Histidine-Rich Cationic Cell-Penetrating Peptides for Plasmid DNA and siRNA Delivery 2 3

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Abstract 6

Amphipathic, pH-responsive, membrane-active peptides such as LAH4 and derivatives thereof have the ability to effectively deliver genes and small interfering RNA (siRNA) into mammalian cells. Their ability to bind and protect nucleic acids and then disrupt membranes when activated at low pH enables them to harness the endocytic machinery to deliver cargo efficiently and with low associated toxicity. This chapter describes protocols for the chemical synthesis of transfection peptides of the LAH4 family, complex formation with nucleic acids, and their use for the in vitro delivery of either plasmid DNA or siRNA into mammalian cell lines. 7 8 9 10 11 12 13

Keywords Gene therapy, Nonviral delivery system, Cationic amphipathic peptides, Plasmid DNA, siRNA, RNA interference, Endosomal release, Histidine, Cell-penetrating peptide 14 15

1 Introduction 16

A large number of monocationic and polycationic compounds have been developed in recent years with the aim of overcoming the bottlenecks for the development of nonviral nucleic acid-based therapeutics and/or laboratory toolkits. Ideally, these compounds should be multifunctional; in particular they should stabilize the nucleic acid and protect it from the desired intracellular compartment (Fig. 1). In the case of plasmid DNA, nuclear delivery is required but for small interfering RNAs (siRNAs), which are used for inducing RNA interference (RNAi) [1], delivery to the cytosol is sufficient. In both cases, complexes are commonly taken up through nonspecific endocytosis [2] and it is the ability to escape from endosomes that often determines the success of a cationic formulation. 17 18 19 20 21 22 23 24 25 26 27 28 29

One way that has been found to increase the efficiency of endosomal escape of the nucleic acids consists in using the “proton 30 31

Fig. 1 Schematic representation of LAH4-mediated transfection of eukaryotic cells. Addition of an excess of LAH4 to plasmid DNA or siRNA generates complexes which exhibit a small excess of positive charges on their surface. Particles interact with the cell membrane, preferentially with proteoglycans, via nonspecific ionic interactions. The LAH4/nucleic acid complexes enter the cell through endocytosis. During acidification of the endosome, histidine residues become protonated and LAH4 interacts with the endosomal membranes in an in-plane alignment. Membrane destabilization occurs, followed by the release of DNA/siRNA into the cytosol

sponge” mechanism. This process involves a transporter-induced swelling of the endosomal compartment during acidification which leads to disruption of the endosomal membrane [3–6]. Various compounds have been described that react to the change in pH encountered in the endosomes [7]. Many of these compounds incorporate the imidazole group found in histidine [8–10] which has a pK_a around 6.1. At increasingly acidic pH such groups are protonated leading to enhanced cationicity of the polyplexes and, potentially, some release of the vector compound and hence such groups may constitute effective triggers for nucleic acid delivering activities.

Our efforts have focused on the development of pH-responsive, cationic amphipathic peptides [11–16]. These peptides have both nucleic acid binding and membrane disruptive

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Fig. 2 Helical wheel diagram for LAH4-L1 (residues 6–23). The primary sequence (*top*) and three-dimensional model (*left*) of LAH4-L1 indicate the positions of the histidine residues and how these are located in the amphipathic helix conformation that the peptide adopts when bound either to DNA or target membranes. The helical wheel projection (*right*) allows one to visualize the distribution of hydrophobic and polar residues with respect to the helical axis. The angle of the positively charged helix face (histidine residues are in ***bold and underlined***), at pH 5, for the LAH4-L1 peptide is 80°

capabilities with their amphipathic α -helix motif being inspired by antimicrobial peptides whose main mode of action involves membrane destabilization [17, 18]. The concept uses histidine pH triggers to release membrane-destabilizing peptides from the peptide–nucleic acid complex (or other cargo) allowing them to interact to and to lyse endosomal membranes. The original sequence, LAH4 (KKALLALALHHLAHLALHLALA LKKA) [11], and later variants, including LAH4-L1 (KKALLAHALHLLALLALHLAHLALKKA) [14, 15], have been designed to adopt an α -helix conformation when bound to nucleic acids [16] and, particularly, lipid bilayers [19] (Fig. 2). The LAH peptides exhibit a hydrophobic surface, comprising a mixture of alanine and leucine residues, and a hydrophilic surface in which the four (or more) histidine residues are located. The segregation of the two types of surfaces and the positioning of the histidine residues (Fig. 2) are crucial to the effective disordering of the membranes and the efficacy of delivery mediated by the peptide [11, 15]. The membrane disordering and disruption are the final elements in a strategy that has proven to be remarkably effective, with both DNA and siRNA delivery that matches and often outperforms commercially available cationic nonviral vectors [11, 13]. Furthermore, we have observed only low cytotoxicity associated with the pH-responsive peptide delivery system.

