Design of a new cell penetrating peptide for DNA, siRNA and mRNA delivery

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Abstract
Background: Delivery systems, including peptide-based ones, that destabilize endosomes in a pH-dependent manner are increasingly used to deliver cargoes of therapeutic interest, such as nucleic acids and proteins into mammalian cells.

Methods: The negatively charged amphipathic alpha-helical forming peptide named HELP (Helical Erythrocyte Lysing Peptide) is a derivative from the bee venom melittin and was shown to have a pH-dependent activity with the highest lytic activity at pH 5.0 at the same time as becoming inactive when the pH is increased. The present study aimed to determine whether replacement in the HELP peptide of the glutamic acid residues by histidines, for which the protonation state is sensitive to the pH changes that occur during endosomal acidification, can transform this fusogenic peptide into a carrier able to deliver different nucleic acids into mammalian cells.

Results: The resulting HELP-4H peptide displays high plasmid DNA, small interfering RNA and mRNA delivery capabilities. Importantly, in contrast to other cationic peptides, its transfection activity was only marginally affected by the presence of serum. Using circular dichroism, we found that acidic pH did not induce significant conformational changes for HELP-4H.

Conclusions: In summary, we were able to develop a new cationic histidine rich peptide able to efficiently deliver various nucleic acids into cells.

KEYWORDS
cationic peptide, cell penetrating peptide, DNA transfection, histidine residues, mRNA vectorization, siRNA delivery

1 INTRODUCTION

Amphipathic peptides are found in many species, including bacteria,1 insects, amphibians, humans and plants.2-4 These peptides show bactericidal, fungicidal, virucidal and even tumoricidal activities. Viral derived amphipathic peptidic motifs with biological activities such as pH-dependent membrane destabilizing effects have also been identified. These latter motifs have attracted great attention in the 1990s because escape of therapeutics from endocytic vesicles into the cytosol is often a limiting step. This is particularly the case for therapeutic nucleic acids, which remain trapped in vesicular compartments when delivered with non-viral vectors. In this latter context, peptides of the N-terminus of rhinovirus HRV2 VP-1 protein,5 but above all peptides derived from the amino-terminal sequence of hemagglutinin of the influenza virus (GLFGAIAGFIEGGWTGMIDGWYG), have been largely used for increasing the endosomal escape of nucleic acids complexed with polymers6-9 or lipids.10,11 These anionic peptides have a disordered conformation at pH 7, however when the pH decreases, the carboxyl groups of aspartic acid and glutamic acid become protonated and the disappearance of the anionic repulsion permits the adoption of an amphipathic α-helical conformation.9,12-14 This in turn allows to the peptides to interact with lipid bilayers to form pores or induce
membrane fusion/lysis. However, these delivery systems require a ternary complex composed of the DNA, a cationic vector for nucleic acid complexation and a negatively charged fusogenic peptide. An early strategy to be explored consisted of the development of multifunctional amphipathic peptides. Szoka’s group attempted to confer both membrane destabilizing capacities and DNA binding properties on the peptides. To achieve this, they started with the fusogenic GALA peptide of artificial sequence (WEAALAEALAEALAEALAEALAEALAEALAA) and replaced the negatively charged glutamic acid residues by cationic lysines. The resulting KALA peptide (WEAKLAKALAKALAKAKALAKACEA) was able to deliver plasmid DNA into cells without addition of another component. A few years later, a similar strategy was applied when the negatively charged amino acids of the anionic fusogenic peptide JTS-1 were replaced by arginines in the ppTG20 peptide. The sequence of this peptide derives from the bee venom melittin from Apis mellifera and some of the hydrophilic residues by glutamic acid. Four glutamic acids were put into the central region of the amphipathic peptide (Table 1) to promote helix destabilization by mutual charge repulsion at neutral pH. Moser found that HELP-induced leakage of haemoglobin from erythrocytes was pH-dependent, whereas, at pH 5.0, the lytic activity was high it became very low when the pH increased.

The present study aimed to determine whether it is possible to transform the HELP peptide into a nanocarrier for nucleic acids by replacing the glutamic acid residues with histidines. Our results show that the HELP-4H peptide possesses robust DNA, small interfering RNA (siRNA) and mRNA delivery capabilities.

