysine (PLL) without specific ligands complexing oligonucleotides (ON). An overview of in vitro and in vivo studies

Component	Cells	Major results	Reference
PLL–ON conjugate	Fibroblast-like cell line (L929)	Antiviral activity ON targeting vesicular (VSV) stomatitis virus N protein	Lemaitre et al. 1987 [22]
PLL–ON conjugate	Fibroblast-like cell line (L929)	Antiviral activity of ON targeting vesicular stomatitis virus (VSV)	Degols et al. 1989 [23]
PLL–ON conjugate	Human T cell line (MT4)[a]	Antiviral activity ON targeting tat protein of the HIV-1	Stevenson et al. 1989 [25]
PLL–ON conjugate	Fibroblast-like cell line (L929)	Antiviral effect VSV, cellular uptake studies	Leonetti et al. 1990 [24]
PLL–ON conjugate-heparin complex	Fibroblast-like cells (L929), cervical cancer cell line (HeLa)	Enhanced antiproliferative effect of ON targeting *c-myc* protein by adding polyanions like heparin to the complex	Degols et al. 1991 [29]
PLL–ON conjugate	Human T cell line (MT4)[a]	Specific antiviral effect against tat-protein of HIV-1	Deglos et al. 1992a [26]
PLL–ON conjugate	Human T cell line (MT4)[a]	Specific antiviral effect against tat-protein of HIV-1	Deglos et al. 1992b [27]
PLL–ON conjugate-heparin complex	Human T cell line (MT4)[a]	Enhanced antiviral effect against HIV-1	Degols et al. 1994 [28]
PLL–ON-alginate complex	In vivo	Oral administration of microparticles in rate- and dog-bioavailability studies	Gonzales Ferreiro et al. 2002 [31]

[a] Human T cell leukaemia virus type 1, transformed human leukaemic CD4+ cell line.

the non-conjugated ON ($EC_{50} = 20$ μM) [26,27]. A ternary complex of PLL–ON and a sulphated polyanion (heparin) enhanced the antiviral effect in a similar cell model [28]. Comparable effects of these ternary complexes with heparin were found in a cell proliferation assay using an ON sequence targeting the mRNA of the oncogen *c-myc* [29].

An innovative concept for an antisense oligonucleotide carrier represented an alginate/PLL-microparticle based on the ionotropic gelation of alginate. Sponge-like microparticles were obtained [30] and tested in vivo for the intestinal delivery of antisense-ON in a rat and a dog model. The ON bioavaibility after intrajejunal administration was found to be between 10 and 25% in rats, but resulted in a poor bioavaibility in dogs (0.42%) [31].

Covalent binding of polyethylene glycol (PEG) to PLL and mixing of these conjugates with ON in a physiological saline formed large micelles with a hydrodynamic diameter of about 60 nm [32,33]. These micelles protected the entrapped ON efficiently against nuclease digestion [34]. Thiolated PEG–PLL with a core structure cross-linked by disulphide bonds and ON also formed micelles spontaneously with a size of about 40 nm in diameter [35]. In a further study self assembly systems with poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) chains bound to PLL and ON were physicochemically characterised [36]. Unfortunately, no in vitro or in vivo data have yet been published for both promising concepts.

2.2. PLL-conjugates and complexes with specific ligands

Specific uptake of drug delivery systems can be mediated by specific molecular structures. Via a receptor mediated endocytosis a ligand-PLL–ON complex has the possibilty to enter the target cell. This strategy can be useful for cancer treatment in the future because receptors of some growth factors, hormones and vitamins are over expressed by tumour cells [37]. These molecules as ligands represent an elegant pathway for conjugated drugs or drug delivery systems as targeting device and cellular uptake enhancer. Common examples for this approach are hepatocytes by asialoglycoproteins and the targeting of the transferrin receptor with drug-transferrin complexes. In the following section the different strategies are discussed. For an overview of in vitro and in vivo studies see Table 2.

2.2.1. Asialoglycoprotein

The asialoglycoprotein receptor is specific for hepatocytes and is also expressed on the surface of cells of some cell lines of hepatic origin [38]. Binding of PLL to the asialoorosomucoid protein (AsOR) and complexation with phosphorothioate

Table 2
Antisense oligonucleotide targeting using poly-L-lysine (PLL) with various ligands. An overview of in vitro and in vivo studies

