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THEMED SECTION: VECTOR DESIGN AND DRUG DELIVERY

REVIEW

Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers

Patrick Midoux[1], Chantal Pichon[1], Jean-Jacques Yaouanc[2] and Paul-Alain Jaffrès[2]

[1]*Centre de Biophysique Moléculaire CNRS UPR 4301 affiliated to the University of Orléans and Inserm, rue Charles Sadron, F-45071 Orléans Cedex 2, France, and* [2]*CEMCA, UMR CNRS 6521, Université Européenne de Bretagne, Université de Brest, Faculté des Sciences et Techniques, 6 avenue Le Gorgeu, F-29238 Brest, France*

DNA/cationic lipid (lipoplexes), DNA/cationic polymer (polyplexes) and DNA/cationic polymer/cationic lipid (lipopolyplexes) electrostatic complexes are proposed as non-viral nucleic acids delivery systems. These DNA-nanoparticles are taken up by the cells through endocytosis processes, but the low capacity of DNA to escape from endosomes is regarded as the major limitations of their transfection efficiency. Here, we present a current report on a particular class of carriers including the polymers, peptides and lipids, which is based on the exploitation of the imidazole ring as an endosome destabilization device to favour the nucleic acids delivery in the cytosol. The imidazole ring of histidine is a weak base that has the ability to acquire a cationic charge when the pH of the environment drops bellow 6. As it has been demonstrated for poly(histidine), this phenomena can induce membrane fusion and/or membrane permeation in an acidic medium. Moreover, the accumulation of histidine residues inside acidic vesicles can induce a proton sponge effect, which increases their osmolarity and their swelling. The proof of concept has been shown with polylysine partially substituted with histidine residues that has caused a dramatic increase by 3–4.5 orders of magnitude of the transfection efficiency of DNA/polylysine polyplexes. Then, several histidine-rich polymers and peptides as well as lipids with imidazole, imidazolinium or imidazolium polar head have been reported to be efficient carriers to deliver nucleic acids including genes, mRNA or SiRNA *in vitro* and *in vivo*. More remarkable, histidylated carriers are often weakly cytotoxic, making them promising chemical vectors for nucleic acids delivery.
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Keywords: non-viral vectors; gene transfer; SiRNA; cationic lipids; cationic polymers; pH-sensitive membrane fusion; pH-sensitive membrane permeation; endosome escape; fusogenic peptides; imidazolinium and imidazolium

Abbreviations: bPEI, branched polyethyleneimine; ChIm, cholesterol-(3-imidazol-1-yl-propyl) carbamate; chol, cholesterol; DF4C11PE, rac-2,3-Di[11-(F-butyl)undecanoyl]glycero-1-phosphoethanolamine; DOIm, 4-(2,3-bis-oleoyloxy-propyl)-1-methyl-1H-imidazole; DOPC, L-α-di-oleoyl phosphatidyl choline; DOPE, L-α-di-oleoyl phosphatidyl ethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA, N-[1-(2, 3-di-oleyloxy) propyl]-N,N,N-trimethylammonium chloride; DOTIM, 1-[2-(9(Z)-Octadecenoyloxy)ethyl]-2-(8(Z)-heptadecenyl) l-3-(2-hydroxyethyl)imidazolinium chloride; DMTIM, 1-[2-(Tetradecanoyloxy)ethyl]-2-tridecyl-3-(2-hydroxyethyl)imidazolinium chloride; DPIm, 4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1H-imidazole; DPTIM, 1-[2-(Hexadecanoyloxy)ethyl]-2-pentadecyl-3-(2-hydroxyethyl)imidazolinium chloride; DS(9-yne)PE, Distear-4-ynoyl L-α-phosphatidylethanolamine; FGF-2, fibroblast growth factor 2; ODN, oligonucleotide; PTEN, phosphatase and tensin homologue deleted on chromosome 10 complex

Introduction

Gene therapy uses the nucleic acids as medicine to cure genetic deficiencies and a large variety of acquired diseases. That concerns not only the large DNA molecules (plasmids DNA; pDNA) but also the small DNA (oligonucleotides; ODN)

Correspondence: Patrick Midoux, Centre de Biophysique Moléculaire CNRS UPR 4301 affiliated to the University of Orléans and Inserm, rue Charles Sadron, F-45071 Orléans Cedex 2, France. E-mail: Patrick.midoux@cnrs-orleans.fr
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and RNA (ribozymes, SiRNA and mRNA). pDNA can encode proteins, antisense ODN, ribozymes and SiRNA. Although, the efficiency of gene therapy has been proved to cure some genetic diseases by using viral vectors to deliver therapeutic genes, the non-viral options are searched. The simplest method is the direct introduction of pDNA into the target cells. This approach is limited because it can only be used for some tissues and requires large amounts of pDNA. Inspired by the strategy of some viruses to gain entry into mammalian cells, researchers are trying to build synthetic viruses. The goal is to replicate with the synthetic molecules all the steps used by the viruses to infect mammalian cells. The main stages are the protection of the nucleic acid, its penetration into the cell, its transfer inside the cytosol and in the nucleus, and for some applications its integration into the genome of the host cell, all these steps being milestones.

Protection and stability of DNA in serum are achieved by pDNA condensation with cationic polymer, cationic lipids or encapsulation in liposomes. Since the pioneering work of Felgner and Behr, the molecular diversity of cationic lipids has become very broad. They are usually formulated as cationic liposomes with a neutral co-lipid like di-oleoyl phosphatidyl ethanolamine [L-α-di-oleoyl phosphatidyl ethanolamine (DOPE)] or cholesterol. Cationic liposomes form electrostatic complexes (so-called lipoplexes) with pDNA. At high positive charge ratios, lipids and pDNA undergo topological transformation into compact quasi-spherical vesicles of 200–300 nm in diameter, in which DNA and lipids adopt an ordered multilamellar structure (Safinya, 2001). Various cationic polymers, including polylysine, polyethyleneimines, polyamidoamine, dendrimer, polyallylamine and methacrylate/methacrylamide polymers have been also proposed for delivering of pDNA. The self assembly of pDNA with cationic polymer in solution induces a strong DNA condensation leading to a large size reduction of a plasmid of thousands base pairs in toroid or rod DNA/polymer complexes (so-called polyplexes). Lipopolyplexes combining DNA, polymer and lipids are lipid vesicles resulting from the encapsulation of polyplexes in liposomes, which are also efficient gene delivery systems (Li *et al.*, 1998; Vangasseri *et al.*, 2005).