### 2 | MATERIALS AND METHODS

#### 2.1 | Compounds

The list of the HELP derived peptides, their sequences and Mw are given in Table 1 (see also Supporting information, Table S1). The efficiency of the peptides was compared with commercial agents available from different suppliers: the cationic lipid 1.2-dioleoyl-3-trimethylammonium propane (DOTAP) (Sigma-Aldrich, Saint Quentin Fallavier, France), the branched polymer polyethyleneimine (PEI) of 25 kDa (B-PEI; Sigma-Aldrich) and the cationic peptides Vpr55–82 (Eurogentec, Seraing, Belgium), LAH4 and LAH4-L1 (Proteogenix, Schiltigheim, France). The lipid 1.2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and chloroquine were purchased from Sigma-Aldrich. The fusogenic peptide JTS-1 (GLFEALLELLELWELLLEA) was synthesized by Proteogenix (Schiltigheim France). All of the peptides were dissolved in sterile MilliQ water (MerckMillipore, Burlington, MA, USA) at 1 mg/ml. B-PEI was used as a 10 mM monomer aqueous stock solution and DOTAP was dissolved at 1.3 mM in water. All the peptides and transfection reagents were stored at ~20°C.

#### 2.2 | Plasmids and siRNAs

The plasmid pSMD2LucΔITR 7.6 kbp and the eGFP-C1 plasmid 4.7 kbp (Clontech-Takara Bio, Saint-Germain-en-Laye, France) were aliquoted

| Peptide | Sequence | pH 8 | pH 2 | ΔG_{\text{wOct}} - ΔG_{\text{wIF}} |
|---------|----------|------|------|-------------------------|
| HELP    | GLGTTLTLEFLLEELLEFlKRRKKQCONH2 | +1   | +5   | 0.265                   |
| HELP-4H | GLGTTLTLLHFLLLHFLKRRKKQCONH2  | +5   | +9   | 0.008                   |
| HELP-4H-D1 | GLGTTLATLAFHFLHFLFAKRKRCONH2 | +5   | +9   | 0.163                   |
| HELP-4H-D2 | RRLGFILLASLALHFALLLHILWKRACONH2 | +3   | +7   | 0.053                   |
| HELP-4H-D3 | GLFTALSLLHFLALHFLWLLKRRKKQCONH2 | +5   | +9   | 0.081                   |
| LAH4-L1 | KKLALALHALLAHLHLAHALKKA-CONH2 | +5   | +9   | 0.105                   |

Nominal charges are given at pH 8 and pH 2. Average hydrophobicity is given on the whole-residue hydrophobicity octanol-interface scale (ΔG_{\text{wOct}} - ΔG_{\text{wIF}}) based on the the free energy of transfer from water to palmitolyloleoylphosphatidylcholine and to n-octanol.
respectively at concentrations of 2 mg/ml and 900 ng/ml in sterile MilliQ water and stored at –20°C. These two plasmids encode respectively for the luciferase gene pGL2 and for the eGFP gene (green fluorescent protein). Both genes are under the control of the human cytomegalovirus immediate-early promoter. The siRNAs used in our experiments were: the siRNA pGL3 that targets the mRNA of the Firefly luciferase gene expressed in AS49-Luc cells (sense sequence: 5’-CUUA CGCUAGAGUCUUGCAdTdT-5’, antisense sequence: 3’- dTdTGAA UGCGACUCAUAGAAGCU-5’; from Eurogentec, Seraing, Belgium) and the control siRNA obtained from Dharmacon that does not match with any human mRNA. Its sequence was not disclosed by the supplier.

2.3 | Circular dichroism (CD)

Peptides were dissolved in 5 mM Tris amine at a final concentration of between 30 and 50 μM. The pH of the samples was titrated down by adding 0.3% or 1% (v/v) HClO4 solution in microlitre amounts. ClO4− has greater optical transparency than HCl (which has substantial absorbance below 200 nm); hence, the replacement of HCl by HClO4 makes a significant improvement on spectral quality in the far-UV region. CD spectra were acquired on a Chirascan™ Spectrometer (Applied Photophysics, Leatherhead, UK) with samples maintained at 310 K. Spectra were recorded from 260 to 180 nm using a 0.5-mm path length and were processed using Chirascan software where a spectrum of the peptide free solution was subtracted and Savitzky–Gorlay smoothing with a convolution width of 5 points applied.

2.4 | Cell culture

Several human cell lines have been used during the project: alveolar basal epithelial cells from a human adenocarcinoma transfected to stably express (AS49-Luc), or not (AS49), the pGL3 luciferase gene; human foetal lung fibroblasts transformed with SV40 (MRC5-V2); human embryonic kidney cells (HEK293) and the human colorectal carcinoma cell line HCT116. The cell lines were cultured at 37°C in a humid atmosphere (5% CO2) in either Dulbecco’s modified Eagle medium (DMEM) or RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum. AS49-Luc cells were cultured in the presence of 0.8 mg/ml of Geneticin (G418; Promega, Charbonnières-les-Bains, France).