Ligands	Cells	Major result	Reference
Asialoorosomucoid	Mouse fibroblasts (NIH-3T3)[a]	Enhanced activity of antisense ON against CAT-gene	Bunnell et al. 1992 [39]
	Human hepatoma cells (HepG2)	Specific inhibition of human hepatitis B virus (HBV)	Wu and Wu 1992 [40]
Asialofetuin	Hepatoma cell line (PLC/PRF/5)	Increased specific cellular uptake	Reinis et al. 1993 [42]
Fucose	Lung carcinoma cells (A549)	Specific inhibition of the ICAM-1 expression	Stewart et al. 1996 [45]
Mannose	Alveolar human macrophages	Receptor mediated specific uptake of the complexes	Liang et al. 1996 [48]
	Alveolar human macrophages	Inhibition of TNFα expression	Rojanasakul et al. 1997 [49]
Mannose and galactose	Mouse, in vivo studies	Complexes were delivered to hepatocytes and macrophages	Mahato et al. 1997 [50]
Epidermal growth factor	Adenocarcinoma cells (A549)	Enhanced cellular uptake of ON via EGF receptor mediated endocytosis	Deshpande et al. 1996 [54]
Folic acid	Human leukemic cells (HL-60)	Enhanced cellular uptake of ON via receptor mediated endocytosis	Citro et al. 1993 [56]
	Human leukemic cells (HL-60)	Enhanced cellular uptake of ON via receptor mediated endocytosis	Citro et al. 1994a [57]
	Human leukemic cells (HL-60)	Activity of ON against the *c-myb* gene	Citro et al. 1994b [37]
	Human melanoma cells (M14)	Improves efficacy of ON against *c-myc*, reduction of cell proliferation	Ginobbi et al. 1997 [58]
Steroids	Breast cancer cells (MCF-7)	Cellular uptake of ON-complexes	Citro et al. 1993 [56]
Transferrin	Human leukemic cells (HL-60)	ON directed towards *c-myb* protein showed inhibition of proliferation	Citro et al. 1992 [63]
	Adenocarcinoma cells (LoVo Dx)	ON directed towards *c-myb* protein showed inhibition of proliferation	Citro et al. 1994a [57]
Adenovirus	Breast cancer cells (SK-BR-3, MCF-7)	Delivery of triplex forming ON to the nucleus of various tumour cell lines	Ebbinghaus et al. 1996 [64]

[a] Genetically modified cells which expressing the asialoglycoprotein receptor.

antisense ON resulted in 50–150 nm large toroid-shaped complexes which were recognised by the liver-specific asiaglycoprotein receptor and were taken up by hepatocytes. The antisense effect was found to be 10 fold higher compared to purified ON alone in a CAT-gene assay [39].

A classic target for antisense oligonucleotides represents the mRNA of viral proteins [8]. Hepatocytes that possesses asialoglycoprotein receptors were permanently transfected with hepatitis B virus (HBV). AsOR was coupled to PLL (59,000 Da) and afterwards complexed with ON directed against the polyadenylation signal of HBV. This carrier was targeted to cells via asialoglycoprotein receptor resulting in specific inhibition of HBV gene expression and replication [40]. Further studies on this PLL–AsOR conjugate showed that plasmids and oligonucleotides were substantially protected from nuclease degradation [41]. Receptor mediated endocytosis was found for the delivery of ON complexed with an asialofetuin–PLL conjugate into hepatocytes. The cellular uptake was twenty times higher compared to free ON [42].

2.2.2. Fucose, mannose and galactose

Cell surface-binding receptors named membrane lectins are targets for drug delivery systems using glycosylated carriers. Membrane lectins with different sugar specificities are expressed at the cell surface of normal and tumour cells [43,44]. When phosphorothioated ON were complexed with PLL carrying 100 fucose residues, the cellular uptake was 15 fold enhanced compared to free ON. A dose-dependent inhibition of ICAM-1 expression in lung carcinoma cells (A549) was obtained ($IC_{50} = 500$ nM), whereas non-fucosylated PLL–ON complexes gave no inhibition in this cell model at a concentration $< 2\mu$M [45].

Macrophages possess mannose specific membrane receptors which can recognise and internalise glycoproteins bearing mannose residues [46,47]. To achieve receptor mediated ON delivery into human alveolar macrophages, PLL was partially substituted with mannose residues and allowed to form complexes with ON. The cytotoxicity of these complexes decreased compared to non-mannosylated PLL–ON conjugates [48]. Additionally, mannosylated PLL were used to transfer ON directed against tumour necrosis factor alpha (TNFα) into the human macrophages. The cellular uptake depended on the incubation time, the concentration of the mannose–ON–PLL complexes and the receptor density on the macrophages cell-membranes. A selective inhibition of the macrophage TNFα expression was indicated [49]. In vivo disposition studies in mice showed that galactosylated and mannosylated PLL–ON complexes can be delivered to hepatocytes and macrophages via galactose receptors (e.g. Kuffer cells) and mannose receptors, respectively [50].

2.2.3. Epidermal growth factors

Many epithelial tumour and brain tumours of glial origin are known to overexpress the surface epidermal growth factor (EGF) [51] while breast and ovary tumours over-express the EGF-related receptor erbB2 [52,53]. EGF–PLL conjugates enhanced the cellular uptake of ON in adenocarcinoma cells compared to free ON treated cells. The uptake of the complexes was shown to occur via the EGF receptor mediated pathway [54].

2.2.4. Folic acid

Folic acid plays an important role in the hematogenesis and is over-expressed by various cancer cells [55]. After binding folic acid covalently to PLL (10,000–20,000 Da) the ligand affinity to the folic acid receptor was not significantly influenced. The cellular uptake of ON complexed with these conjugates was found to be enhanced via a receptor mediated endocytosis. Additionally, an increased stability was shown against the nuclease degradation of the ON complexed with the folic acid–PLL conjugate compared to free ON [56,57]. In subsequent studies, inhibitory effects of ON directed against the mRNA of the oncogene *c-myb* were determined resulting in an improved down-regulation of the *c-myb* expression [37]. The same strategy was employed to introduce phosphorothioate modified ON directed towards the proto-oncogene mRNA of the *c-myc* protein into human melanoma (M-14) cells. In this cell model using the previously described delivery system the complexed ON were capable to impair protein synthesis and function [58].