These systems are usually captured by the cells and internalized into vesicles by endocytosis mechanisms. While for several types of cationic lipids, the uptake of lipoplexes proceeds mainly by the clathrin-dependent pathway, it appears that the polyplex uptake pathway is more dependent on the polymer and the cell type (Midoux *et al.*, 2008). The major and best-characterized route of endocytosis in most mammalian cells is mediated by the clathrin-dependent pathway. Internalized molecules rapidly experience a drop in pH from 7.4 on the cell surface to 6.0 in the lumen of early endosomes, with a reduction to pH 5 during their progression to late endosomes and lysosomes where degradation takes place. The caveolae-mediated endocytosis process has been also identified for lipoplexes and polyplexes internalization. Overall, it appears that polyplex uptake occurs both by the clathrin-dependent pathway and a clathrin-independent (cholesterol-dependent) pathway. These two entry mechanisms are not exclusive and can occur simultaneously in the same cell. Next, pDNA must rapidly and quantitatively escape into the cytosol to be imported in the nucleus. The low capacity of DNA to escape from endosomes is regarded as the major limitations of the transfection efficiency of lipoplexes and polyplexes. Several strategies have been explored to improve this crucial step. They include the incorporation of fusogenic lipids in cationic liposomes, the association of fusogenic peptides with polyplexes and lipoplexes and the generation of a proton sponge effect inside endosomes. Among these strategies, the introduction of the histidine and imidazole in the cationic polymers, peptides or cationic lipids has led to the generation of new powerful nucleic acids carriers that are detailed in this review.

Fusogenic lipids

Helper or co-lipids are frequently added to the cationic liposomal formulations to increase the lipoplex fusogenicity but also to decrease their cytotoxicity (Table 1). DOPE and L-α-dioleoyl phosphatidyl choline (DOPC) are among the first tested helper lipids (Gruner *et al.*, 1988; Farhood *et al.*, 1995). DOPE has the propensity to form H_{II} phase structure known to induce supramolecular arrangements leading to the fusion of lipid bilayer at a temperature upper than 5–10°C. Its incorporation into the lipoplexes also helps the formation of H_{II} phases that is supposed to destabilize endosomal membrane. Because some drawbacks have been observed for DOPE lipoplexes after administration in the blood circulation, cholesterol is usually preferred for *in vivo* transfection (Sakurai *et al.*, 2001). The presence of one unsaturation in the acyl chain of DOPE is a crucial factor for membrane fusion activity (Talbot *et al.*, 1997). Nevertheless, the replacement of oleyl chains has been investigated. However, lipids with saturated chains containing poly-fluorinated moities as in DF4C11PE (rac-2,3-Di[11-(F-butyl)undecanoyl]glycero-1-phosphoethanolamine) enhanced also the transfection efficiency of lipopolyamine lipoplexes (Boussif *et al.*, 2001; Gaucheron *et al.*, 2001). HL1, a DOPC analogue with an ester group in ω position of the alkyl chains was found to be more efficient than DOPE to transfect *in vitro* CHO and L6 cells with the 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipoplexes (Prata *et al.*, 2008). The replacement of the double bond of the oleic chains of DOPE by a triple bond illustrated by Distear-4-ynoyl L-α-phosphatidylethanolamine [DS(9-yne)PE] has been also carried out to tune the temperature of the Lα-H_{II} phase transition in order to produce more stable lipoplexes (Fletcher *et al.*, 2006; 2008).

Fusogenic peptides

Amphiphilic anionic peptides derived from the N-terminal segment of the HA-2 subunit of the haemagglutinin of the influenza virus such as IFN7 (GLFEAIEGFIENGWEGMIDG-WYG) and E5CA (GLFEAIAEFIEGGWEGLIEGCA) have been used to increase by several orders of magnitude the transfection efficiency of polyplexes (Wagner *et al.*, 1992; Midoux *et al.*, 1993) or of lipoplexes (Kichler *et al.*, 1997; Wagner, 1999; Zhang *et al.*, 2001). Some artificial peptides such as GALA have been also used (Li *et al.*, 2004; Sasaki *et al.*, 2008). These peptides allow the membrane fusion and permeation

Table 1 Chemical structure of selected helper lipids with a glycerol unit

Chemical structure

R^1	R^2	Name	Reference
	NH_3	DOPE	1,2
	$N(CH_3)_3$	DOPC	1
	NH_3	DF4C11PE	3
	NH_3	DS(9-yne)PE	4,5
	$N(CH_3)_3$	HL1	6

(1) Farhood *et al.* (1995); (2) Gruner *et al.* (1988); (3) Gaucheron *et al.* (2001); (4) Fletcher *et al.* (2006); (5) Fletcher *et al.* (2008); (6) Prata *et al.* (2008).
DF4C11PE, rac-2,3-Di[11-(F-butyl)undecanoyl)glycero-1-phosphoethanolamine; DOPC, L-α-di-oleoyl phosphatidyl choline; DOPE, L-α-di-oleoyl phosphatidyl ethanolamine; DS(9-yne)PE, Distear-4-ynoyl L-α-phosphatidylethanolamine.

between pH 5.5 and pH 5.0 after glutamic acid protonation and formation of α-helix. However, the acid-membrane destabilization properties of those anionic peptides are almost inactive after conjugation onto another molecule as well as in the presence of serum (Midoux *et al.*, 1995; Moore *et al.*, 2008). The fusogenic peptide of the glycoprotein H from herpes simplex virus was also found to improve the endosomal release of DNA/Lipofectamine lipoplexes and transgene expression up to 30-fold in human cell lines (Tu and Kim, 2008).