Notably, the LAH4 peptides have also been shown highly efficient for delivering a variety of other cargoes into mammalian cells including CpG oligonucleotides [20], proteins [20, 21], adeno-associated viruses [22], lentiviruses [23–25], and quantum dots [26].

Table 1
Sequence of some of the most efficient LAH/Dap derivatives

Peptide	Sequence ^a	DNA	siRNA	Protein	Ref.
LAH4	KKALLALALHHLAHLALHLALALKKA	yes	yes	yes	[11, 13]
LAH4-L1	KKALLAHALHLLALLALHLAHLALKKA	yes	yes	nd	[13, 15]
LAH6-L1-80	KKHLLAHALHLLALLALHLAHLAHLKKA	yes	nd	nd	[14]
LADap(Me)6-L1	KKXLLAXLLXLLALLALXLLXALKXXK	yes	yes	nd	[27]

^apH responsive residues are marked in bold. X monomethylated 2,3-diaminopropionic acid (Dap), *nd* not done

It is also worth mentioning that not only histidine residues are able to confer endosomolytic properties to LAH peptides. Indeed, we found that the pKa of the β-NH2 in 2,3-diaminopropionic acid (Dap) is sufficiently lowered, when Dap is incorporated into peptides, and that its protonation state is sensitive to the pH changes that occur during endosomal acidification [12]. Integration of monomethylated Dap allowed to further improve the efficiency of these modified peptides [27]. Peptide analogs of LAH4 peptides that are based on oligoureas have also been shown highly effective in DNA transfection [8]. A short list of some of the most efficient LAH/Dap peptides that we have developed in the last years are shown in Table 1.

We believe that our peptide-based system has a number of advantages that makes it attractive to researchers who rely on effective delivery of nucleic acid (or other) cargoes with few side effects and, accordingly, we are pleased to provide detailed protocols for the production, characterization, and use of this emerging delivery system.

2 Materials

2.1 Peptide Synthesis

The peptide LAH4 (MW 2777) with the sequence KKALLA-LALHHLAHLALHLALALKKA-NH₂ [11] or derivatives such as LAH4-L1 [15] can be purchased from a number of peptide synthesis services (e.g., ProteoGenix, Schiltigheim, France; GeneCust, Luxembourg; and many others). We would recommend requesting a custom synthesis and purchasing the peptide as desalted grade as the most cost-effective solution. Alternatively, it can be synthesized and prepared in any laboratory that has access to a solid-phase peptide synthesizer (e.g., ABI433 automatic peptide synthesizers, Weiterstadt, Germany) or the expertise and setup to go through the synthetic cycles manually.

Materials needed for peptide synthesis:

1. Automatic peptide synthesizer (e.g., ABI433, Weiterstadt, Germany). 105
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2. Access to preparative reverse-phase HPLC instrumentation and MALDI mass spectrometric analysis. HPLC is performed for example on a 300 Å Prontosil column (Bischoff, Leonberg, Germany) of 20 mM diameter and 250 mM length (or similar). We have purified LAH4 peptides also on columns of dimension 7.8×100 mM and the conditions change accordingly. Furthermore, during the acetonitrile shortage during the economic crisis in 2008 it has proven advantageous that methanol can be used instead of acetonitrile. 107
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3. Solvents and Fmoc-protected amino acids (Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, and Fmoc-His(Trt)-OH, for example, from Novabiochem Merck Darmstadt, Germany; Bachem, Heidelberg, Germany; or Applied Biosystems, Weiterstadt, Germany) according to the instructions given for your automatic peptide synthesizer, including HBTU (2-(1H-benzo-triazole-1-yl)-1,1,3,3-tetra-methyluronium-hexafluorophosphate, Novabiochem, Merck, Darmstadt, Germany), Dipea (*N*-ethyl-diisopropylamine, Sigma, St. Louis, MO, USA), TentaGel R RAM, or TentaGel S RAM resin (load 0.18–0.25 mmol/g; Rapp Polymer, Tübingen, Germany). 116
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4. 5% H₂O, 1% triethylsilane (97%, Sigma), in trifluoroacetic acid (TFA) (99.9% Carl Roth, Karlsruhe, Germany) for the cleavage reaction. 128
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5. Diethyl ether (stabilized with BHT for analysis, SDS (Solvents Documentation Syntheses), Peypin, France). 131
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6. HPLC-grade acetonitrile, water, and TFA. When using methanol as an alternative to acetonitrile make sure that the impurities do not absorb at 214 nm, thereby interfering with the detection of chromatogram. 133
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If the peptide is prepared by automated peptide synthesis it is recommended to follow the detailed protocols that are provided by the manufacturers of such instrumentation for the setup of the synthetic cycles, including the solvents, resins, and Fmoc-protected amino acid residues. Furthermore access to preparative and analytical reverse-phase (C₄, C₈, or C₁₈) HPLC and mass spectrometric characterization of the final product are necessary. As solid-phase peptide synthesis is an elaborate science, which fills many textbooks and libraries, discussing the details and pitfalls of the technique is beyond the scope of this paper. Nevertheless, in **Note 1** a short outline of the fundamental idea is provided. So far the LAH4 peptides and their derivatives could be obtained in good yields (several 100 mg of crude product) using standard synthesis cycles of automated peptide synthesizers. In our hands the crude product

contains about 90% of the LAH4 sequence and can be used after
desalting for preliminary transfection assays without apparent loss
in efficiency when compared to the purified product. However, it
should be noted that even a 99% yield of each individual cycle
results in truncation products, where one residue is missing and
an overall yield of the full sequence reduces to about 80% (0.99^{25}).
Therefore, the shorter sequences can be separated from the desired
product using preparative HPLC and identified by MALDI mass
spectrometric analysis.