2.2.5. Transferrin

All actively metabolising cells require iron ions which are taken up by the cells as a transferrin–iron complex by means of receptor-mediated endocytosis [59–61]. To exploit this ubiquitous and efficient transport mechanism for the delivery of ON into cells, PLL was covalently linked with human tranferrin. These transferrin–PLL molecules formed electrophoretically stable complexes with ON [62]. Exposure of human leukemic (HL-60) cells to transferrin–PLL conjugates complexed with antisense ON directed towards *c-myb* encoded mRNA resulted in a rapid and profound inhibition of proliferation and loss of cell viability much more pronounced than that occurring in cells which were incubated with free ON. This effect was incubation time and concentration dependent. The pathway for the uptake of this ON delivery system again was found to be a receptor-mediated endocytosis [63]. Similar results were obtained with the same target protein in a colon adenocarcinoma cell line (LoVoDx) by the same group [57].

2.2.6. Steroids

Estradiol and progesterone were linked convalently to PLL. The cellular uptake of these steroid–PLL conjugates complexed with ON was shown in breast cancer (MCF-7) cells. Further studies of this ON delivery system unfortunately are not available at this time [56,57].

2.2.7. Viral protein–PLL conjugates

Triplex forming ON have the ability to specifically inhibit a single gene but must cross the cell membrane, escape the endosomal vesicle and additionally have to traverse the nuclear membrane to arrive at their target molecules. For this approach adenovirus-PLL–ON complexes were designed and an efficient delivery of the ON to the nucleus was found via receptor-mediated endocytosis in different breast cancer cell lines [64].

3. Peptides with membrane translocating properties

The use of peptides which are able to build up stable, non-covalent complexes with ON often results in enhanced stability and protection against enzymatic digestion. During the last years, peptides possessing various additional beneficial properties have attracted many scientists in order to overcome the poor cellular uptake of ON and their inability to reach the nucleus [65–71].

3.1. Arginine rich peptides

3.1.1. Protamine

Studies on protamine were started in the year 1868 by Friedrich Miescher [72] A long period of research work was necessary to characterise the group of arginine rich, strongly basic, aliphatic peptides, present in the sperm cell nuclei of fish with a molecular mass of approximately 4000–6000 Da [73]. Now protamine and its salts are well established as pharmaceutical excipients of sustained release formulations of insulin and for some cases as stabilisers of vaccines. Additionally, protamine represents an antidote for heparin intoxications [74]. In the last three decades protamine salts were used often in combination with liposomal preparations to deliver plasmid DNA into cells [74–77]. Antisense technology offer the possibility of highly selective pharmacological manipulation of gene expression, as described previously. ON can bind to the cationic protamine via electrostatic interaction and build complexes [72] which can deliver the ON into various cells.

Wagner et al. linked one to three protamine molecules to one transferrin molecule to create a drug delivery system for ON similar to the transferrin–PLL conjugates. These conjugates were recognised and taken up by receptor-mediated endocytosis in avian erythroblasts. This drug delivery system showed low cytotoxic side-effects in this cell line [62].

ON mixed with protamine free base solution formed nanoparticles spontaneously with a diameter of 100–200 nm. The total amount of ON could be entrapped in the particle matrix using a two-fold mass excess of protamine [78]. These nanoparticles were taken up from human promonocytic leukaemia cells (U 937) carrying an antisense ON directed towards the proto-oncogene *c-myc*. A significant reduction of cellular growth was found in a cellular proliferation assay [79]. These nanoparticles, so called proticles, could also deliver ON to the African green monkey kidney cells (Vero cells). The fluorescence labelled ON were seen in the cytoplasm and in the nucleus of these cells. The cytoxicity was found to be negligible, and the ON were protected against nuclease degradation [80]. This nuclease stability of ON was also found in nanoparticles prepared with various protamine salts and treated with rat small intestine homogenates. These measurements were carried out to prove the utility of this carrier system for gastrointestinal administration [81]. Additionally, for this approach different macromolecules (sodium chenodeoxycholate and sodium caprate) were incorporated in the nanoparticles to promote complex dissociation [82]. Disadvantages of the ON–protamine nanoparticle are (i) their potential to aggregate in aqueous solutions under isotonic conditions and (ii) a poor dissociation of the nanoparticles captured intra-cellularly in the endosomes. To overcome these problems human serum albumin was added to the complexes, which inhibited the precipitaton of the particles [83]. The albumin-protamine–ON complexes showed an enhanced release of ON into the cytoplasm compared to the binary system of ON and protamine. However, also the binary composed nanoparticles showed an antisense effect in a artifical HIV-1 cell model [84]. Further studies on the albumin-protamine–ON nanoparticle demonstated low cytotoxicity and an increased intracellular dissosiation of ON out of the nanoparticles. Fluorescence labelled ON were detected by confocal laser scanning microscopy in the entire cytoplasm using mouse fibroblast. The antisense effect of ON delivered by this drug delivery system was comparable with a commercially available liposomal ON carrier system (DOTAP) using an excitotoxicity cell model, verified by western blot analysis [85].

Protamine sulphate was also used as a cationic component in negatively charged liposomes to deliver chimeric ON to hepatocytes (HepG2) [86]. An increased cellular uptake and a nuclear transport of these ON were found [87,88].

