Gene Delivery

Deutsche Ausgabe: DOI: 10.1002/ange.201504884
Internationale Ausgabe: DOI: 10.1002/anie.201504884

A Cell-Penetrating Foldamer with a Bioreducible Linkage for Intracellular Delivery of DNA

Céline Douat, Christopher Aisenbrey, Stéphanie Antunes, Marion Decossas, Olivier Lambert, Burkhard Bechinger, Antoine Kichler, and Gilles Guichard**

Abstract: *Despite significant advances in foldamer chemistry, tailored delivery systems based on foldamer architectures, which provide a high level of control over secondary structure, are curiously rare among non-viral technologies for transporting nucleic acids into cells. A potent pH-responsive, bioreducible cell-penetrating foldamer (CPF) was developed through covalent dimerization of a short (8-mer) amphipathic oligourea sequence bearing histidine-type units. This CPF exhibits a high capacity to assemble with pDNA and mediates efficient delivery of nucleic acids into the cell. Furthermore, it does not adversely affect cellular viability and was shown to compare favorably with a cognate peptide transfection agent based on His-rich sequences.*

Among the huge diversity of non-viral transport systems conceived to deliver genes into cells for therapeutic purpose,[1] naturally occurring and synthetic cationic cell-penetrating α-peptides (CPPs)[2] present favorable features that account for their rapid recent development.[3,4] Compared to purely cationic amphiphilic lipids and linear or branched polymers, CPPs have a well-defined composition that can be readily optimized by sequence manipulation and they are also potentially less cytotoxic. Complexes based on non-covalent charge interactions prevail over covalent CPP/nucleic acid (NA) conjugates, which facilitates synthetic accessibility by allowing the use of standard scalable synthetic procedures. Endocytosis is the main pathway by which CPP/NA complexes are taken up into the cells. Because of the low capacity of DNA to escape from endosomes, there is a need to endow CPPs with specific properties to enable endosomal escape and release of DNA into the cytoplasm.[5] It was postulated very early that CPPs containing multiple pH-responsive histidine (His) residues could provide an elegant solution to this problem by allowing pH-dependent membrane permeation and/or endosomal buffering (the so called proton-sponge effect).[4a,6] One remarkable example is the LAH4 peptide, a cationic amphipathic His-rich 26mer CPP (sequence: KKALLALALHHLAHLALHLALALKKA) with the ability to strongly promote the delivery of pDNA into various cell lines.[6b] Transfection efficiency was found to strikingly depend on the number of histidine residues and on their distribution along the polar face of the putative α-helical structure. Nonetheless, despite these recent important achievements, the use of synthetic peptides such as LAH4 still suffers from limitations, in part owing to their poor biostability.

Synthetic folded oligomers (foldamers)[7] can provide peptide and protein mimics that manifest unusual properties, including diminished susceptibility to degradation by proteases, high control over secondary structures, and a propensity to form assemblies in aqueous solution. Consequently, developing pH-responsive foldamer-type architectures that reproduce key structural features of CPPs such as LAH4 may represent a significant step towards the development of original transporters for NA delivery. Surprisingly, attempts to design foldamer-based delivery systems have thus far been limited, with no application to NA delivery and most current reports being restricted to investigation of cellular internalization in the absence of cargo molecules.[4b,8] In this work, we employed aliphatic oligoureas, a class of peptidomimetic foldamers composed of ethylene diamine type units connected by urea bonds, which are known to adopt a robust helical fold reminiscent of the α-helix.[9] In contrast to α-peptides, the helicity of oligoureas is largely independent of their primary sequence, a feature that greatly facilitates structure–activity relationship studies. We previously reported the design of short 8-mer oligourea sequences that mimic globally amphiphilic α-helical host-defence peptides.[10] Remarkably, these oligoureas show a high helical content in the vicinity of phospholipid membranes, display potent antimicrobial activity through a mechanism involving permeabilization of the bacterial membrane, and exhibit high resistance to proteolytic degradation. We reasoned that it might be possible to convert such antimicrobial foldamer sequences into pH-responsive delivery systems with high DNA transfer capacities and low toxicity by combining the

[*] Dr. C. Douat, S. Antunes, Dr. G. Guichard
Univ. Bordeaux, CBMN, UMR 5248
Institut Européen de Chimie et Biologie (IECB)
2 rue Robert Escarpit, 33607 Pessac (France)
E-mail: g.guichard@iecb.u-bordeaux.fr
Dr. C. Douat, S. Antunes, Dr. M. Decossas, Dr. O. Lambert, Dr. G. Guichard
CNRS, CBMN, UMR 5248, 33600 Pessac (France)
Dr. A. Kichler
Laboratoire "Vecteurs: Synthèse et Applications Thérapeutiques"
UMR 7199 CNRS—Université de Strasbourg, Labex Medalis
Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch cedex (France)
E-mail: kichler@unistra.fr
Dr. C. Aisenbrey, Dr. B. Bechinger
Membrane Biophysics and NMR, Chemistry Institute
University of Strasbourg-CNRS UMR7177
4, Rue Blaise Pascal, 67008 Strasbourg (France)
Dr. M. Decossas, Dr. O. Lambert
Univ. Bordeaux, CBMN, UMR 5248
All. Geoffroy Saint-Hilaire, 33600 Pessac (France)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201504884.

defined helical side-chain distribution of oligoureas with the key side-chain composition of LAH4.