2.2 Gel Mobility Shift Assay

1. Agarose (ultrapure agarose from Invitrogen, Cergy Pontoise, France).
2. TBE buffer: 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0.
3. Loading buffer 6×: 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in water.
4. SYBRSafe DNA gel stain (Invitrogen, Cergy Pontoise, France).
5. Electrophoresis unit.

2.3 Plasmid DNA and siRNA

1. pCMV-Luc is an expression plasmid encoding the firefly luciferase gene under the control of the human cytomegalovirus (CMV) immediate-early promoter. Plasmid DNA is resuspended in sterile water at 1 mg/mL and is stored at -20°C .
2. The siRNA-Luc (from Sigma-Aldrich, Saint-Quentin Fallavier, France) sequence that we use for knock down of luciferase has been previously published [1]: sense oligonucleotide: 5'-CGUACGCGGAUACUUCGATT-3'; antisense oligonucleotide: 5'-UCGAAGUAUCCGCGUACGTT-3'. Aliquots of a 10 μM stock solution are stored at -20°C .
3. The sequence of the siRNA-eGFP (Sigma-Aldrich, Saint-Quentin Fallavier, France) that we use as control siRNA has been published previously by Caplen et al. [28]: sense oligonucleotide: 5'-GCAAGCUGACCCUGAAGUUCAU-3'; antisense oligonucleotide: 5'-GAACUUCAGGGUCAGCUUGC CG-3'. Aliquots of a 10 μM stock solution are stored at -20°C .
4. Limit the freeze-thaw cycles of each tube. To this end, make small aliquots of DNA and siRNA.

2.4 Dynamic Light Scattering

1. Dynamic light scattering (DLS) measurement system. We have chosen the Zetasizer Nano-S instrument (Malvern Instruments, UK) since it has the ability to measure molecular systems, from some hundreds of Daltons to micrometer-size aggregates.

	2. Low-volume cuvettes. High-quality quartz cuvettes are the best for the purpose of the described measurements since they minimize the light scattered at the air—and liquid—window interfaces (Hellma, Germany).	195 196 197 198
	3. Suspension of 60 nm latex nanospheres (Duke Scientific) for calibration of the DLS instrument.	199 200 201
2.5 Cell Culture	1. 24-well culture plates (from Costar).	202
	2. DMEM supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin.	203 204
	3. Sterile (0.2 µM filtered) 150 mM NaCl solution.	205 206
2.6 Luciferase Assay	1. Lysis buffer: 25 mM Tris–phosphate, 8 mM MgCl ₂ , 1 mM dithiothreitol, 1 mM EDTA, 15% glycerol, 1% Triton X-100, pH 7.8.	207 AU2 208 209
	2. Assay buffer: 25 mM Tris–phosphate, 8 mM MgCl ₂ , 1 mM dithiothreitol, 1 mM EDTA, 15% glycerol, and 2 mM of ATP—the latter component of the buffer has to be added just before performing the luciferase assay (store aliquots of 1 mL of ATP at 40 mM in water at –20 °C).	210 211 212 213 214
	3. Make aliquots of 50 mL of the lysis and assay buffer and store them at –20 °C.	215 216
	4. D-Luciferin sodium salt (Invitrogen, Cergy Pontoise, France): 167 µM in water. Make aliquots of 10 mL, protect the tubes from light, and store them at –20 °C.	217 218 219
	5. Luminometer (e.g., Victor ² , PerkinElmer, Courtaboeuf, France).	220 221 222
2.7 Protein Assay	1. Detergent-compatible protein determination kit (e.g., the Bradford protein assay from Bio-Rad, Marnes-la-Coquette, France).	223 224 225
	2. Transparent 96-well plate.	226
	3. A 96-well plate spectrophotometer able to read at 595 nm.	227 228
2.8 MTT Cell Viability Assay	1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; from Sigma) at 5 mg/mL in PBS (GIBCO).	229 230
	2. A 96-well plate reader capable of measuring optical absorbance at $\lambda = 570$ nm.	231 232 233
2.9 Flow Cytometry	1. Fluorescent FITC-siRNA-Luc: A fluorescein group is coupled to the 3 end of the antisense strand of the duplex (such a modification can be made by suppliers as for example Sigma).	234 235 236
	2. Phosphate-buffered saline (PBS).	237