3.1.2. Synthetic arginine rich peptides

In addition to the native arginine rich peptide protamine various artificial arginine rich sequences were synthesised to investigate their potential to deliver ON into cells [89].

Conjugates consisting of oligoarginine peptides containing three, five, or seven arginines, linked to ON were prepared [90]. Based on this chemistry, three aginine rich peptides each consisiting of nine D-arginine, nine L-arginine or six L-arginine were synthesised and afterwards conjugated with a fluorescein labelled ON. Limited data for these peptides are published so far, however, it was demonstrated that these conjugates were taken up by hepatocytes (HepG-2 cells) and an accumulated fluorescence was observed in the endosomes of the cells [91].

3.2. Histidine rich peptides

Poly-L-histidine mediates an acid-dependent fusion and leakage of negatively charged liposomes after protonation of the imidazole group of histinyl monomers [92]. Based on this finding partially histidinylated oligo-lysine were synthesised to transfer ON into cells and afterwards mediate an endosomal escape into the cytosol. A 10-fold enhancement of the ON uptake in the prescence of histidylated oligo-lysine was found. Furthermore, an increase of ON in the nucleus was detected. Histidinylated oligo-lysine increased the biological efficiency of antisense ON directed against the ICAM-1 gene in a TNFα-induced ICAM-1 expression assay and antisense ON directed against the HIV-1 gag gene in a constitutive expression model [10,93,94].

3.3. Fusogenic peptides

The main interest regarding a successful application of ON lies in peptides having lytic, membrane-destabilising-often called fusogenic-properties or nuclear localising signal (NLS) peptides. A number of small protein domains, commonly termed as protein transduction domains (PTDs), show the ability to cross biological membranes efficiently and independently of transporters or specific receptors. Moreover, they are also capable of promoting the delivery of macromolecules like ON into cells. Therefore, they offer the potential to be utilised for delivering oligonucleotides. However, these PTD display limitations in that they mostly require covalent linking to the ON [95].

3.3.1. Binding strategies

Today, the main interest to exploit these sequences lies on the development of covalently attached peptide–ON conjugates [16,67,96–100]. Two chemical schemes are widely adopted. One attempt is to introduce a suitable tether containing a reactive group (e.g. $-NH_2$ or $-SH_2$) to the ON and to add the peptide postsynthetically as an active intermediate, followed by carrying out the coupling reaction in aqueous medium. An alternative approach is to carry out the conjugation in a linear mode on a single solid-phase support [96]. In contrast to the use of complexes, such covalent conjugates of peptides and ON are discrete chemical entities of known stoichiometry [101] resulting in novel functional DNA and RNA [102]. The efficiency of such peptides is generally high and, hence, very low concentrations of the peptides are needed to observe any biological effects [103].

The poor permeability and the selective nature of cell membranes to hydrophilic oligonucleotides severely limits their therapeutic potential. But the discovery of short peptides that can cross membranes efficiently is opening up new possibilities for the intracellular delivery of such agents [104].

3.3.2. Cellular uptake mechanisms

As an endocytotic uptake mechanism is generally believed to be the conventional mode of cellular entry of ON [12,13,66,98,105–107] there exist various severe limitations according to this pathway that need to be overcome.

Once ON have been taken up by cells via adsorptive or receptor-mediated endocytosis, they remain entrapped in endosomal compartments which represents an end-point for them unless the chosen drug delivery vector enables their escape [16,108,109]. Recently, Shadidi and co-workers explored the potential of the cancer cell binding peptide LTVSPWY to specifically deliver antisense ON. They designed a fluorescein-conjugated ON against the ErbB2 receptor and coupled it via a disulphide bridge to the LTVSPWY peptide. The data suggest this peptide to be useful for the tumour treatment with antisense ON in the future [110].

Another approach for direct cytoplsmatic delivery of ONs applies a synthetic signal import peptide (IP) derived from a naturally occuring signal peptide from Kaposi fibroblast growth factor. This peptide in conjugation with polylysine formed complexes with ONs resulting in an enhanced effectiveness regarding the cellular uptake. The effect was found to be concentration- and cell-dependent and the mechanism by which the peptide facilitates ON uptake appears to be a non-endocytotic process [111].

In an excellent review about peptide-based gene delivery with special attention on plasmids, Mahato describes the strategies to achieve endosomolysis via peptides in order to escape lysosomal degradation and, further on, to enter the nucleus for subsequent expression [108].

One strategy is to utilise conformation changes of peptides at acidic endosomal pH so that transient permeabilisation of cell membranes occurs before the content of the endosomes is delivered to lysosomes [11,103,112].

Besides causing destabilisation and fusion of bilayer membranes in a pH-sensitive manner, many viruses release their genome through the action of mostly hydrophobic peptide sequences, which are a part of different viral proteins, also causing disruption of the endosomal membranes by lysis and/or fusion [108,113]. Such sequences are known to destabilise and penetrate cellular membranes, and furthermore, to act as NLSs [96,111,114].

Commonly considered mechanisms of membrane destabilisation are the formation of pores and membrane rupture. Pore formation occurs without affecting general membrane properties as a self-assembly process of appropriate peptides in the membrane.

On the other hand, peptides altering the membrane properties of the lipid bilayer lead to membrane rupture via rearrangement of lipid packaging. For further information excellent reviews are available [108,115].