2.5 | Preparation of the complexes

The peptide/DNA complexes were prepared by mixing equal volumes of DNA and peptide in 150 mM NaCl. For example, 2.25 μg of DNA in 50 μl of 150 mM NaCl were mixed with increasing amounts of peptide in 50 μl of 150 mM NaCl. After vortex mixing for 20 s, the mixture was kept for 20 min at room temperature to allow the formation of the complexes. Culture medium was then added to obtain a final volume of 750 μl; next, 250 μl was added to each well of the triplicate. The peptide/siRNA complexes were prepared by following the same protocol as that for DNA, with the exception that the amounts of nucleic acid and the volumes of preparation of the complexes were smaller; for a triplicate, 500 ng of siRNA/15 μl of 150 mM NaCl were mixed with increasing amounts of peptide/15 μl of 150 mM NaCl. The final concentration of siRNA was always maintained at 50 nM/well.

2.6 | Size analysis by dynamic light scattering

For this, 8 μg of HELP-4H and 1.5 μg DNA (w/w ratio of 5.3/1) were each diluted in 25 μl of 150 mM NaCl. After the solutions were mixed and left for 20 min at room temperature, 50 μl of NaCl was added and particle size was measured using a Zetasizer nano ZS (Malvern Instruments, Malvern, UK). The same protocol was used for measuring the size of the complexes prepared in water. The sample for Zeta potential measurement was prepared by adding 900 μl of water to the 100 μl of complexes generated in 150 mM NaCl.

2.7 | Transfection of plasmid DNA and siRNAs

The transfection assays were carried out using 48-well plates. These experiments were performed in biological duplicates or triplicates (the same test was performed on two or three biological samples; i.e., we used two or three distinct wells per condition in the cell culture plates). Key experiments were performed at least twice. The cells were seeded into the plates 1 or 2 days before transfection, aiming to achieve a confluence of 60–80% for DNA transfections and 30–40% for siRNA transfections. For transfections realized in the absence of serum, a serum-free culture medium was mixed with the complexes to obtain a final volume of 250 μl per well. After removing the culture medium, the complexes were deposited into the wells and the plates were placed into the incubator. After 2.5 h of incubation, the transfection medium was replaced by fresh medium containing 10% serum. Then, the cells were incubated at 37°C until the end of the experiment. For the experiments conducted in the presence of percentages of serum higher than 10%, pure serum was directly added into each well using a micropipette to obtain the desired percentage. After 3 h of incubation at 37°C, the medium was changed with fresh medium supplemented with 10% serum. The transfection experiments involving chloroquine at a final concentration of 100 μM were performed as described above except that the drug was added after dilution of the complexes with RPMI, just prior to the addition of the transfection medium to the cells. The fusogenic peptide JTS-1 (1 mg/ml solution) was added to preformed peptide (or polymer)/DNA complexes. After an incubation time of 10 min, the transfection volume was adjusted to the desired volume with culture medium. For the complexes with DOPE, the procedure used DOPE, diluted in ethanol containing traces of chloroform (10 μl per ml of ethanol), which was added to the cationic vector prior addition of the DNA solution.
2.8 | mRNA transfection

The mRNA expressing luciferase (CleanCap® FLuc mRNA) was from Tebu-Bio (Le Perray-en-Yvelines, France). The transfection experiments were performed in 96-well plates. The HCT116 cells were seeded into the plates the day before transfection (24,000 cells/well) to achieve a confluency of 70%. To prepare the peptide/mRNA complexes, 0.6 μg of mRNA in 15 μl of 150 mM NaCl was mixed with increasing amounts of carrier diluted in 15 μl of 150 mM NaCl. The mixture was then kept for 15–20 min at room temperature to allow the formation of the complexes. Then 120 μl of serum-free culture medium was added to the complexes to achieve a final volume of 150 μl. After removing the culture medium, the complexes were added to the cells (75 μl per well) and the plates were incubated at 37°C. After 2.5 h, the transfection medium was replaced by medium supplemented with 10% serum. Then, the cells were incubated at 37°C until the end of the experiment (around 24 h). The luciferase quantification was conducted as described below.