3. Flow cytometer (e.g., FACSCalibur from Becton Dickinson, Le Pont-De-Claix, France). 238
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3 Methods 241

3.1 Peptide Synthesis

1. We have prepared LAH4 and related peptides by solid-phase peptide synthesis on either Millipore 9050 or ABI433 automatic peptide synthesizers using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry which has also allowed us to introduce amino acid residues labeled with stable isotopes for NMR structural studies, amino acid replacements, or the addition of optical dyes [19, 29]. As the detailed protocols and the solvents used depend on the synthesizer they shall not be specified here in detail but reference is made to the corresponding user manuals. In short, LAH4 is typically prepared following the standard protocols of the automatic synthesizer using a fourfold excess of Fmoc-protected amino acids during chain elongation with activation by HBTU and Dipea using for example a TentaGel R RAM or a TentaGel S RAM resin (0.18–0.25 mmol/g). The typical scale of the synthesis is 0.25 mmol. After cleavage of the peptide product by exposing the resin to TFA and in the presence of scavengers such as triethylsilane the crude product (several 100 mg) is precipitated in ice-cold diethylether and LAH4 purified by preparative HPLC using an acetonitrile/water gradient typically ranging from 5 to 60% acetonitrile in the presence of 0.1% TFA and a detection of the absorbance at $\lambda = 214$ nm. The peptide typically elutes at 40% acetonitrile using for example a C4 reversed-phase column of 2 cm diameter and 25 cm length at a flow between 7 and 10 mL/min (*see Note 2*). At the end of the run the acetonitrile concentration is raised to 95% to clean the column. 242
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2. The identity and high purity of the product should be verified by MALDI mass spectrometry and analytical HPLC. 269
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3. After lyophilization the TFA counterions are exchanged by at least three cycles of dissolving the peptide in 5% acetate (v/v) and lyophilization (*see Note 3*). 271
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4. Peptides are resuspended in sterile water at 1 mg/mL (*see Note 4*). 274
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3.2 Gel Mobility Shift Assay

The agarose gel mobility shift assay is a method to determine the capacity of a given compound to complex nucleic acids. More precisely, it allows one to determine the minimal amount of compound required to retard the migration of plasmid DNA or siRNA during agarose gel electrophoresis. Indeed, the complexation leads 277
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Fig. 3 Gel retardation experiment with LAH4/DNA complexes. The following conditions were used: *Lanes 1–9*: (1) naked DNA, (3–9) LAH4/DNA complexes with w/w ratios equal to 0.5, 1, 1.5, 2.5, 5, 7.5, 10, respectively

to the formation of particles which are unable to migrate through the agarose mesh [13, 16].

1. Prepare a 1% agarose gel ($\geq 1.3\%$ for siRNA) by dissolving agarose in 100 mL of TBE buffer and boiling the suspension at 100 °C using a microwave oven. After cooling down to about 50 °C, add 8 μ L of SYBRSafe to the suspension.
2. Fill the electrophoresis unit, and position a comb into the gel. Using a Pasteur pipette remove bubbles from the gel and then wait until agarose has solidified. Place the gel in the electrophoresis unit and remove the comb. Take care to add enough TBE buffer to cover the gel.
3. Samples containing 500 ng pDNA or 750 ng of siRNA are prepared using the same buffer conditions than those used for transfection. Therefore, dilute the pDNA/siRNA in 10 μ L of 150 mM NaCl.
4. Take a tube containing a 1 mg/mL solution of LAH4 (or derivative) from the freezer. After thawing, vortex vigorously the tube—an additional short sonication using a bath sonicator may be performed—before withdrawing the desired amount of peptide from the tube.
5. Dilute increasing amounts of peptide in 10 μ L of NaCl 150 mM (typically, use peptide–nucleic acid weight/weight ratios ranging from 0.5:1 to 10:1).
6. Mix the nucleic acids and the peptide, and incubate for about 15 min at room temperature.
7. Add 3 μ L of loading buffer to each sample, mix, and then load the samples into the wells of the agarose gel. Also include a sample containing nucleic acids without peptide.
8. Electrophoresis conditions: 100 V for 20–30 min.
9. Visualize the agarose gel stained with SYBRSafe using an UV illuminator. An example of gel is shown in Fig. 3: the results indicate that addition of a cationic peptide such as LAH4 inhibits in a dose-dependent manner the migration of DNA (*see Note 5*). In this experiment, complete retardation was

obtained at an LAH4/DNA w/w ratio of 2.5. This ratio of 2.5 corresponds to 0.59 LAH4 peptide per base pair or expressed as a positive/negative charge ratio to +1.5 (*see* **Notes 6** and **7**).

3.3 Plasmid DNA

Plasmid DNA is purified using a commercially available kit (e.g., NucleoBond from Macherey-Nagel, Hoerd, France). The concentration of the DNA solution is determined by spectrophotometry at $\lambda = 260$ nm taking into account that one absorbance unit corresponds to 50 $\mu\text{g}/\text{mL}$ of double-stranded DNA. The optical density is also taken at $\lambda = 280$ nm (*see* **Note 8**).