A feature common to several fusogenic peptides is that they promote negative membrane curvature in membranes when present in low concentrations [113]. These peptide signals,

usually ranging between 9 to 30 amino acid residues in length, as already mentioned, additionally have the capability to deliver other biological molecules into cells [116–118].

Niidome et al. employed several kinds of α-helical and otherwise structured peptides to understand what structure of the peptide is required for binding to an oligonucleotide. They suggested that amphiphilic α-helix peptides were best for binding to oligonucleotides. The large hydrophobic regions in the amphiphilic structure of such peptides seems to be necessary for the binding and forming of aggregates with the oligonucleotides [119,120]. Moreover, the majority of peptides with membrane translocating properties show an amphiphilic structure and α-helical structure. Investigations on the secondary structure could verify an α-helical structure for many cases, but there also exist other studies suggesting that α helicity is not an essential requirement for peptides to translocate across cellular membranes [103]. β-sheet forming peptides seemed not to be appropriate candidates for this purpose due to their tendency to aggregate in water and, therefore, very limited solubility.

Nonetheless, Krause et al. synthesised a water soluble amphipathic 26-mer β-sheet peptide which showed good internalisation into endothelial cells [121,122]. Chaloin et al. also noted an antiparallel β-sheet form for two amphipathic carrier peptides in the presence of lipids. Their results in this study suggest that the β-sheet is responsible for the translocating properties of the peptides they used, but also questions the conformational state of signal peptides when associated to hydrophilic sequences like ON [123].

Similar observations were made with penetratin. For this peptide the original α-helical structure maintained in the parental Antennapedia homeodomain can adopt a β-sheet structure in the presence of a charged lipid monolayer [70, 124].

3.3.3. Artificial fusogenic peptides

Pioneering studies with cell-penetrating peptides mimicking viral fusogenic peptides were initiated in the group of Szoka. In the late eighties they published the first results from their studies with a pH-sensitive peptide composed mostly of repeating GALA (Gly-Ala-Leu-Ala) units [125–127]. In the following years GALA was studied extensively for the use in gene delivery. An overview of such sequences is given in Table 3.

There is a consensus that GALA undergoes a conformational change to an amphipathic α-helix when the pH is reduced via neutralisation of the glutamic acid residues. At neutral pH GALA is a water-soluble peptide with an aperiodic conformation. As the pH is lowered to 5.0 it starts interacting with bilayers where it exhibits high affinity of binding to neutral and negatively charged membranes and causes aqueous content release [128,129]. Hughes et al. examined the use of GALA as an adjuvant to enhance the effectiveness of antisense ON. They noticed a 35–40% reduction in CAT expression when using AS ON in co-incubation with the GALA peptide [130]. Also another fusogenic peptide derived from the influenza virus-HA was studied. This peptide is known to facilitate endosomal gene transfer [131], but in the study only a minimal ON activity enhancement was published [130].

The replacement of glutamic acid residues in GALA with lysine residues led to KALA (Lys-Ala-Leu-Ala), which also represents an amphipathic peptide when present in an α-helical conformation. KALA undergoes a pH-dependent random coil to amphipathic α-helical conformational change as the pH is increased from 5.0 to 7,5 [132]. Therefore, it is more interestingly for the purpose of ON delivery through the plasma membrane at physiological pH [16].

Wyman et al. showed that KALA assisted internalisation of fluorescent ON into the adherent monkey kidney fibroblast cell line CV-1 as compared to the poor internalisation of naked ON.

Recently, Jeong et al. complexed ON/PEG conjugates with KALA. The ON segments and KALA ionically interacted and formed an inner polyelectrolyte complex core, while the PEG fraction constituted a surrounding corona. This combination resulted in stable micelles, which were transported more efficiently into cells than the ON itself [133].

Gottschalk et al. developed another amphipathic, pH-dependent endosomal releasing agent, referred to as JTS-1, designed for the purpose of assembling DNA–peptide complexes [134]. The hydrophobic face of this fully synthetic pore-forming agent contains strongly apolar amino acids, while negatively charged glutamic acid residues dominate the hydrophilic face at physiological pH. Jääskeläinen et al. used JTS-1 as a membrane-active component in a lipid-based delivery system for antisense-oligonucleotides directed against the luciferase gene. In this study, the presence of JTS-1 was essential for significant antisense-effects [135,136].

3.3.4. Viral derived peptides

Deshpande et al. described the ability of the influenza HA2 peptide, like Polymyxin B, to promote the internalisation of ON via the EGF receptor pathway. When A549 cells were incubated with complexes of fluorescently labeled ON and the EGF–PLL conjugates of either of these peptides, a significant dose-dependent increase in intracellular fluorescence was observed [54].

Another peptide which also forms complexes with nucleic acids and exhibits membrane fusion and permeabilisation activities at neutral pH like melittin is K5. K5 has five K (Lys) residues in place of the five G (Glu) residues in the anionic fusogenic E5 peptide [137]. E5, an anionic 20 amino acid peptide is also derived from the *N*-terminal segment of hemagglutinin subunit of the influenza virus hemagglutinin and causes fusion transiently at acidic pH [112,138].