Proton sponge effect

The induction of a proton sponge effect inside endosomes containing DNA is illustrated by chloroquine (Zenke *et al.*, 1990; Midoux *et al.*, 1993). The accumulation of this weak base inside acidic vesicles counterbalances the accumulation of proton generated by a specific ATPase, neutralizes the lumen of endosomes and lysosomes and increases their osmolarity. As a consequence, the vesicles swell and their content is delivered into the cytosol. This behaviour is also found with polyethylenimine (PEI) that contains protonable amines acting as a weak base in acidic medium and make it a remarkable nucleic acids carrier *in vitro* and *in vivo* (Boussif *et al.*, 1995; Demeneix and Behr, 2005).

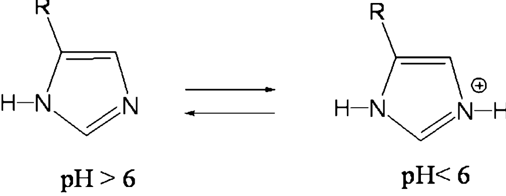

Figure 1 pH dependence of the imidazolium protonation state.

Histidylated polylysine

Due to its protonation at pH 6.0, the imidazole ring of histidine is also a weak base, suitable to design cationic polymers exhibiting the capacity to condense DNA and to favour the espace of pDNA from endosomes (Figure 1). Histidylated polylysine (HpK) was the first example of a long series of histidine or imidazole vectors. HpK prepared by substitution of poly(L-lysine) of 190 degrees of polymerization with histidine residues has been found to be 3–4.5 orders of magnitude more efficient to transfect HepG2 cells than polylysine (Midoux and Monsigny, 1999). Depending on the cell type, the optimal transfections were obtained with HpK containing 63–100 (33–50%) histidyl residues. The transfection efficiency was dramatically reduced when cells were incubated in the presence of bafilomycin A1, a specific inhibitor of the ATPase

Table 2 Histidine-rich polymers and peptides

Name	*Type*	*Reference*
HpK, HIS, His-pLK	Histidylated polylysine	1–4
HoK	Histidylated oligolysine	4,5
PLH-g-PLL	N-Ac-poly(L-hisidine)-graft-poly(L-lysine)	6
Im-pK	Imidazole polylysine	7,8
KGH6	Dendrimer	9
PDHP	poly(DMAEA/His(Boc)-OMe)phosphazene	10
HK; HHK4b; HHHK8b	linear and branched copolymers of histidine and lysine	11–16
His6 RPCs	$(CH_6K_3H_6C)_n$	17,18
CDPim	Histidylated β-cyclodextrin	19,20
UCA	Imidazole-modified chitosan	21–23
Im-PEI	Imidazole-modified bPEI	24
$PLH-NH_2$, $PVIm-NH_2$, PVIm-Bu	Aminated poly(L-histidine), aminated poly(1-vinylimidazole), alkylated poly(1-vinylimidazole)	25–28
cRGD-hk	cRGD-$[(H)KKKK]_6$	29
HoKC	K[K(H)KKK]$_5$-K(H)KKC	30
LAH4; LAH4-L1	$K_2AL_2ALALH_2LAHLALHLALALK_2A$; $K_2AL_2AHALHL_2AL_2ALHLAHALK_2A$	31,33
Tat-10H & C-5H-Tat-5H-C	Tat peptide covalently fused with 10 histidine residues	34
$O_{10}H_6$	Ornithine & histidine peptides	35
MS($O_{10}H_6$)	$K_{10}H_6$-coated microspheres	36,37
(KHKHKHKHKK)$_6$-FGF2	recombinant polymer/protein fusion	38,39

(1) Midoux and Monsigny (1999); (2) Fajac *et al.* (2000); (3) Bello Roufai and Midoux (2001); (4) Pichon *et al.* (2001); (5) Pichon *et al.* (2000); (6) Benns *et al.* (2000); (7) Putnam *et al.* (2001); (8) Putnam *et al.* (2003); (9) Okuda *et al.* (2004); (10) Yang *et al.* (2008); (11) Chen *et al.* (2000); (12) Chen *et al.* (2001); (13) Chen *et al.* (2002); (14) Leng and Mixson (2005); (15) Leng *et al.* (2005); (16) Leng *et al.* (2008); (17) Read *et al.* (2005); (18) Stevenson *et al.* (2008); (19) Mishra *et al.* (2006); (20) Bartlett and Davis (2007); (21) Kim *et al.* (2003); (22) Jin *et al.* (2006); (23) Jin *et al.* (2008); (24) Swami *et al.* (2007); (25) Asayama *et al.* (2004); (26) Asayama *et al.* (2007a); (27) Asayama *et al.* (2007b); (28) Hakamatani *et al.*, (2008); (29) Aoki *et al.* (2001); (30) Yu *et al.* (2004); (31) Kichler *et al.* (2003); (32) Mason *et al.* (2006); (33) Mason *et al.* (2007); (34) Lo and Wang (2008); (35) Chamarthy *et al.* (2003); (36) Kovacs *et al.* (2005); (37) Jia *et al.* (2006); (38) Hatefi *et al.* (2006); (39) Canine *et al.* (2008)
bPEI, branched polyethyleneimine; HpK, histidylated polylysine; PDHP, Poly(DMAEA/His(Boc)-OMe)phosphazene; RPCs, reducible polycations.

responsible to accumulation of protons inside the endosome lumen. In the presence of bafilomycin A1, the lumen is neutral and thus the protonation of imidazole ring of HpK cannot occur preventing its membrane destabilization properties. While the endosomal escape mechanism of DNA mediated by HpK is not yet known and is supposed to occur via a proton sponge effect like chloroquine and PEI, membrane fusion and permeation induced by HpK can be also involved. Indeed, membrane fusion and permeation in acidic medium have been demonstrated for poly(L-histidine) (Wang and Huang, 1984) and the histidine-rich peptide H5WYG (Midoux *et al.*, 1998). The delivering capacities of HpK differ depending on the nucleic acids type. Indeed, HpK was not at all able to transfect the 25-mer ODN. In contrast, HoK, a poly(L-lysine) of 19 degrees of polymerization was an efficient carrier for ODN delivery into the cytosol after highly substitution (≥80%) with histidine residues (Pichon *et al.*, 2000). Conversely, HoK was not efficient for DNA transfection. Since HpK and HoK publications, several histidine-rich polymers and peptides have been reported to be efficient carriers for DNA and SiRNA delivery. They are listed in Table 2.