3.4 Dynamic Light Scattering

The protocol described below outlines (1) the preparation of the peptide (here LAH4 as an example) and the DNA stock solutions, (2) the measurement of the size of the transfection complexes, and (3) the critical analysis of the recorded data.

3.4.1 Preparation of LAH4 and DNA Stock Solutions

The procedure for preparation of LAH4 and DNA stock solutions constitutes a major factor for the success of size measurement of LAH4–DNA complexes and should be done step by step as described below.

1. Weight the appropriate amount of peptide and prepare a 1 mg/mL stock solution of LAH4 in the desired buffer (e.g., 10 mM acetate, pH 5) (*see* **Notes 4** and **9**). Mix by gently tapping and shaking the tube. Sonication of the LAH4 solution for periods of about 10–30 s will help to remove any visible aggregated powder.
2. Perform a first size measurement on the LAH4 stock solution by DLS (as explained in the following sections) in order to confirm the solubility of the peptide at low pH. If this is not the case, ultracentrifugation of the solution at $100,000 \times g$ for 1 h has to be performed in order to remove the remaining aggregates and impurities bigger than about ≈ 100 nm (*see* **Note 9**).
3. Prepare a 1 mg/mL stock solution of DNA following the same protocol described for LAH4 with the exception of the sonication step.

3.4.2 Size Measurement of LAH4–DNA Complexes

1. Prepare a minimum of 40 μL of complexes by mixing the right amount of the peptide and the DNA stock solutions in an Eppendorf tube. Note that the concentrations of the stock solution were chosen high enough so that the complexes could be detected by our experimental system.
2. Transfer the LAH4–DNA mixture in the low-volume quartz cuvette. Place the cuvette in the sample holder of the DLS measurement system.
3. Set the working temperature and the related physical parameters needed by the program to perform the mathematical

procedure of data inversion. Refractive index of the buffer and of the complexed molecules at $\lambda = 633$ nm have to be known. At 25 °C, the index of refraction of pure water ($n = 1.33$) and of pure proteins ($n = 1.45$) can be considered as good approximations for the solvent and the complex, respectively. Prior to the measurements the geometry of the cuvette and the viscosity of the buffer have also to be entered in the program.

4. Record a first measurement after equilibration of the sample in the cuvette. Perform at least four more acquisitions to ensure the reproducibility of the measurements and the stability of the sample over time.
5. After measurements, the sample can be recovered for use in other experiments.

3.4.3 Analyzing the Quality of the Data

A careful analysis of the results has to be performed in order to avoid artifacts and to prevent misinterpretation and/or overinterpretation of the data. For this, it is useful to recall some basics of the functioning of the DLS instrument and of the mathematical algorithm applied to the data.

The software controlling the Zetasizer Nano S performs an analysis of the time autocorrelation function of the scattered light to extract the size distribution of the dissolved particles. The basic idea behind the experiment is that the scattering of a light beam depends on the size of the particles in solution and consequently their velocity. If the particles are small, they diffuse fast and the “memory” is only short lived. In other words the correlation in the diffraction properties between two intervals separated by a given period of time is low. If a particle is large, it diffuses slowly and the correlation remains even if the separating time period is extended. According to the general theory, the intensity of the autocorrelation function is proportional to the square of the electric field–field time autocorrelation function, denoted as $g^{(1)}(t)$. The latter takes complex forms depending on the size, shape, and composition of the scattering particles.

The averaged intensity hydrodynamic radius $\langle R_H \rangle$ and the polydispersity index (PDI) values (an estimate of the distribution width around $\langle R_H \rangle$) are calculated from a mathematical analysis implemented into the Malvern software. For this, the correlation functions are compared to a monoexponential decay $g^{(1)}(t) = \exp(-Gt)$, supposing a monodisperse population of spherical particles. In this case, the particle’s hydrodynamic radius is proportional to the invert of the time relaxation of the decay. The decay rate is equal to $G = Dq^2$, where D is the diffusion coefficient of the particles and q the magnitude of the scattering wave vector that depends on λ_0 , θ , and the refractive index of the solvent. The Stokes–Einstein relation, $D = k_B T / 6\pi\eta \langle R_H \rangle$, where k_B is Boltzmann’s constant and η is the dynamic viscosity, then relates the diffusion coefficient to

$\langle R_H \rangle$. PDI values are calculated from the deviation of the data from the monoexponential decay. With this background in mind, analyze carefully the experimental data in the following manner:

1. Compare the average intensity over time of the signal arising from the transfection complexes (known as SLS, for static light scattered) with that of a “blank measurement” recorded with the buffer only, in the same quartz cuvette. If the intensity of the latter is much higher, the molecules in the former are probably strongly aggregated involving very large size particles (up to several micrometers). In this condition special attention has to be given to the analysis of the data and to possible artifacts generated by the inversion algorithm.
2. Inspect the autocorrelation function measured on your samples. If the value at $t = 0 \mu s$ is close to zero, the density of the complexes is too low to be measured. The peptide and DNA concentrations of the stock solutions have to be increased in order for the oligomers to be detected. Alternatively sedimentation of large size aggregates might have led to the depletion of the molecules in solution that cannot longer be observed by the system.
3. The PDI indicates the deviation of the sample when compared to an ideal monodispersed sample. For PDI values from 0 to 0.2, the sample solution can be considered as monodisperse. If $PDI > 0.7$, the sample is highly polydisperse and the values of the hydrodynamic radius given by the instrument are probably meaningless. Short periods of sonication of the sample may help to homogenize the solution [30].