To this end, we initially designed seven urea-based sequences presenting different patterns of side-chain distribution and different chain lengths. The first series of oligoureas (**1**–**4**; Figure 1) differ from previously reported cationic amphiphilic 8-mer antimicrobial sequences by the

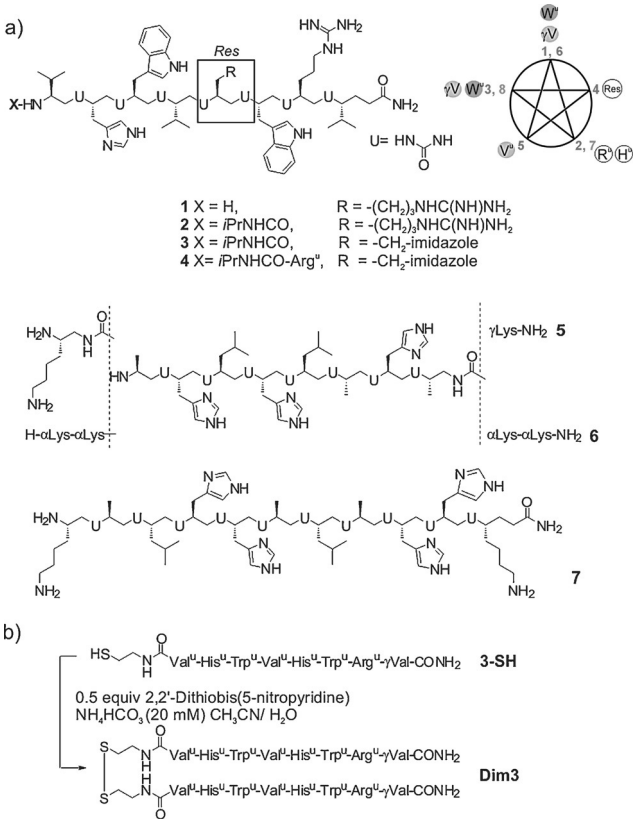

Figure 1. a) Structures of the pH-responsive amphiphilic oligoureas designed for the cellular delivery of NA. Foldamers **1**–**4** are derived from a previously reported antibacterial 8-mer oligourea sequence and **5**–**7** recapitulate the main features of the LAH4 CPP. b) Chemical synthesis of **Dim3** through disulfide condensation of **3-SH**, an analogue of **3** bearing a terminal cysteamine moiety.

presence of His- and Arg-type units (His^u, Arg^u) on the polar surface to maximize interaction with DNA and cellular delivery. The second set of oligoureas (**5**–**7**) ranging in size from 10 to 12 residues was designed to reflect more precisely the side-chain composition and distribution of the LAH4 peptide, that is, Ala and Leu side chains for the hydrophobic face, three to four central imidazole side chains, and extra Lys-type units at both ends of the sequence (Figure 1). All compounds were synthesized on solid support using Boc chemistry as previously described[11] and were obtained in satisfactory yield and high purity after RP-HPLC purification (see the Supporting Information).

With His^u-rich foldamers **1**–**7** in hand, we next investigated their ability to condense and transfect DNA into cells. A gel-shift assay was first carried out to determine the amount of oligomers **1**–**7** required to retard the migration of a luciferase expression plasmid (p-luc) during agarose gel electrophoresis (Figure S9 in the Supporting Information). A luciferase expression assay was then performed using the HEK293 human cell line, with the LAH4 peptide as a positive control.[12] In this assay, oligoureas **1**–**7** were used either alone or in the presence of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or $ZnCl_2$ as additives. DOPE is a fusogenic helper lipid that is used to synergistically enhance the transfection efficiency of various DNA/vector complexes.[13] Similarly, Zn^{2+} has been shown to promote membrane fusion and DNA delivery by His-rich polypeptide vectors when added to the transfection medium.[14] Although oligomers **1**–**7** were all found to bind DNA in the gel-shift assay, significant differences were observed in their ability to successfully deliver *p*-luc into cells (Figure 2a and Figure S10).