Histidine-rich polymers

First, 25% of the ε-amino group of a 8 kDa poly(L-lysine) has been coupled with a 18-mer poly(L-histidine) (Benns *et al.*, 2000). The transfection of 293T cells with the resulting PLH-g-PLL polymer complexed with pDNA encoding the β-galactosidase gene was threefold to fourfold higher than with polylysine. The PLH moiety had a buffering capacity at pH 6.5–5.0. Another polylysine derivatives (Im-pK) has been prepared by coupling several 4-imidazoleacetic acid residues to the ε-amino groups of a 3.43 kDa poly(L-lysine) (Putnam *et al.*, 2001; 2003). When complexed with pDNA encoding the luciferase gene, the polymer with an imidazole content of 86.5 mol% gave a high level of transfection of human hepatocarcinoma (HepG2), mouse macrophage (P388D1) and smooth muscle (CRL1476) cell lines. The luciferase activities were close to that obtained with branched polyethyleneimine (bPEI) of 25 kDa but with a lower cytotoxicity.

Histidine-rich dendrimers have been also synthesized. The terminal cationic groups of a dendritic poly(L-lysine) of the fifth generation (KG5) were substituted with histidine residues (Okuda *et al.*, 2004). The resulting KGH6 dendrimer in which all the terminal amino-acids were histidines showed a buffering effect between pH 4 and pH 6. KGH6 polyplexes exhibited higher level of transfection in COS-1, HeLa and CHO cell lines than polyplexes made with the lysine counterpart dendrimer. Note that transfections were efficient only when polyplexes were formed at pH 5.0.

Poly(DMAEA/His(Boc)-OMe)phosphazene (PDHP) has been synthesized by coupling several His(Boc)-OMe and 2-dimethylaminoethylamine (DMAEA) residues on the poly(dichlorophosphazene) polymer (Yang *et al.*, 2008). The resulting histidylated polymer of 17.1 kDa contained a His(Boc)-OMe : DMAEA molar ratio of ~0.4:l.6. PDHP polyplexes with a size around 110 nm at the PDHP/DNA ratio of 10:1 (w/w) gave a higher transfection efficiency of 293T cells than polyplexes made with the histidine-free polymer and bPEI. Moreover, the cytotoxicity of PDHP polyplexes was low.

Another kind of polymer are the linear and branched copolymers of histidine and lysine (so-called HK, HHK, HHHK copolymers) (Chen *et al.*, 2000; 2001; 2002; Leng and Mixson,

2005; 2008). They enhanced the transfection efficiency of liposomes in the presence of serum in several breast cancer cell lines and a non-small cell lung cancer cell line. From these series, HHK4b, $[KHKH_2KH_2KH_2KH_2KH_2KHK]_4$-KKK, a 83-mer branched copolymer of ~10 kDa, was the most effective (Chen *et al*., 2002). Although HHK4b was an effective carrier for pDNA, it was not efficient for SiRNA *in vitro*. In contrast, highly branched HK peptides were effective carriers of SiRNA and not particularly effective for pDNA. So as above-mentioned for HpK and HoK, the delivering capacities of a given polymer can differ depending on the nucleic acids type. From the highly branched HK peptides series, HHHK8b appeared as a very efficient vector for *in vitro* and *in vivo* SiRNA delivery (Leng *et al*., 2005). This polymer consists of a 163 mer of ~23 kDa made of a $[KH_4NH_4]_4$-KKK core branched copolymer with eight terminal $H_3KH_3KH_3KH_3$ branches. HHHK8b complexed with Raf-1 SiRNA has decreased Raf-1 mRNA and induced apoptosis in cell lines *in vitro*. The systemic injection of HHHK8b/Raf-1 SiRNA polyplexes has allowed inhibition of the growth of MDA-MB-435 xenografts (Leng *et al*., 2008). This remarkable effect was accompanied by a reduced toxicity in normal tissues.

The oxidative polymerization of a 10-mer oligolysine ($CK_{10}C$) via a disulfide bridge forms linear reducible polycations (RPCs) exhibiting a DNA unpackaging capacity (Read *et al*., 2003). The disulfide bridges can be cleaved in a reducible medium like the cytosol leading to the release of pDNA. Indeed, RPCs can condense pDNA; a 10-mer oligolysine cannot. In a second generation, reducible polymers containing histidines (His6 RPCs) were prepared by oxidative polymerization of the $CH_6K_3H_6C$ peptide via a disulfide bridge. His6 RPCs exhibited both DNA condensation via the lysine residues, pH-buffering capacity due to the presence of histidine residues and unpackaging of DNA in a reducible medium (Read *et al*., 2005). As above-mentioned, the delivering capacities of those polymers also differ depending on the nucleic acids type. A His6 RPC of 162 kDa was most effective for pDNA and mRNA transfection whereas a His6 RPC of 80 kDa was for SiRNA (Stevenson *et al*., 2008).

Cyclodextrins are natural biocompatible cyclic oligosaccharides composed of 6, 7 or 8 D(+)-glucose units linked by α-1,4-linkages and named α-, β-, or γ-cyclodextrin respectively. Davis *et al*. have developed a β-cyclodextrin-containing polycation (CDP) made by copolymerization of 6A,6D-dideoxy-6A,6D-di(2-aminoethanethio)-β-cyclodextrin (Gonzalez *et al*., 1999; Hwang *et al*., 2001). An imidazole-containing variant (CDPim) has been synthesized in order to contain pH-buffering elements. CDPim polyplexes were almost 10 times more effective than CDP polyplexes. This type of polymer has been continuously improved and used for successful pDNA and SiRNA transfections as well as for tumour targeting and imaging (Bartlett *et al*., 2007; Heidel *et al*., 2007).