3.5 Cell Culture

1. The day prior to transfection, use trypsin to detach actively dividing cells from the culture flask (Corning).
2. After addition of 15 mL of DMEM supplemented with 10% of serum, the cells are counted.
3. Finalize the protocol of the experiment in order to determine how many wells are needed. Notably, transfection experiments are usually done using duplicates or triplicates.
4. The required volume of cell suspension to obtain a confluence of 50–80% the following day is then calculated (for 911 and 911-Luc cells, we use 200,000 cells/well) (*see Note 10*). Dilute the cell suspension with a volume of culture medium which allows the addition of 1 mL of culture medium into each well of the 24-well plate.
5. The cells are then incubated at the appropriate cell culture conditions (a humidified tissue culture incubator at 37 °C and 5% CO₂).

3.6 Cell Transfection

Before starting the DNA/siRNA transfection experiment, the cells should be examined using a microscope. This observation allows for verification of the confluence of the cells and also of the (good) shape of the cells. The peptide–DNA or siRNA complexes are generated just before transfection of the cells. For a transfection performed in duplicate, the following protocols are used.

3.6.1 DNA Transfection

1. The tube containing the plasmid DNA is thawed and gently vortexed. Take 4 µg of plasmid DNA (for one duplicate) and dilute them in 100 µL of sterile (0.20 µM filtered) 150 mM NaCl.
2. The tube containing a 1 mg/mL solution of LAH4 is thawed. The tube is then vigorously vortexed—a short sonication using a bath sonicator may be additionally performed—before withdrawing the desired amount of peptide (*see Notes 11 and 12*). Complete to 100 µL with sterile 150 mM NaCl.
3. Mix the DNA with the peptide, centrifuge very shortly to pull down all the drops, and let the tube at room temperature for about 15 min.
4. Add to the mixture serum-free medium (if applicable) to obtain a final volume of 1 mL; 0.5 mL of the transfection mixture are then put in each well of the duplicate.
5. Remove the culture medium from the cells by aspiration. If the cells that are used adhere well, they may be rinsed with PBS in order to remove all serum containing proteins from the wells (*see Note 13*).
6. Add 0.5 mL of LAH4/DNA transfection medium into each well of the duplicate and incubate at 37 °C for 2–4 h.
7. Remove carefully the transfection medium and replace it with 1 mL of complete culture medium (DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% of fetal calf serum).
8. Incubate at 37 °C for 28–48 h, depending on the cell type.
9. Analyze the cells for luciferase expression (*see Notes 14 and 15*) and protein content or proceed for the MTT cell viability assay (*see below*).

3.6.2 siRNA Transfection

1. Thaw tubes containing the siRNA-Luc. Take 0.46 µg (3.5 µL of the 10 µM siRNA solution) of siRNA-Luc (to obtain a final concentration in the wells of 50 nM). Complete to 30 µL with NaCl 150 mM.
2. The tube containing a 1 mg/mL solution of LAH4 is thawed. The tube is then vigorously vortexed—a short sonication step using a bath sonicator may be additionally applied—before

- withdrawing the desired amount of peptide (*see* **Notes 11** and **12**). Complete to 30 μ L with NaCl 150 mM (*see* **Note 14**). 499 500
3. Of note, transfection complexes of related composition should be prepared with a control siRNA such as a siRNA-GFP. This control is important in order to ascertain that the silencing is gene specific. 501 502 503 504
 4. Mix the siRNA with the peptide, centrifuge very shortly to pull down all the drops, and let the tube at room temperature for about 15 min. 505 506 507
 5. Add to the mixture serum-free medium (if possible) to obtain a final volume of 0.7 mL; 0.350 mL of the transfection mixture are then added to each well of the duplicate. 508 509 510
 6. Remove the culture medium from the cells by aspiration. If the cells that are used adhere well, the cultures may be rinsed with PBS in order to remove serum containing proteins from the wells. 511 512 513 514
 7. Add 0.350 mL of LAH4/siRNA transfection medium into each well of the duplicate and incubate at 37 °C for 2–4 h. 515 516
 8. Remove carefully the transfection medium and replace it with 1 mL of complete culture medium (DMEM supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% of fetal calf serum). 517 518 519 520
 9. Incubate at 37 °C for 28–48 h, depending on the cell type. 521
 10. Analyze the cells for luciferase expression and protein content or proceed for the MTT cell viability assay (*see* below) (*see* **Notes 16** and **17**). 522 523 524 525