Also the analogous anionic amphiphilic E5CA sequence is based on the subunit of the influenza virus. This was used by Pichon et al. to deliver antisense oligonucleotides directed against the HIV-1 gene. In the presence of

Table 3
Overview: cell-penetrating peptides

Name	Origin	Peptide sequence	References
GALA	Artificial	WEAALAEALAEALAEHLAEALAEAEALEALAA	Parente et al. 1988 [129]
KALA	Artificial	WEAKLAKALAKALAKHLAKALAKALAKACEA	Wyman et al.1997 [132]
JTS-1	Artificial	GLFEALLELLESLWELLLEA	Gottschalk et al. 1996 [134]
FLUOS-peptide-NH_2 (not further termed)	Artificial	KLALKLALKALKAALKLA	Oehlke et al. 1998 [174]
HA	Influenza virus	GLFEAIAGFIENGWEGMIDG	Lear et al. 1987 [175]
		GLFEAIAGFIENGWEGMIDGWYG	Hughes et al. 1996 [130]
K5	Influenza virus	GLFKAIAKFIKGGWKGLIKG	Murata et al. 1991 [176]
E5	Influenza virus	GLFEAIAEFIEGGWEGLIEG	Murata et al. 1991 [176]
H5WYG	Influenza virus	GLFHAIAAHFIHGGWHGLIHGWYG	Midoux et al. 1998 [142]
E5CA	Influenza virus	GLFEAIAEFIEGGWEGLIEGCA	Midoux et al. 1995 [112]
E5WYG	Influenza virus	GLFEAIAEFIEGGWEGLIEGWYG	Freulon et al. 2000 [141]
INF-1	Influenza virus	GLFEAIAGFIENGWEGMIDGGGC	Plank et al. 1994 [131]
INF-7	Influenza virus	GLFEAIEGFIENGWEGMIDGWYG	Plank et al. 1994 [131]
adenoviral core peptide μ (mu)	Adeno virus	MRRAHHRRRRASHRRMRGG	Keller et al. 2002 [146]
MPM (membrane permeable motif) also known as: IP/K-FGF	Kaposi fibroblast growth factor (K-FGF)	AAVALLPAVLLALLAP	Lin et al. 1995 [177], Dokka et al. 1997 [111]
Melittin	Venom of *Apis mellifera*	GIGAVLKVLTTGLPALISWIKRKRQQ	Midoux et al. 1995 [112]

the E5CA peptide at pH 6.0, fluoresceinylated ON were rapidly taken up by different types of adherent cells, such as HepG2, HeLa, Rb-1 or COS cells, and diffused into the nucleus, where a large part of the internalised ON was located. The uptake was dependent on the concentration of the E5CA peptide and on the duration of the incubation at low pH [139]. To improve the efficiency of E5CA Freulon et al. synthesised two peptide derivatives which are closer to the natural sequence of the influenza virus fusion peptide-E5WYG and (E5WYGG*AA*)$_2$KA (*A* stands for β-alanine). They compared those monomeric and dimeric peptides with respect to their ability to introduce ON into the cytosol and nuclei of several types of cultured cells compared to E5CA. The monomeric peptide, unlike the dimeric species, efficiently permeabilised cells incubated at pH 6.2 or slightly lower. In the presence of E5WYG, ON were mainly found in the nucleus, while in the presence of the dimeric peptide they colocalised with the peptide into vesicles [140]. Finally, Freulon et al. reported that the dimerisation of the E5WYG peptide leads to greater efficiency when the dimerisation device includes a longer spacer arm made of G*AA* residues, but to 10-fold less efficiency when the dimerisation device includes a shorter spacer, a glycyl residue [141].

Midoux et al. synthesised a peptide named H5WYG that permeabilises cell membrane at a slightly acidic pH but not at neutral pH [142]. H5WYG is also analogous to the *N*-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin in which G (Gly)-4, G (Gly)-8, E (Glu)-11, T (Thr)-15, and D (Asp)-19 were replaced by histidyl residues and M (Met)-17 was exchanged against a leucyl residue. They designed this peptide based on the following observations: as mentioned above, the efficiency of an eicosamer containing five glutamyl residues (E5) could be considerably enhanced either by transforming it into a dimer [141,143] or by adding the tripeptide WYG (Trp-Tyr-Gly) in a C-terminal position (E5WYG) [141]. This enhanced activity was related to the presence of the additional tripeptide sequence WYG which is also seen in native fusogenic peptides of the influenza hemagglutinin subunit HA-2 [131]. But an anionic peptide such as E5WYG requires a more acidic pH to permeabilise membranes [139]. Knowing that the imidazole group of histidine has a pK of around pH 6.0 and thus becomes cationic in a slightly acidic medium, they hypothesised a change of lysyl by histidyl residues in K5 peptide to be useful. Additionally, H5WYG rather than E5WYG or H5 was expected to efficiently destabilise the endosomal compartments early upon uptake [142]. In a more recent study the same group also described the effect of H5WYG on the oligonucleotide transfer. They found out that this peptide could be used to easily load the cytosol and the cell nucleus with ON by lowering the extracellular pH of the medium containing both ON and H5WYG. Without decreasing the pH the peptide concentration inside the endocytotic vesicles was too weak to allow membrane destabilisation [144]. In slightly acidic medium no permeabilisation of plasma membranes, but a selective disruption of endosomal vesicles will occur. This is of importance, as it has to be emphasised, that many of the other peptides, cationic or anionic, are inefficient when the transfection step is conducted in the presence of serum.