Chitosan is a widely used cationic polysaccharide to develop gene delivery systems. This is a biodegradable, biocompatible and non-toxic cationic polymer obtained from deacetylation of chitin. Chitosan has been also modified with imidazole rings to improve its endosomal escape capacity (Kim *et al*., 2003). The substitution of chitosan amine functions was carried out with urocanic acid comprising an imidazole ring. The transfection efficiency of HEK293 cells was the highest with UAC70 corresponding to imidazole substitution degree of ~37 mol% of the chitosan amine functions. Compared with the high cytotoxicity induced by PEI of 25 kDa (only 20–40% cell viability), transfection with UAC70 has kept the viability of HEK293 and A549 cells around 90%. Aerosol delivery of UAC70 complexed with pDNA encoding GFP gave efficient transfection into lung cells of mice (Jin *et al*., 2006). UAC70 was used to deliver the PDCD4 tumour suppressor gene (i.e. the programmed cell death protein 4). Remarkably, PDCD4/UAC70 polyplexes have facilitated apoptosis with consequence on cell proliferation and tumour angiogenesis in lung of K-*ras* null mice. Recently, repeated aerosol deliveries of UAC70 complexed with PTEN (phosphatase and tensin homologue deleted on chromosome 10 complex), another tumour suppressor gene was applied in K-*ras* null mice to suppress cell growth and increase survival via Akt-signalling pathway (Jin *et al*., 2008). The treatment led to significant suppression (~50%) of pulmonary tumorigenesis in the lung. Thus UAC70 appears as an efficient carrier for gene delivering in lung via aerosol with fewer side effects than repeated exposure of PEI.

To increase the buffering effect of PEIs, imidazolylated bPEI of 25 kDa and 750 kDa have been synthesized (Swami *et al*., 2007). In COS-1 and HEK293 cells, the transfection efficiency was three times higher than bPEI when 39 mol% of primary and secondary amines of bPEI were substituted with imidazolyl residues. The buffering capacity of this polymer (IP) was greater than bPEI but was reduced when 5 mol% of IP was further coupled with PEG8000 (IPP). Again, the imidazolyl substitution of bPEI decreased the cytotoxicity. The cell viability which was 37% with bPEI polyplexes, reached 78% with IPP polyplexes.

In the above-mentioned histidylated polymers, cationic polymers have been substituted with histidyl or imidazolyl groups. Conversely, aminated poly(L-histidine) ($PLH-NH_2$; 8 kDa) and aminated poly(1-vinylimidazole) ($PVIm-NH_2$; 8.1 kDa) have been synthesized (Asayama *et al*., 2004; 2007a,b). These polymers induced membrane fusion and permeation at pH 6.0. They formed complexes with pDNA, but their low content in cationic charges did not allow transfection. The stability of polyplexes was improved by partial alkylation of PVIm with 1-bromobutane (PVIm-Bu), which generates quaternary imidazole groups (Hakamatani *et al*., 2008). Consequently, the transfection efficiency of HepG2 cells with DNA/PVIm-Bu polyplexes was two orders of magnitude higher than with DNA/$PVIm-NH_2$ polyplexes and even reached gene expression levels close to bPEI polyplexes.

Histidine-rich peptides

Peptides have the advantages to be more homogenous than polymers, but their DNA condensation capacity is lower. So peptides rich in lysine and histidine have been designed. cRGD-$[(H)KKKK]_6$ (cRGD-hk) comprised a DNA-binding domain made of oligolysine, histidyl residues for cytosolic delivery and a tumour-homing signal made of a cyclo RGD peptide (Aoki *et al*., 2001). cRGD-hk polyplexes with the luciferase gene showed efficient transfections into hepatocar-

cinoma (HepG2 and PLC) and pancreatic cancer (Hs700T and MIAPaCa-2) cell lines. The transfections were inhibited in the presence of bafilomycin A1 providing evidence for a cytosolic gene delivery mediated by histidine protonation. *In vivo* potency of cRGD-hk polyplexes as a tumour-targeting vector has been reported in nude mice bearing PLC or MIAPaCa-2 tumours upon systemic administration. A similar peptide, $K[K(H)KKK]_5$-K(H)KKC (HoKC), was found to enhance the transfection efficiency in tumour of DNA/immuno-liposome complexes (Yu *et al.*, 2004).

Histidine-rich amphiphatic peptide antibiotics have been described to exhibit DNA transfer capacities into mammalian cells (Kichler *et al.*, 2003; Mason *et al.*, 2006; 2007). LAH4 is an amphiphatic peptide rich in alanine and leucine. It bears lysines at its ends to condense DNA and histidines inside the sequence to favour endosomal escape. LAH4/DNA polyplexes were able to transfect cells in the absence of serum. From a series of LAH4 derivatives, high transfection efficiencies were obtained only with peptides containing four to five histidine residues in the central region of the peptide sequence. The transfection activity did not depend on the number of histidine but did depend on their position inside the peptide sequence. Structural investigations indicated that the position of the histidine residues within the polar face of the peptide is important. The LAH4-L1 derivative with a different location of two histidine residues in the sequence showed enhanced capacity in membrane disruption because the angle subtended by the histidine residues is 80 degrees. This peptide was also more efficient for transfection in the presence of serum.

The RKKRRQRRRR peptide (Tat) is a cell-penetrating peptide known to enhance the cellular uptake of a large variety of molecules including nucleic acids (Brooks *et al.*, 2005). However, its use for DNA delivery is limited by its inability to deliver DNA in the cytosol and the instability of peptide/DNA complexes due to the low number of cationic charges. So, the C terminus of Tat has been elongated with 5 (Tat-5H), 10 (Tat-10H) and 20 (Tat-20H) histidine residues (Lo and Wang, 2008). The highest levels of gene expression (up to 7000-fold improvement over the controls) were observed with Tat-10H and were comparable to that obtained for bPEI of 25 kDa. The contribution of histidine residues to this improvement was evidenced by a significant transfection inhibition in the presence of bafilomycin A1. Another histidine-rich Tat peptide (C-5H-Tat-5H-C) has been designed in which five histidine residues were put at the C and the N terminus of the Tat peptide. Cysteine residues at the ends allow the formation of inter-peptide disulfide bonds by air oxidation upon pDNA condensation with the peptide. As previously described, the stability of polyplexes is enhanced in serum (McKenzie *et al.*, 2000). Again, the histidine-rich peptide was less toxic than bPEI of 25 kDa. Indeed, C-5H-Tat-5H-C peptide did not displayed any significant effects on cell viability *in vitro* at N/P ratio up to 20, whereas bPEI caused 60% of cell death at the same N/P ratio. C-5H-Tat-5H-C polyplexes were as efficient as bPEI ones to transfect cells of the nervous system *in vivo* (Lo and Wang, 2008).