3.7 Luciferase Assay

To determine luciferase activity, the following protocol is used: 526

1. Remove carefully the culture medium from the 24-well plates. 527
2. Add 250 μ L of lysis buffer to each well. 528
3. After 10 min, the cell lysate is recovered and transferred into 1.5 mL Eppendorf tubes 4. Centrifuge the tubes for 5 min at 10,000 $\times g$ to pellet debris. 529 530 531
4. Take 50 μ L of the supernatant of each tube and transfer them into the wells of a white 96-well plate. 532 533
5. Measure the bioluminescence using a luminometer which automatically injects 100 μ L of buffer assay and 100 μ L of luciferin solution. 534 535 536
6. Read luminescence over 10 s. 537
7. Remove the luciferase background (obtained with the lysate from nontransfected cells) from each value. 538 539
8. Calculate the light units for 10 s/250 μ L of sample. 540

9. After having measured the protein content (*see* below), express the efficiency as light units/10 s/mg (or µg) of protein.

3.8 Protein Content

The protein determination procedure described here is valid for the Bradford protein assay from Bio-Rad (*see* **Note 18**).

1. Transfer 2 µL of cell lysate to a transparent 96-well plate. Of note, add a control with 2 µL of lysis buffer in order to obtain the background value.
2. Add 200 µL of Bradford reagent which was diluted five times in water.
3. Mix gently.
4. Read absorbance at $\lambda = 595$ nm and determine the protein content in each sample by using a bovine serum albumin standard curve.

3.9 MTT Cell Viability Assay

This assay is used to measure the cell viability (*see* **Note 18**). It therefore allows for an evaluation of the cytotoxicity of the transfection complexes.

1. The transfection should be performed using the same experimental conditions as those used for the luciferase assay (DNA or siRNA).
2. Instead of removing the medium and lysing the cells after 28–48 h of transfection, add to the medium MTT reagent at a final concentration of 0.5 mg/mL per well.
3. Cells are incubated at 37 °C and 5% CO₂ for 3 h.
4. Remove the medium from the cells.
5. Add 500 µL DMSO to each well to dissolve the formazan crystals.
6. Transfer 100 µL from each sample into a transparent 96-well plate and measure absorption at $\lambda = 570$ nm.
7. Untreated cells serve as control (=100% cell viability).

3.10 Flow Cytometry for the Evaluation of the Delivery Efficiency of the siRNA

By using a fluorescently labeled siRNA and flow cytometry it is possible to quantify the amount of nucleic acid that is associated with or endocytosed by the cells.

1. Complexes are made fluorescent by using a fluorescently labeled siRNA-Luc (an FITC group is conjugated to the 3' end of the antisense strand).
2. After preparation of the complexes as described for cell transfection, they are added to 911-Luc cells (final siRNA concentration = 50 nM).

3. After 3 h of incubation at 37 °C, cells are washed with cold PBS and harvested in 1 mM EDTA in PBS and 10,000 cells are then analyzed by flow cytometry.

4 Notes

1. Solid-phase peptide synthesis is based on a cycle of repetitive (and therefore potentially automated) steps where one amino acid after the other is connected to the growing peptide chain. The synthesis starts with the most C-terminal residue of the sequence which is attached to beads of a solid resin and progresses toward the N-terminus (i.e., reverse to the conventional reading of polypeptide sequences). By attaching the intermediate and final products to the solid support it is possible to freely exchange the solvents simply by washing steps where the resin is retained by a ceramic filter funnel (or column) without the need of recrystallization or other types of intermediate purification.
A synthetic cycle starts with one amino acid being attached to the resin by its carboxyl group and the amine being protected (e.g., Fmoc). In a first step the protection group is cleaved from the chain which liberates the amino group for the following reaction. In parallel the amino acid N-terminal ($n-1$) to the one already on the resin (n) is activated chemically; thus its carboxyl group reacts with the amine of the residue already coupled to the solid support. The amine group of this amino acid is protected (e.g., by a Fmoc group); thus it cannot polymerize with its like. By mixing an excess of this activated amino acid with the chain on the resin and incubation of this reaction mixture by several minutes the polypeptide chain grows by one residue, and a new cycle can start by removal of the protection group.
Ideally the reaction yield of each step is >99%, but critical steps may occur where the cleavage of the protection group or the addition of the next amino acid residues is hindered (e.g., sterically). In such cases, increased temperatures, double couplings, extended couplings, a change in chemistry and/or solvents, or the use of pseudoproline residues may be helpful (e.g., [31]) and references cited therein.
2. The optimal flow to obtain best resolution on this type of column would be about three times increased, but this is not always possible as the resulting back pressure of the HPLC system and column exceeds the capacity of the instrument.
3. More recent studies suggest that a more reliable method consists in at least three cycles of dissolving 1 mg/mL peptide in 2 mM HCl (respecting a few-fold excess over the TFA ions)

and lyophilization [32]. If the peptide is purchased from a commercial source make sure you have information about the counterions. Note that the molecular weight to be considered during the preparation of a solution is much different if you have TFA ($2777 + 9 \times 113$), acetate ($2777 + 9 \times 59$), or Cl^- ($2777 + 9 \times 36$) counterions to nine cationic sites of the polypeptide sequence.