2.9 | Determination of luciferase activity

The dosage of the luciferase was determined as described previously. Briefly, the transfected cells were first lysed by adding 100 μl of lysis buffer (8 mM MgCl₂, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 0.6% Triton X-100, 15% glycerol and 25 mM Tris-phosphate buffer, pH 7.8) per well after having removed the culture medium. After 15 min of incubation, the lysates were transferred into Eppendorf tubes and centrifuged at 18,000 g for 5 min. Then, 1–5 μl of the supernatant were deposited in wells of a white 96-well plate. The dosage of the luciferase activity was realized with a luminometer after sequential addition of 50 μl of assay buffer and 50 μl of a luciferin solution. The results are expressed in relative light units (RLU) and are normalized for the amount of proteins present in each sample and are expressed in RLU/1 s/mg protein. Of note, the light units/s measured with untreated cells are considered as background and therefore these values are subtracted from each sample. The amount of protein was determined by a Bradford assay (Bio-Rad protein assay kit; Bio-Rad, Marnes-la-Coquette, France). This measurement was made using 4 μl of supernatant. To each sample, 200 μl of Bradford reagent, diluted at 1/5 in MilliQ water, was then added. The colorimetric assay was performed by measuring the optical density at 595 nm using a spectrophotometer FLX-Xenius (Safas, Monaco). The amount of protein was determined using a calibration curve obtained using albumin as model protein.

2.10 | Cell viability assay

Cell viability was determined using the “Cell growth determination kit, MTT based” (Sigma-Aldrich). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in quadruplicate in 48-well plates. To this end, 55,000 MRCS-V2 cells were seeded in 48-well plates in DMEM supplemented with 10% serum. The next day, the cells were transfected. Twenty-four hours after transfection, the cells were incubated for 3 h at 37°C in the presence of 500 μl of medium containing MTT reagent (0.5 mg/ml). The enzymatic reaction was stopped by removing the culture medium and by adding dimethylsulfoxide. The absorbance was then measured at 570 nm (and 690 nm) and the percentage of cell viability was calculated relative to untreated cells (the control “untreated cells” gives the value of 100% viability). The MTT assay was performed in triplicate: the last well of the quadruplicate was used to determine the transfection efficiency because it is not possible to perform the MTT and luciferase assays on the same sample.

2.11 | Membrane integrity assay with lactate dehydrogenase (LDH)

Membrane permeabilization was determined with the “LDH Cytotoxicity Assay” kit, in accordance with the manufacturer’s instructions (Thermo Scientific, Illkirch-Graffenstaden, France). LDH is a cytosolic enzyme. This enzyme is not able to cross the plasma membrane. Measurement of the amount of LDH present in the supernatant of cells allows for the detection of membrane alterations. To determine whether our peptides possessed permeabilizing properties, we determined the amount of LDH released into the medium by measuring the optical density at 490 nm (and 680 nm) of an aliquot of each sample after 2.5 h of transfection. Of note, the LDH assay was performed using the same transfection plates that were used for the MTT assay. The supernatants of lysed cells (lysis buffer supplied with the kit) allows 100% release of LDH to be achieved, whereas untreated cells provide the background release of LDH. The LDH assay was performed in quadruplicate.

2.12 | Confocal microscopy

30,000 A745-Luc cells were plated into an eight-well chamber slide (Lab-Tek, Santa Cruz, CA, USA). The next day, cells were transfected using 2.83 μg of HELP-4H/167 ng of siRNA-cyanine5/well (= 17 μg of peptide/μg of siRNA). After 2.5 h of incubation in serum-free medium, transfection medium was removed and replaced with complete medium. After another 90 min, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde and treated with DAPI (4',6-diamidino-2-phenylindole) for nuclear staining. The cells were then imaged using a DMI 4000 microscope (Leica, Wetzlar, Germany) and a ×63 objective.

3 | RESULTS

3.1 | Design of HELP derived peptides

Using the bee venom melittin peptide as template, Moser designed a new amphipathic α-helical forming peptide named HELP (Table 1). The design of the peptide was performed in such a manner
that the four glutamic acid residues inserted in the central region of the peptide promote helix destabilization by charge repulsion at pH 7.4. Interestingly, however, although no pH-dependent helix-random coil transition was observed, Moser\textsuperscript{28} found that the peptide had a pH dependent membrane leakage activity (high lytic activity at pH 5.0 that became low when the pH increased). Considering that endosomal escape of nucleic acids is most often a limiting step in the transfection process, we considered whether minimal sequence modification of HELP could allow to obtain a cell penetrating peptide able to deliver nucleic acids.