The $O_{10}H_6$ peptide containing two linear clusters of ornithine and histidine residues has been synthesized (Chamarthy *et al.*, 2003). Using a plasmid encoding β-galactosidase, $O_{10}H_6$/DNA polyplexes showed less cytotoxicity than K_{16} and $K_{10}H_6$ polyplexes, and a superior transfection efficiency of JAWSII cells, a murine dendritic cell line and murine bone marrow-derived DC *in vitro*. Furthermore, the transfected dendritic cells were capable of triggering antigen-specific T cell response when mice were immunized with gene-transfected JAWSII cells. In order to increase the stability of DNA/$O_{10}H_6$ and ODN/$O_{10}H_6$ complexes in the biological fluid, anionic microspheres (MS) of 100 nm were coated with $O_{10}H_6$ and then nucleic acids were adsorbed on their surface. MS($O_{10}H_6$)-ODN particles were resistant to digestion/disruption by serum and heparin and promoted accumulation of ODN in splenic $CD11c^+$ DC (Kovacs *et al.*, 2005). MS($O_{10}H_6$) particles were used also to transfect murine dendritic cells with pDNA encoding murine IL-10 in an anti-inflammatory approach (Jia *et al.*, 2006).

Lastly, genetic engineering techniques have been used to prepare a recombinant polymer/protein fusion comprising the fibroblast growth factor 2 (FGF-2) protein fused with a tandem repeating units of six HK peptides (KHKHKHKHKK) (Hatefi *et al.*, 2006). FGF-2 acted as a ligand of FGF-2 receptors present on tumour cells, the lysine residues as DNA binding and histidine residues as endosome escape elements. $(KHKHKHKHKK)_6$-FGF-2 polyplexes mediated transgene expression in several cell lines expressing the FGF receptors. In this vector, the lysine residues were dispersed. Another recombinant protein $(KKKHHHHKKK)_6$-FGF-2 was recently prepared in which the lysine residues were set in clusters mimicking histones (Canine *et al.*, 2008). The comparison of these two constructs in terms of DNA condensation and gene transfer efficiency showed that the construct with KH units in clusters was able to condense pDNA into more stable particles with size of 150 nm and resulted in five times higher transfection efficiency.

Lipids with imidazole moiety

Glycerol-, non-glycerol-, lipophosphoramidate- and cholesterol-based cationic amphiphiles with a lysine, arginine or histidine residue as polar head have been reported and evaluated for cell transfection (Montier *et al.*, 2008). Those containing an imidazole moiety are listed in Table 3. The first example concerns pH-sensitive cationic liposomes. The imidazole moiety was coupled to either cholesterol (cholesterol-(3-imidazol-1-yl-propyl) carbamate, ChIm; 1), 1,2-di-oleoyl deoxyglycerol (rac-2,3-Di[11-(F-butyl)undecanoyl]glycero-1-phosphoethanolamine, DOIm; 2) or 1,2-di-palmitoyl deoxyglycerol (4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1H-imidazole, DPIm; 3) (Budker *et al.*, 1996). ChIm/DOPE, DOIm/DOPE and DPIm/DOPE liposomes that did not exhibit permanent cationic charges at physiological pH were able to bind to DNA and to encapsulate it after imidazole protonation at acidic pH. Compared with N-[1-(2, 3-di-oleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA)/ DOPE lipoplexes, DPIm/DOPE lipoplexes were found to give the highest transfection efficiencies in several cell lines. Additionally, the transfection inhibition (44% and 60% in NHI3T3 and HepG2 cells respectively) observed in the presence of bafilomycin A1 suggests that acidic changes in liposome

Table 3 Lipids with an imidazole moiety

Chemical structure	*Reference*	*Chemical structure*	*Reference*
1		R^1 = C17 :1 **2** R^1 = C15 :0 **3**	1
R^1 = C12:0 ; R^2 = C12:0 **4** R^1 = C18:0 ; R^2 = C12:0 **5**	2	R^1 = C16:0 ; R^2 = H **6** R^1 = C16:0 , R^2 = CH_3 **7**	3
G = H **8** G = Ts **9**	4	**10**	5
R^1 = C16:0 ; R^2 = NH_3, Cl ; *n*=1 **11** R^1 = C16:0 ; R^2 = NH-His ; *n*=1 **12** R^1 = C16:0 ; R^2 = NH-His ; *n*=2 **13**	6	R^1 = C18:1Δ^9 ; R^2 = CO_2CH_3 **14** R^1 = C18:1Δ^9 ; R^2 = H **15**	7
R = C14:0 **16** R = C16:0 **17** R = C18:0 **18**	8	R = $C_{11}H_{23}$ **19** R = $C_{15}H_{31}$ **20**	9
21			10

(1) Budker *et al.* (1996); (2) Heyes *et al.* (2002); (3) Kumar *et al.* (2003); (4) Singh *et al.* (2004); (5) Denoyenle *et al.* (2006); (6) Karmali *et al.* (2006); (7) Mevel *et al.* (2008a); (8) Obata *et al.* (2008); (9) Tarwadi *et al.* (2008); (10) Shigeta *et al.* (2007).

properties may also contribute to the endosomal release of DNA. As illustrated by lipids 4 and 5, histidine was linked via an amide bond to the amine function of 2,3-dialkyloxypropaneamine (Heyes *et al.*, 2002). These cationic lipids, which possess one primary amine function, are positively charged at physiological pH. Glutamic acid has been used as a scaffold to produce lipids 16, 17 or 18 containing an amino-acid residue (Obata *et al.*, 2008). These histidylated lipids were obtained upon the linkage of two lipid chains and one histidine onto glutamic acid. It has been observed that these histidylated derivatives exhibited a lower capacity to bind DNA than derivatives containing lysine or arginine. The weakness of the interaction with DNA was probably due to the lower pKa of α-amino group of the histidine residue compared with that of the amino groups of the side chains of lysine and arginine. Hence, the transfection efficiencies were lower than those observed for derivatives with a lysine or arginine group. Cholesterol has been also used to design histidylated amphiphiles (Singh *et al.*, 2004). Histidine was linked to the cholesterol unit via an ethylene bridge, and the imidazole heterocycle was either non-substituted (8) or N-substituted with a tosyl group (9). 8/DOPE lipoplexes exhibited higher transfection activity than lipoplexes made with the tosylated analogue 9. Moreover, the transfection efficiency dropped when it was performed in the presence of bafilomycin A1, providing evidence for a cytosolic gene delivery mediated by histidine protonation. To introduce a cell-targeting element, histidylated cholesterol was linked to galactose (21) and 21/DOTAP lipoplexes showed much higher transfection activity in HepG2 cells expressing galactose receptors than its analogues that did not possess the histidine group (Shigeta *et al.*, 2007).