Of all the foldamers, oligourea 8-mer **3** proved to be the best one and, when used in combination with $ZnCl_2$, it exhibited good transfection efficiency that was only 5-fold lower than that of LAH4 (Figure 2a). Surprisingly, compound **4**, which differs from **3** only by the presence of an additional Arg^u residue at the terminus, was found to be rather less potent in this luciferase expression assay (Figure 2a and Figure S10) and none of the longer oligomers **5**–**7**, which were designed to more closely mimic the LAH4 sequence, led to significant transfection levels even in the presence of additives (data not shown).

Overall, these results demonstrate that 1) the presence of cationic residues at both extremities of oligoureas **5**–**7** does not facilitate DNA delivery into HEK293 cells, 2) the Trp^u residues in **3** likely play a positive role in the mechanism of DNA transfection as previously shown for cognate antibacterial oligoureas, and 3) the presence of only two His^u residues (as in **3**) is sufficient when combined with $ZnCl_2$ to mediate DNA condensation and uptake by cells.

To further improve the transfection efficacy of **3**, we thought to design longer oligomers that would more closely match the total number of polar and hydrophobic side chains of LAH4, while retaining the original side-chain distribution pattern of **3**. Instead of preparing a single-chain molecule with a sequence repeat, we chose to covalently dimerize sequence **3** through the formation a disulfide bridge. Expected advantages of a covalent dimerization approach include chemical modularity and increased stability of the resulting CPF/DNA complex.[15] Moreover, disulfide bonds are more prone to cleavage in the intracellular environment, where the glutathione concentration is much higher (100–1000×) than in the extracellular milieu.[16] The introduction of a bioreducible covalent crosslink such as a disulfide could thus promote effective vector unpacking once the complex is internalized in the cell and could reduce toxicity.[17,18] To test this hypothesis, we prepared **Dim3** through dithiol condensation of **3-SH**, an analogue of **3** bearing a terminal cysteamine moiety (see Figure 1b), and evaluated its capacity to associate with and transfect *p*-luc into HEK293 cells. Gel-shift experiments revealed that at an equivalent mass ratio, **Dim3** was more effective than monomeric **3** in complexing pDNA (Figure 2b). We found that the helical content was not modified by the dimerization, as revealed by a comparison of the CD spectra of **3** and **Dim3** recorded in phosphate buffer (40 mM)

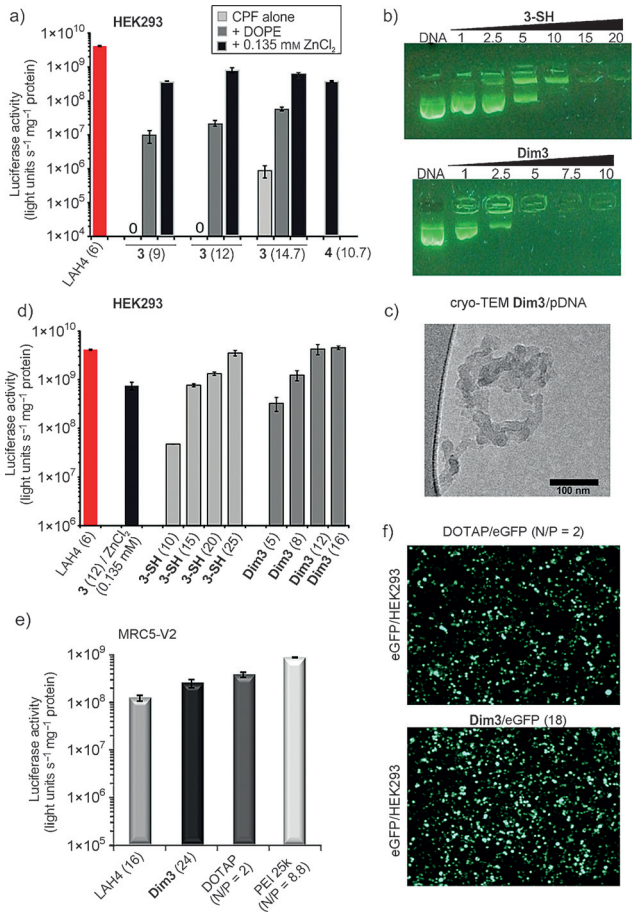

Figure 2. a) Transfection of HEK293 cells with *p*-luc in the presence of LAH4 and various oligoureas at different CPF/DNA mass ratios (values in brackets) without or with additives (0.135 mM $ZnCl_2$ or 3.3 μg DOPE/μg DNA). Luciferase levels were measured after 28 h ($n=2$). b) Gel-shift assay with *p*-luc (1 μg) and increasing amounts (from 1 to 20 μg) of **3-SH** or **Dim3**. DNA was visualized after staining with SYBR safe; the values above the gels correspond to the mass ratio of CPF/DNA. c) cryo-TEM of **Dim3**/pDNA assemblies. d) Comparison of the transfection efficiency of **3** + $ZnCl_2$, **3-SH**, and **Dim3** on HEK293 cells after 28 h of transfection ($n=2$). The amount of vector per μg DNA is given in brackets. e) Transfection levels obtained on MRC5-V2 cells after 28 h ($n=3$). 2.25 μg of *p*-luc were mixed with either LAH4, **Dim3**, DOTAP, or 25 kDa-PEI (experiments performed in triplicate). N/P corresponds to the amine/phosphate ratio. f) eGFP expression 48 h after transfection of HEK293 cells with eGFP/DOTAP or **Dim3** complexes ($n=2$).