Taking into account the four cationic amino acids (KKRK motif) at the C-terminus of HELP, this means that the net global charge of the peptide is near neutrality (±1). Because the C-terminus is already cationic, we considered whether replacing the four glutamic acids with histidines (to give HELP-4H) could transform this peptide into a carrier for different nucleic acids, in particular DNA, siRNA and mRNA. Table 1 shows that, by exchanging the four glutamates for histidines, the nominal charge increases at both neutral pH (±5) and acidic pH (±9).

In addition, three other derivatives were designed, all of which contained four histidines in the core of the peptide (Table 1). Compared with HELP-4H, the D1 derivative has four leucine residues that were replaced with alanines in order to reduce the hydrophobicity of the peptide. Also, the two glutamine residues were removed. In the D2 derivative, two positively charged amino acids were present at each terminus instead of having all four cationic amino acids at the C-terminus. Also, three point mutations were introduced compared to D1: phenylalanine in place of glycine, serine in place of threonine and tryptophan in place of phenylalanine. Lastly in the D3 derivative, compared to HELP-4H, three leucine residues were replaced with alanines and point mutations were introduced at positions 3, 7 and 19.

When analyzing the average hydrophobicity as a function of pH, we can see that the glutamic acid/histidine exchange makes HELP-4H more hydrophobic than HELP at pH 8 but more hydrophillic than HELP at pH 2 (Table 1). This effect will be concentrated at the centre of the peptide.

Also of note, all of the HELP-4H peptides (and LAH4-L1) increase their hydrophilicity as the pH drops (in contrast to HELP). Lastly, it should be noted that HELP-4H-D1 is more hydrophilic than the other HELP-4H peptides (even though the glutamines are removed), which emphasizes that the four leucine to alanine switches have an important effect. With this exception, the HELP-4H peptides are generally more hydrophobic than LAH4-L1.

### 3.2 Evaluation of the DNA transfection capacities of the peptides

The DNA transfection activity of the four HELP derived peptides was tested on HEK293 using the reporter gene luciferase. As shown in Figure 1A, HELP-4H revealed to be the most efficient peptide among the four derivatives, followed by peptide D3, whereas the D1 and D2 derivatives were significantly less efficient. As the transfection efficiency can be cell-type dependent, the four peptides were also tested on the HCT116 cell line. The results show that the transfection efficiency of the four HELP derivatives on these cells is comparable (see Supporting information, Figure S1). Although peptide D3 displayed almost the same transfection potential than HELP-4H, we decided to focus on this latter peptide for the rest of the study because its sequence directly derives from the parent peptide HELP.

We first evaluated the capacity of the HELP-4H peptide to complex plasmid DNA. To this end, a gel mobility shift assay was performed using increasing amounts of vector mixed with a constant amount of DNA. The results showed that approximately 2 μg of HELP-4H/μg DNA was required for complete retardation (see Supporting information, Figure S2). If we assume that the peptide has five positive charges at neutral pH (Table 1), this corresponds to a N/P ratio of 1, meaning that, at this ratio, the amount of cationic charges of the peptide equals the amount of negative charges provided by the phosphate groups of the DNA.

The efficiency of HELP-4H was next compared with that of the cell penetrating peptide Vpr55–82 derived from the HIV-1 accessory protein viral protein R described as having high DNA transfection capabilities\textsuperscript{30,31} and with that of the cationic polymer poly-lysine with a degree of polymerization of 215 (pLys215). As shown in the Supporting information (Figure S3A), HELP-4H was slightly more efficient than the Vpr derived peptide, performing 300× better than polylysine. When the reporter gene GFP was used with HELP-4H, we found that a high percentage of HEK293 cells expressed the transgene (average 50%, with n = 2) (Figure 1B; see also Supporting information, Figure S3B).

We then evaluated the DNA transfection activity of HELP-4H on three other human cell lines, namely Human adenocarcinoma cells (A549), human foetal lung fibroblasts transformed with SV40 (MRC5-V2) and HCT116 cells. For comparison, we included LAH4\textsuperscript{22} or LAH4-L1,\textsuperscript{24} two histidine-rich amphipathic peptides that we previously developed as a vector for nucleic acids. Both peptides were revealed to have DNA and siRNA delivery capacities that match or even outperform different commercially available cationic vectors.\textsuperscript{22,32} The best HELP-4H conditions were determined for each cell line by testing several concentrations. The new peptide performed almost as well as LAH4/LAH4-L1 on the three cell lines (Figure 1C; see also Supporting information, Figure S4), demonstrating the good transfection capabilities of HELP-4H.