To further increase the cationic charge of the polar head and thereby the ability of histidylated or imidazolylated lipids to bind and to compact DNA, a second permanent cationic function (at physiological pH) has been incorporated. For example, cationic lipids 6 and 7 characterized by two lipid chains (C16:0) attached on the same nitrogen atom possess two permanent cationic charges at physiological pH (primary amine and ammonium) and a pH-dependent one (imidazole heterocycle) (Kumar *et al.*, 2003). The transfection efficiencies were cell type-dependent. 6/chol (cholesterol) lipoplexes were more efficient for the transfection of HepG2 and HeLa cells, while 7/chol lipoplexes were more efficient in 293T7 cells. Interestingly, Fluorescence Resonance Energy Transfer experiments indicated that the fusogenic character of 6/chol liposomes increased when the pH dropped from 7.4 to 6 in agreement with the imidazole ring protonation. This observation attested that the imidazole heterocycle has suitable physico-chemical properties that could be helpful in assisting pDNA release from the endosome to the cytosol. We have reported that histidylated lipopolyplexes combining these 6/chol liposomes with PEGylated HpK and a melanoma antigen mRNA were efficient carriers for mRNA-based anti-melanoma vaccination in mice (Mockey *et al.*, 2007). MART1 mRNA/PEG-HpK polyplexes that form small dense and spherical aggregates of ~50 nm with a ζ-potential of +7 mV were encapsulated into 60–100 nm 6/chol liposomes with a ζ-potential of +31 mV. The systemic injections of MART1 mRNA lipopolyplexes have delayed significantly the growth of B16 melanoma. The immune response was characterized by a CTL-mediated anti-B16 melanoma response. Note that only MART1 mRNA formulated as lipopolyplexes induced an immune response. MART1 mRNA alone, MART1 mRNA polyplexes and MART1 mRNA lipoplexes did not induce any protection against B16 melanoma growth.

The incorporation of lipid 6 into the membrane of hepatocyte-specific Sendai virosomes containing only the fusion protein has been found to enhance by a factor 4 the membrane fusion activity of the virosomes with the target cell membrane (Verma *et al.*, 2005). The incorporation of several histidine groups into the cationic lipid polar head has been done also with the expectation that acidic pH-mediated membrane fusion activity will be higher than with monohistidylated lipids. The cationic lipids 11, 12 and 13 comprised one, two or three histidine functionalities in the head group region of the amphiphiles respectively (Karmali *et al.*, 2006). Despite the fact that the effective interaction between these cationic lipids and pDNA required high charge ratio, 11/chol and 13/chol lipoplexes were efficient to transfect cells *in vitro* even in the presence of high amount of serum (up to 50%). However, no linear correlation was evidenced between the transfection efficacies and the number of histidine functionalities in the polar head group regions for these histidylated cationic amphiphiles.

Additionally, the synthesis of novel lipopeptides possessing only one alkyl chain and up to six amino-acid functionalities and their transfection evaluation have been reported recently (Tarwadi *et al.*, 2008). The polar head was characterized by one cysteine, one to four histidine and one to three lysine residues as illustrated by the lipopeptides 19 and 20 containing three histidine and three lysine moieties. It was expected that lysine residues will stabilize lipoplexes, cysteine residues will form small lipoplexes after dimerization of lipopeptides, and histidine residues will facilitate the endosomal escape. The lipoplex stability was indeed enhanced by the increase of the number of lysine residues, and histidine functionalities were needed to observe high transfection efficiencies in COS-7 cells presumably by inducing the endosomal escape of pDNA.

In the above-mentioned histidylated lipids, the imidazole ring was included into the polar head of amphiphiles that also contained a permanent cationic charge at physiological pH. In contrast, the following neutral lipids bear a single imidazole polar head without any cationic group. They were designed as pH-sensitive fusogenic helper or co-lipids and used in combination with a cationic lipid. Lipids 14 and 15 have been recently developed with a lipophosphoramidate structure and a polar head that incorporated a α-carboxymethyl-histidine or a histamine moiety respectively (Mevel *et al.*, 2008a). These new helper lipids have improved the transfection by a factor of up to 100 compared with lipoplexes made with DOPE. Once again the fusogenic character of lipid 15 included in these formulations increased when the pH dropped from 7.4 to 6.0. These observations lead us to formulate the hypothesis that the unprotonated imidazole, which has one donor and one acceptor sites for hydrogen bonding, could be involved in a hydrogen bond network. This supramolecular network could stabilize the liposome or lipoplexes. After imidazole protonation, this heterocycle has

two donor sites for hydrogen bonding that could induce disruption of the supramolecular network and increase the fusogenic character of the formulation.

Lastly, bolaamphiphiles with a lysine or a histidine residue have been synthesized (Denoyenle *et al.*, 2006). These bolaamphiphiles are dissymmetric with one neutral polar head (a glycoside unit) and a cationic part possessing either a lysine or histidine residue as illustrated by the lipid 10. Unfortunately, the bolaamphiphiles with the histidine residue showed low capacity to bind DNA.

Cationic lipids with imidazolinium or imidazolium polar head

All the lipids reported in Table 3 possess one or several imidazole groups that have the ability to acquire a cationic charge when the pH of the environment drops bellow 6. For lipids listed in Table 4, the chemical modifications of imidazole-type heterocycles have been achieved to design compounds exhibiting a permanent cationic charge. Indeed, the presence of an alkyl or aryl chain on each nitrogen atom of the heterocycle produces either an imidazolinium or imidazolium cation. These two heterocycles differ by the presence of one or two unsaturations within the ring (Figure 2). It is worth noting that in both cases the cationic charge is distributed between the two nitrogen atoms due to mesomeric structures.