4. We recommend the storage of the peptide powder over longer time periods at -20°C . Therefore, before usage the container and the powder have to be first equilibrated thoroughly to reach room temperature, which can take many minutes, even hours. In order to avoid the condensation of water into the peptide powder and its recipient, it is important that during this process the vial remains tightly sealed. Thereafter, weight the appropriate amount of peptide and resuspend LAH4 at a final concentration of, e.g., stock solution of 1 mg/mL. Before transferring the vial with the peptide back into the cold assure that it is tightly sealed with, for example, Parafilm. Avoid repeated freezing and thawing of the stock solution. Prepare rather small aliquots (500 μL in 1.5 mL Eppendorf tubes).
5. If the condensation of the nucleic acids is very efficient, then the nucleic acids are no more accessible to SYBRSafe. This, in turn, results in the absence of a nucleic acid stain in the agarose gel. This is for example the case in Fig. 3 with the highest LAH4/DNA ratio.
6. The peptide LAH4 has five positive charges at neutral pH: indeed, the C-terminus is amidated and therefore it does not present a negative charge. Also of note, at acidic pH, the imidazole groups of the histidine residues become protonated and this adds four positive charges to the peptide (net charge = +9 at pH 5).
7. In our hands, 2.5 to 5 times more peptide of the LAH4 family is required to retard the migration of siRNAs when compared with plasmid DNA. This indicates that complex formation and interactions of the cationic peptides with plasmid DNA are different when compared to siRNA [13].
8. The quality of plasmid DNA may influence the transfection process and only siRNA and DNA of high quality should be used. It is recommended that the optical density ratio OD260/OD280 is ≥ 1.8 .
9. For biophysical experiments we prefer to use an LAH4 stock solution at low pH, where the peptide is monomeric [30], and therefore, mixes with DNA in the most homogenous manner. In this manner, changing back and forth between acidic and neutral pH gives reproducible DLS measurements for the transfection complexes.

10. It is usually recommended to transfect cells at a confluence of 50–80%. However, the optimal cell density for efficient DNA or siRNA transfection may vary between cell types. In any case, it is important to keep the same seeding protocol for a given cell type between the experiments. It is also important, especially when using primary cells, to use cells having approximately the same number of passages.
11. The optimal peptide–nucleic acid ratio for transfection has to be determined for each new cell line.
12. The optimal peptide–nucleic acid ratio may be different for a given cell line for plasmid DNA and siRNA. For example, in our hands using 911 cells, the optimal weight/weight peptide–nucleic acid ratio for siRNA delivery is higher than the one for plasmid DNA transfection.
13. The DNA and siRNA transfection process with LAH4 and derivatives is reduced in the presence of serum [14]. Therefore, when possible, avoid addition of serum—or at low %—during the 2–3 h of incubation.
14. Optimization of each step of the transfection process should be undertaken if the DNA/siRNA transfection efficiency is low. Besides testing various peptide–nucleic acid ratios, other parameters may be changed: concentration of nucleic acids per well; duration of incubation of the complexes with the cells; buffer conditions employed during complexation (e.g., water seems to give better results with the LAH4 peptides for the delivery of siRNA [13]); confluence of the cells; percentage of serum in the medium during the 2–4 h of incubation. Another way to improve the transfection consists in not replacing the transfection medium after the 2 to 4 h incubation step. In this case, add culture medium containing serum into the wells.
15. It has been shown for PEI–DNA complexes that just after addition of the complexes to the cells, a short centrifugation step of the culture plates at 1000 rpm ($=138 \times g$) for 5 min increases the transfection efficiency [33]. This step may also be performed in order to increase the transfection of DNA/siRNA by LAH4 and derivatives.
16. When using cells expressing a reporter gene such as luciferase for measuring the efficiency of siRNA knockdown, one has to take into account the fact that the efficiency may vary from one cellular clone to another. Indeed, the number of integrated copies probably influences the level of expression and thus also the efficiency of silencing. Cells expressing a reporter gene are very useful for a first evaluation of the siRNA transfection efficiency of compounds. However, for further experiments, the best manner to evaluate the siRNA delivery efficiency of a

system consists in targeting endogenous genes such as GAPDH.

17. For siRNA experiments, it is important to choose the amount of peptide which gives the highest transfection efficiency associated with the lowest cytotoxicity. Indeed, cell toxicity may lead to nonspecific knockdown of genes.
18. For determination of the amount of protein, use a detergent-compatible detection kit. Notably, the total cellular protein content is also an indicator of cell viability.

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