### 3.3 Size and zeta potential of the complexes

Because the size of DNA particles varies depending on the buffer used to generate the complexes, we measured, by dynamic light scattering, the size of HELP-4H/DNA complexes prepared either in salt-free conditions or in 150 mM NaCl. In the absence of salt (complexes prepared in water), we found that particles have a size around 100 nm...
When generating the assemblies in 150 mM NaCl, they were larger with a diameter above the micrometer (1586 nm). Thus, as observed previously for transfection agents like PEI33 and LAH4,25 the presence of salt induces aggregation. Next, the zeta potential of the complexes was measured and was found to be positive (+29.2 mV) (see Supporting information, Figure S5). This latter result was expected because the 5.3/1 w/w ratio used corresponds to a +/−/C0 ratio of +2.65.

We next considered whether the small complexes generated in water possess the same transfection properties as the nanoparticles prepared in sodium chloride. The results show that the efficiency of both complexes is similar when using higher w/w ratios (see Supporting information, Figure S6). Although the complexes generated in 150 mM NaCl were larger, we maintained this latter condition for the following transfection experiments to be able to compare the results with the previous ones.

3.4 Transfection in the presence of serum

Many peptidic carriers, including LAH434 and LAH4-L1,34 display a DNA transfection efficiency that is reduced in the presence of serum. Therefore, we tested the gene delivery efficiency of HELP-4H in the presence of 10% serum using three different cell lines (MRC5-V2, HEK293 and HCT116). The results show that when optimizing the peptide/DNA ratio, no (or only slight) reduction of the transfection activity is observed (Figure 2A; see also Supporting information, Figure S7). Of note, as reported earlier for DOTAP based lipidic formulations,35 a slight increase of the vector/DNA mass ratio is needed to compensate the serum effects (Figure 2A; see also Supporting information, Figure S7A,B). In agreement with previous reports, the activity of LAH4 activity was altered when serum is present (see Supporting information, Figure S7A,B). When the percentage of serum was increased to 25% and 50%, we found that the transfection activity on MRC5-V2 cells remained very significant (Figure 2B). This was confirmed when transfecting HEK293 cells in the presence of 25% serum with the GFP reporter plasmid: the average GFP positive cell population was 71% (n = 2) (see Supporting information, Figure S7C,D).

3.5 Cytotoxicity of HELP-4H

The cell toxicity of the peptide was quantified on MRC5-V2 cells in the presence of 10% serum using an MTT assay. The results show that there is a light cell cytotoxicity when higher amounts of peptide are used (Figure 3A) but the values are in the same order than those found for other cationic vectors. In parallel to the MTT assay, during the same experiment, we determined the permeabilizing activity of
the peptides by measuring the release of LDH from the cells 2.5 h after having started the transfection. LDH is a cytoplasmic enzyme that cannot cross the plasma membrane; thus, the amount of LDH found in the medium is proportional to membrane permeabilization. The positive control for this experiment (100% released LDH) is the amount of LDH found in the medium after complete lysis of non-transfected cells. As shown in Figure 3B, only low leakage of LDH from the cytoplasm into the culture medium was observed. Of note, one well of the quadruplicate was used to verify that the transfection efficiency worked well (data not shown).

3.6 | Endosomal escape

Next, we considered whether inclusion of helper agents could increase the gene delivery capabilities of HELP-4H. First, we tested whether the weak base chloroquine, known to enhance the transfection activity of polymers like poly-lysines36 has an effect. As shown in Figure 4, chloroquine slightly improved the transfection efficiency of HELP-4H, whereas it increased the efficiency of a poly-L-lysine by more than 10-fold, with an average degree of polymerization of 215.