Pichon et al. hypothesised that ONs bearing a KDEL (Lys-Asp-Glu-Leu) motif should be routed in compartments containing a KDEL receptor and enter the cytosol via translocation throughout one of these membranes more easily. And although peptide-substituted ON were internalised 4-fold less than the peptide-free ON, they were 5-fold more efficient in inhibiting the target gene. This large

increase in efficiency was shown to be due to the peptide motif [145].

Keller et al. biophysically characterised the interaction of the cationic adenoviral core complex peptide μ with a dodecameric ON compared to protamine (see also Section 3.1). They used a combination of fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry and dynamic light scattering to determine binding affinity, thermodynamic of binding and stability of the two peptide–ON particles preparations. Broad similarities were shown in this study for the features mentioned above. Like protamine, strong affinity and its ease of formulation over broad peptide–ON ratios renders μ peptide an interesting candidate for the use in peptide-based oligonucleotide delivery [146].

Another peptide particle formulation was reported by Normand et al. who showed that the addition of VP22, a structural protein of herpes simplex virus, to ON induced spontaneous assembly of novel particles incorporating both protein and ON. Brewis et al. extended this observation by constructing a fusion protein containing a soluble subdomain of VP22 together with a short peptide called Bak (MGQVGRQLAIIGDDINRRY). The resulting novel particles were stable in serum, not toxic and were efficiently taken up by many cell types. Interestingly, the particles could be disrupted by light activation, resulting in redistribution of the VP22 protein and ON throughout the cell, with the ON accumulating in the nucleus. Delivery of antisense ON to the transcript of the raf kinase by this route resulted in functional activity and inhibition of cell proliferation [147,148]. In the next section we will focus on the effect of nuclear localisation promoted by peptides in more detail.

3.4. Peptides with a nuclear localisation sequence (NLS-peptides)

One strategy to improve the nuclear uptake of an ON to achieve biological effects is to take advantage of the cellular nuclear import machinery.

The nucleus represents a highly restricted cellular compartment housing the majority of cellular genetic material. It contains the components and enzymes necessary to maintain, pack, unpack and ‘transcribe’, and process genetic material. Macromolecules like proteins needed inside the nucleus are transported through nuclear pore complexes (NPC), cylindrical proteinaceous structures, about 120 nm in diameter and 70 nm thick, that perforate the nuclear envelope. NPCs control the passage of molecules in and out the nucleus. This is, except during mitosis, the only way macromolecules can enter the nucleus. The inner pore of a NPC allows free, passive diffusion of molecules of up to 9 nm in diameter. Additionally an active transport of small molecules up to 25 nm is possible [149]. Larger molecules require so called nuclear localisation signals (NLS) that are recognised by cytoplasmatic transport receptors and mediate the nuclear uptake [108].

Synthetic peptides containing NLS can now bind to ON so that the resulting ON–NLS–peptide hybrid can be recognised as a nuclear import substrate by specific intracellular receptor proteins [150]. Although the contribution of this pathway remains unclear, the conjugation of NLS to molecules like oligonucleotides has been proven to be useful in several cases [130,139].

However, the nuclear entry effects of NLS peptides linked to ON are still open to dispute [151]. NLS sequences are typically less than 12 residues in length and rich in basic amino acids [152] resulting in a net positive charge [153,154]. The NLS sequences identified so far, principally contain one (monopartite) or two (bipartite) clusters of four or more arginine (R) or lysine (K) residues. Beyond this, it has to be noted, that there exist many exceptions and variations [108]. Optimum translocation through membranes is achieved by peptides with a length of six to nine arginine residues. Generally, peptides containing arginine residues are thought to translocate more efficiently than those containing lysine ones [154,155]. Despite this highly basic character, they are able to cross cell membranes to reach the nucleus within a few minutes [154,155]. Furthermore, an advantageous feature common to several fusion peptides is that they promote their membrane translocating properties when present in low concentrations [113].

The most studied and therefore best known NLS sequences are peptides derived from viruses [156,157] like Tat [71,103,118,158–161] or Antennapedia homeodomain protein derived ones [71,103,118,159,161–165], but arginine oligomers seem to be far more efficient than those peptides [166].

3.4.1. NLS peptides derived from HIV and SV40

Morris et al. developed a 27 residue peptide vector called MPG, which contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen. A summary of the most used sequences is given in Table 4. When MPG is mixed with oligonucleotides in solution, they rapidly associate into complexes with tight non-covalent interactions. Efficient delivery of the MPG–ON complex into the nucleus occurred in less than one hour by simply overlaying this complex onto mammalian cells. Their results led to the suggestion that rapid self-assembly between MPG and oligonucleotides involves mainly electrostatic interactions. This takes place between the negatively charged nucleic acids and the positively charged moiety of MPG, mainly the lysine residues of the NLS [167].

Such a mechanism, involving electrostatic interactions has also been suggested by Gottschalk et al. for another lysine-rich peptide mediated ON transfection technique [134].