To the best of our knowledge, DOTIM 22 and its analogues 1-[2-(Hexadecanoyloxy)ethyl]-2-pentadecyl-3-(2-hydroxyethyl)imidazolinium chloride (DPTIM) 23 and 1-[2-(Tetradecanoyloxy)ethyl]-2-tridecyl-3-(2-hydroxyethyl) imidazolinium chloride (DMTIM) 24 were the first cationic lipids comprising an imidazolinium polar group, which have been evaluated for DNA compaction and gene delivery (Solodin *et al.*, 1995). Liposomes made with a mixture of 22 and cholesterol gave the best DNA formulation tested by systemic injection (Mounkes *et al.*, 1997). The biodistribution of 22/chol lipoplexes was examined by fluorescence and electron microscopy from 5 min to 24 h after systemic administration in CD-1 mice (McLean *et al.*, 1997). After 4 h, lipoplexes were found into endosomes and lysosomes in different cell types including endothelial cells. The uptake by capillary endothelial cells was great in lung, ovary and anterior pituitary. Another study has shown also that most of lipoplexes were initially deposited in the lungs after intravenous injection in ICR mice (Niven *et al.*, 1998).

imidazolinium imidazolium

Figure 2 Chemical structure of imidazolinium and imidazolium.

Table 4 Cationic lipids with an imidazolium moiety

Chemical structure			*Reference*
	R = C12:0 R = C14:0 R = C18:1Δ^9	22 23 24	1
	R = C18:1Δ^9, *n* = 2; X = Br R = C18:1Δ^9, *n* = 3; X = I	25 26	2
	R = C18:1Δ^9, Z = P R = C18:1Δ^9, Z = As	27 28	3
	R = C12:0	29	4

(1) Solodin *et al.* (1995); (2) Mevel *et al.* (2008b); (3) Mevel *et al.* (2007); (4) Huang *et al.* (2008).

Recently, we have used the N-methyl-imidazolium moiety to design the cationic polar head of the lipophosphoramides 25 and 26. Lipid 25 proved to be very efficient for *in vitro* transfection of HEK293-T7 cells when it was formulated with DOPE (Mevel *et al.*, 2008b). Moreover, liposomes made in a 1:1 molar ratio with lipid 25 and the co-lipid 15 (i.e. lipophosphoramidate with a histamine moiety) form negative lipoplexes that have showed very efficient transfection in Achille tendon upon local administration, compared with DOTAP lipoplexes and PEI polyplexes (P. Midoux, C. Pichon, J.-J. Yaouanc, M. Mevel, J.-J. Clement and P.-A. Jaffrès, unpubl. data).

The imidazolium cationic group was used to produce lipophosphoramides 27 and 28. These dicationic lipids have been efficient for *in vitro* transfection experiments, but they were less efficient than other dicationic lipophosphoramides without the imidazolium cationic group (Mevel *et al.*, 2007). Finally, the association of imidazolium group and cyclen has recently been explored to design the polar head group of the cationic amphiphile 29 characterized by the presence of only one short lipid chain (Huang *et al.*, 2008).

The imidazolium group still offers numerous interesting perspectives to design molecules with the capacity to bind and to compact DNA. Biomaterials such as silica nanoparticles and carbone nanotubes have been recently introduced to design novel non-viral gene delivery systems. As an illustration, one recent study has reported the immobilization of imidazolium group on the surface of magnetically active iron oxide to bind DNA thus offering potential application for gene delivery (Naka *et al.*, 2008).

Conclusion

This review presents a complete list of polymers, peptides and lipids containing histidine or imidazole that have been designed as nucleic acids carriers. On one hand, polymers, peptides and lipids containing histidine or imidazole residues have been developed to induce membrane destabilization in acidic media, that is, at a pH close to that of lumen of the endocytic vesicles (endosomes) in order to favour nucleic acids delivery into the cytosol. Although the supposed mechanisms (i.e. membrane permeation, membrane fusion or proton sponge effect) have not been yet identified, most of these carriers are ineffective in the presence of bafilomycin A1 that inhibits the endosome acidification. On the other hand, cationic lipids containing an imidazole head group with a permanent positive charge (either an imidazolinium or imidazolium cation) have also given some promising efficiencies. The transfection efficiencies are often close to those obtained with the gold standard transfection reagents. The number of these new carriers is now great. Their transfection efficiency *in vitro* depends on the cell types. Although some of them have proved their efficiency *in vivo*, it is too early to point out the most appropriate structures. Only comparative studies conducted with some of them will determine which will be the best carriers for a given application. Overall, the cytotoxicity of these histidylated or imidazolylated carriers especially polymers was often found much less high *in vitro* and *in vivo*, suggesting that protonated imidazole rings are less cytotoxic than protonated amine groups. The transfection levels are still below than that of viruses, which exhibit a powerful capacity to escape from endosome, while polyplexes and lipoplexes are largely sequestrated in endosomes/lysosomes vesicles. The nucleic acids are the other part of these delivery systems. In contrast to RNA and ODN that have their targets in the cytosol or can pass easily through the nuclear pores, pDNA must be actively imported in the cell nucleus, for gene expression. For this purpose, a promising strategy could be the use of pDNA containing κB sites, which has recently been found to increase the number of plasmid copies in the nucleus and the transfection efficiency of polyplexes (Mesika *et al.*, 2001; 2005; Breuzard *et al.*, 2008). Indeed, the use of the peptide nuclear localization signal of the SV40 large T antigen did not give the expected benefits to help pDNA import into the nucleus (Wagstaff and Jans, 2007). Nevertheless, the endosome escape of pDNA remains a critical bottleneck. Histidylated polymers, peptides and lipids have been designed to favour this crucial step. Several lines of evidence like the inefficiency of transfections in the presence of bafilomycin A1 indicate that they destabilize endosomes. However, their capacity to favour endosome escape has not been yet determined at the cellular level. The quantification of this step that is difficult to set up and requires the development of specific intracellular probes will open new improvements of the use of the imidazole ring in this field.

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