DOPE is often used to increase the transfection capacities of cationic lipids. Here, we found that addition of DOPE improved the activity of the cationic amphipatic peptide and also that of the polylysine (Figure 4). Lastly, we considered whether the addition of the fusogenic amphipathic peptide JTS-1, which was shown to have a pH-dependent lytic activity on erythrocytes,18 can boost the activity of HELP-4H. Although this anionic peptide increased the transfection of pLys215 by more than 20-fold, it severely decreased the activity of HELP-4H, suggesting that JTS-1 may disorganize the complexes as a result of its negative charges. Interestingly, using the same conditions, LAH4 behaved in a very similar manner to HELP-4H (Figure 4). It was previously reported that the acidification of the endosomes plays an important role in the transfection process of various vectors including LAH422 and polyethylenimines (PEI).37 Indeed, inhibition of the acidification of the endosomes by inhibitors of the H+ ATPase pump, such as bafilomycin A138 or concanamycin A,39 significantly decreases the transfection efficiency of these two carriers. Because HELP-4H contains histidine residues that can be protonated upon acidification of endosomes, we investigated the impact in terms of transfection efficiency after inhibition of this process. MRC5-V2 cells were transfected in the presence of either bafilomycin A1 or
Transfection in the presence of helper agents. Transfection of HCT116 cells was performed in duplicate in 48-well plates using 0.75 μg DNA/well. HELP-4H was used at 2.66 μg/μg DNA, LAH4 at a w/w ratio of 8/1 and pLys215 at w/w = 1.66. The complexes were incubated for 2.5 h in serum-free medium. Thereafter, the medium was removed and replaced with serum-containing medium. Luciferase activity was measured one 24 h post-transfection and is expressed as total light units/1 s/mg protein, and the average of the duplicates is shown. Error bars represent the SD. Chloroquine was used at a final concentration of 100 μM. For generating the ternary complexes, we added respectively 5 μg of DOPE and 10 μg of JTS-1 (for a duplicate) to the HELP-4H, pLys215 and LAH4 complexes.

Concanamycin A. As a positive control, we used B-PEI 25 kDa and as a negative control we chose the monocationic lipid DOTAP because the protonation state of this lipid does not change within the pH range of 4 to 7 (amine is already fully protonated at neutral pH). The results show that the efficacy of B-PEI is reduced 13- and 85-fold with baflomycin A1 and concanamycin A, respectively, whereas DOTAP is only marginally affected by these drugs (less than 2-fold) (see Supporting information, Figure S8A). Interestingly, the efficiency of HELP-4H was only reduced by around 3-fold, suggesting that acidification of the endosomes is not essential for HELP-4H to allow endosomal release of the nucleic acid. Similar results were obtained when using HCT116 cells: showing a dramatic decrease of B-PEI, whereas DOTAP and HELP-4H were almost unaffected by the presence of concanamycin A (see Supporting information, Figure S8B). Altogether, the results obtained in the presence of proton pump inhibitors suggest an intracellular mechanism of action of HELP-4H that does not strongly depend on the protonation of the imidazole groups of the histidines once the complexes are in endocytic vesicles.

3.7 | CD

Next we considered whether a pH-dependent helix-random coil transition occurs with the HELP-4H peptide. CD spectroscopy shows that LAH4-L1 undergoes a pH dependent transition from alpha-helix to disordered and previous work indicates that this is associated with disaggregation of the peptide (Figure 5A,B).40,41 Indeed, the amphipathic alpha-helix conformation of LAH4-L1 is only achievable in solution if the hydrophobic surface can be stabilized by another hydrophobic surface (i.e., from another peptide). For HELP-4H, the same effect is not conserved and spectra consistent with alpha-helix are observed at both high and low pH (Figure 5C). Although a small, pH dependent, increase in the negative band at 208 nm is detected, possibly associated with decreased light scattering (Figure 5C), based on the CD at 220 nm, the alpha-helix conformation is retained throughout the pH range tested (Figure 5D). Interestingly, this was also the case of the parent peptide HELP, which showed no significant pH-triggered conformational changes at different pH values.28 The fact that HELP-4H maintains its alpha-helix in a pH independent manner may explain why its transfection activity is only poorly affected by the presence of proton pump inhibitors.

The distribution of the hydrophilic and hydrophobic amino acids in the helix is represented in the helical-wheel projection provided in the Supporting information (Figure S9).

Taken together, we were able to show that HELP-4H has robust DNA transfection capabilities in a variety of cell lines. As a result of the high therapeutic potential of other nucleic acids, in particular of siRNAs and mRNA, we considered whether our peptide was also able to deliver them efficiently.

3.8 | siRNA and mRNA delivery

The capacity of the different peptides to introduce siRNAs into cells was evaluated using A549 cells that have been engineered to stably express the reporter gene luciferase (A549-Luc). As siRNA, we used siLuc, which is able to silence the luciferase expression through RNA interference. The selectivity of the siLuc induced luciferase silencing is evaluated by comparison with a control siRNA (siCtrl) transfected with the peptide using the same procedure. This control proves the absence of unspecific gene down-regulation by the delivery system.