Table 4
Most commonly used NLS-sequences

Name	Source of peptides	Membran-active protein	Main motif
Tat	Human immunodeficiency virus type 1 (HIV-1)	gp41 (*N*-terminus)	GRKKRRQRRRPPQC
Ant	Homeodomain of Antennapedia (*Drosophila*)		RQIKIWFQNRRMKWKKC
SV-40	Simian virus 40 large T-antigen	gp32	PKKKRKV
Influenza nucleoprotein	Influenza virus		NSAAFEDLRVLS
NLS of NF-κB	Transcription factor NF-κB	NF-κB p50	VQRKRQKLM

For efficient in vivo delivery of antisense ON, Toth et al. also used a SV40 T antigen nuclear signal peptide fragment as part of a more specific cellular approach. They developed a two step system where the ON is first electrostatically bound to the peptide coupled to a ligand of a cellular receptor. The ON were then delivered inside the cells by adding a transfection agent. Hence, in this study the function of the SV40 peptide sequence is to bind antisense ON and bring them to the cellular membrane of the targeted cells. The SV40 T antigen derivative has been shown as a nuclease protecting agent in the range from one ON for five peptides to one ON for nine peptides [168].

3.4.2. NLS peptides derived from transcription factors

Ragin et al. synthesised different fluorescently labelled NLS sequences derived from transcription factors NF-κB, TFIIE-β, Oct-6, TCF1-α, SV40, HATF-3, and *C. elegans* SDC3. They chose these sequences to evaluate the effect of varying the distribution of charged and uncharged residues within the peptides on cellular uptake of MCF-7 breast carcinoma cells. Their NLS sequence of NF-κB was successfully used to deliver covalent adducts of proteins and oligonucleotides to MCF-7 cells [153].

Antopolsky et al. used another nuclear localisation sequence of the transcription nuclear factor κB in addition to a 17-mer membrane permeable motif to build up peptide–ON phosphorothioate conjugates with membrane translocation and nuclear localization properties. These conjugates are capable of inhibiting the luciferase gene expression in a cell-free model transcription–translation system. But when applied to living cells some of the conjugates (the peptides used to conjugate differ in their end groups) failed to show any serious antisense effect.

Taken together, these data give rise to the conclusion that mere conjugation of antisense ON and peptides possessing membrane penetration and nuclear localising properties does not necessarily result in a reliable delivery system which works well with a number of cell types [114].

3.4.3. NLS peptides combined with other peptides

Recently, Kubo et al. conjugated antisense oligonucleotides among other functional molecules to peptides such as NLS, nuclear export signals (NES) and artificial amphiphilic α-helical and β-sheet peptides. NLS sequences investigated in this study were derived from SV40 T antigen, HIV-1 tat protein and the influenza nucleoprotein. Conjugated NLS-ON showed antisense activities several times higher compared to native or phosphorothioate DNA. Therefore, they expect their conjugates to have promising antisense properties [169]. In another publication they described that ON conjugates with NLS peptides were successfully delivered into the nucleus, but showed insufficient membrane permeability. An improvement was achieved by combining fusion peptides and NLS. ON conjugated with both of them have shown to be effectively taken up into cytoplasm and also to be delivered into the nucleus. Additional beneficial effects like increased stability of ON against nuclease digestion were also observed [170].

The use of peptides having miscellaneous membrane-translocating properties to assist the delivery of ON seems to be an interesting, promising approach towards enhancing their biological effects. The advantages of utilising fusogenic peptides as part of a delivery system for ON are promising: enhanced efficiency although showing the advantages of a non-viral system, applicable to a wide range of cells and rapid cytoplasmic delivery [103]. But there are also a number of potential limitations which can cause complications. Toxicity is the most severe complication expected so far. An example is Melittin, a toxic 26-residue peptide from the venom of the European honeybee *Apis mellifera* [171] and its conjugate with dioleoyl chain [172] which has been considered for gene transfection. Rozema et al. studied the ability of a maleic anhydride derivative of melittin to aid in the delivery of phosphorodiamidate morpholino oligonucleotides. This delivery strategy relies on the co-endocytosis of both the ON and the melittin. However, this event was only possible at relatively high concentrations in vitro, and in vivo applications remain impossible to date [173].

4. Conclusion

Synthetic cationic peptides like PLL were utilised for many years to enhance cellular uptake of ON–PLL complexes via non-specific endocytosis. The ON, entrapped in these complexes, were efficiently protected against nuclease degradation. Various ligands were linked to PLL to promote specific cellular uptake via receptor-mediated endocytosis. Similarly to these, native peptides e.g. protamine can be used as an excipient for ON delivery.

Furthermore, arginine- and histidine-rich peptides enhanced an endosomal escape of the ON into the cytoplasm. Fusogenic peptides have membrane-destabilising and lytic properties, and can increase also the ON release into the cytoplasm. Additionally, nuclear localisation sequence motifs can mediate the transport of ON to the nucleus (NLS-peptides).

Today, many antisense ON have entered clinical trials, however, these formulations consist of mostly unbound ON without any delivery device. It will be interesting in the future to see what biotechnology and pharmaceutical technology can do to improve the efficacy/efficiency of oligonucleotide drugs.

The use of siRNA is a new and promising strategy for the antisense technology. Although, in most of the studies presented in this review, single stranded DNA was used, these ON were with or without chemical modifications. To our mind all these excellent findings will also help to develop and to optimise carriers for the very promising siRNA approach, generating non-toxic, specific and efficient oligonucleotide drug delivery systems.

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