In the absence of serum, HELP-4H could induce a very good silencing because luciferase expression was reduced by more then 90%, whereas control siRNA (siCtrl) had no effect on the expression level of the reporter gene (Figure 6A). When serum was present, the silencing was less efficient but reached nevertheless 60% compared to untreated cells (Figure 6B).

Next, the delivery of the siRNA into A549-Luc cells was also investigated using confocal microscopy. The results indicate that HELP-4H has the capacity to deliver significant amounts of siRNAs into the cells (red fluorescence). Most of the fluorescence localizes near the nucleus, probably in vesicular compartments, in particular endosomes (Figure 6C). These results are consistent with previous studies showing that only a very minor fraction of siRNAs is released from endosomes and this siRNA fraction remains undetectable by standard fluorescence microscopy methods.42

We then considered whether our peptide is able to switch off the expression of a gene in another cell line. For this, we used kidney tumor cells, 786-O-Luc, which stably express the pGL4.50 luciferase.43 The results provided in the Supporting information (Figure S10) indicate that HELP-4H allowed for a very important and specific decrease (90%) of the luciferase levels in this cell line. It was noted that the optimum w/w ratio peptide/siRNA is the same as that identified for the A549-Luc cell line (17/1).
Lastly, we considered whether HELP-4H can allow expression of a protein after mRNA delivery. Using a luciferase expressing mRNA, we transfected HCT116 cells with HELP-4H and DOTAP. The results indicated that both delivery systems allowed for a high expression level of luciferase (Figure 6D).

Taken together, our results show that HELP-4H is able to deliver efficiently different types of nucleic acids into cells.

4 | DISCUSSION

Peptides have an interesting potential in medicine, not only because their building blocks are biodegradable, but also as a result of their small size, as well as the facility of large-scale production and product characterization. During the past three decades, a new family of short peptides that efficiently cross biological membranes, known as cell penetrating peptides (CPPs), has emerged. Their discovery is promising for the development of approaches that aim to increase the intracellular delivery of compounds with therapeutic potential but low membrane permeability, such as drugs, peptides, proteins and nucleic acids. Among the CPPs, arginine-rich peptides are often used. Unfortunately, these peptides, whether complexed or not to a cargo, are efficiently taken up by the cells but endosomal escape is rather low. As a consequence, more efficient CPPs remain actively sought after.

In the present study, we developed a new CPP based on the sequence of the fusogenic peptide HELP. Because the C-terminus is cationic, we considered whether simple replacement of the glutamic acids by histidine residues can transform this fusogenic peptide into a carrier able to deliver different nucleic acids into mammalian cells. Among the four derivatives that were designed and tested, HELP-4H was the most promising one. This latter peptide proved useful not only for the delivery of plasmid DNA, but also siRNA and mRNA. This point is interesting because not all vectors able to deliver DNA are efficient for the delivery of other nucleic acids, in particular of siRNAs. For example, this is the case for PEI, which belongs to the most efficient synthetic transfection agents for DNA but is a rather poor siRNA delivery agent. Also of note, HELP-4H not only demonstrated good transfection capabilities, but also was able to keep its transfection capabilities in the presence of serum. When the structure of the peptide was analyzed using CD, it was found that, similar to the HELP parent peptide, HELP-4H showed no significant pH-triggered conformational changes.

We also considered whether the addition of helper agents could enhance the transfection efficiency of HELP-4H. Among the three compounds [chloroquine, JTS-1 and DOPE] that were tested, DOPE proved to be the most efficient (Figure 4). Notably, the helper activity of this zwitterionic lipid relies on its capacity to increase endosomal escape of the complexes through its fusogenic activity. Interestingly, DOPE was also shown to enhance the nucleic acid transfection efficiency of non-lipidic formulations, in particular hydrophobized dodecalysines and oligourease foldamers.

Finally, it should be emphasized that, when we decided to insert histidine residues into HELP, this was to confer “proton-sponge” capacities on the peptide, similar to those of PEIs or LAH4 peptides. Surprisingly, however, the activity of HELP-4H in the presence of proton pump inhibitors such as bafilomycin A1 or concanamycin A was almost unaltered, suggesting a mechanism of action differing from that of LAH4. This property of HELP-4H is interesting and may reveal advantageous compared to the LAH4 peptides: indeed, not all cellular entry pathways lead to acidification of the endocytic organelles. Exactly how this new peptide acts to release the nucleic acids into the cytosol remains to be elucidated.
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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SA, CD, BP and AJM performed the experiments. AJM analyzed data and contributed to the writing of the manuscript. AJM and BF contributed to the design of the study. AK designed and supervised the study and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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