at pH values of approximately 7.4 (Figure S7). We also used dynamic light scattering (DLS) to measure the size and surface charge of **Dim3**/DNA complexes because both parameters influence the cellular uptake of the particles. In the absence of salt, we found a majority of particles with a size of around 100 nm, together with some larger aggregates. Under salt-rich conditions, the complexes were larger, with a diameter of 700 nm. Thus, as for other transfection agents, including PEI[19] and LAH4,[20] the presence of salt induces aggregation. Finally, as expected, the surface charge of the complexes generated at the optimal w/w ratio for in vitro transfection was found to be positive (above +30 mV). Accordingly, cryo transmission electron microscopy (cryo-TEM) of a mixture of pDNA diluted in water and **Dim3** suspended in water revealed strings of small globular objects forming structures of about 100–200 nm in diameter (Figure 2c), while larger complexes were observed under salt-rich conditions (Figure S16). These globules indicate that **Dim3** firmly interacts with pDNA.

Remarkably, **Dim3** was found to outperform cognate **3** as a DNA delivery agent, with a level of luciferase expression upon transfection of HEK293 cells that compares favorably with that with LAH4 (Figure 2d). It is noteworthy that **Dim3** mediates effective gene delivery in the absence of any additives such as DOPE or $ZnCl_2$ (Figure S11). Interestingly, **3-SH** was also found to complex DNA and to exhibit significant transfection efficiency, albeit at higher a oligourea/DNA mass ratio compared to **Dim3** (Figure 2b,d and Figure S13). These results strongly suggest that **3-SH** spontaneously oxidizes into **Dim3** upon DNA condensation.[18b] The transfection potential of **Dim3** was next assessed with two other human cell lines, namely A549 and MRC5-V2 cells. Besides LAH4, two commercially available transfection reagents, a monocationic lipid *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP) and a branched 25 kDa polyethylenimine (PEI 25k),[21] were included as standards in these assays. The results obtained with the two cell lines show that **Dim3** is roughly as robust for cell transfection as the three other reagents (Figure 2e and Figure S12 for A549 cells). Concurrently, the ability of **Dim3** to mediate DNA uptake into the cell and promote gene expression was confirmed by fluorescence microscopy following transfection with a green fluorescent protein (GFP) expression plasmid. As shown in Figure 2f, the level of GFP expression 48 h after transfection was higher with **Dim3** than with DOTAP.

Next, cell viability in the presence of **Dim3**/p-luc complexes was evaluated on MRC5-V2 cells by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, and the results were compared with those measured in the presence of PEI and DOTAP (Figure S14). We found that **Dim3** and DOTAP did not reduce the cell viability, whereas PEI 25k exhibited some cytotoxicity (8% cell death).

This investigation was completed at the cellular level by the determination of the pH-dependency of **Dim3** action. It has been shown that inhibition of endosome acidification by a specific inhibitor of vacuole membrane H^+-ATPase such as concanamycin A significantly diminishes the transfection efficiency of PEI.[22] We thus asked whether the activity of **Dim3**, which contains imidazole groups that could become protonated during acidification of the endosomes, is also sensitive to such inhibitors. The results indicate that in the presence of this drug, the transfection efficiency of PEI and **Dim3** was diminished by 15- and 13-fold, respectively (Figure S15), whilst the efficiency of DOTAP, which is already protonated at pH 7, was almost unaltered. Consequently, these results underline the fact that acidification of the endocytic vesicles, and thus protonation of the imidazole groups, is required for optimal activity of **Dim3**. This is in good agreement with the p*K*a values determined for the two histidine-like units of oligourea **3**.[23]

In summary, we have developed an effective pH-responsive urea-based CPF for gene transfection through efficient thiol-mediated dimerization of a short (8-mer) amphiphilic cationic oligourea foldamer. **Dim3** is not only able to bind and shuttle DNA into cells but demonstrates transfection efficiency that compares favorably with LAH4, a His-rich peptide CPP with high transfection ability. Importantly, **Dim3** does not require the addition of helper lipid or Zn^{2+} to be active. In addition, **Dim3** displays no apparent cellular toxicity and is accessible with moderate synthetic effort. Future work aimed at optimizing this foldamer sequence to enable the transfection of a wider range of nucleic acids (e.g., siRNA) with cell-specific targeting moieties will be reported in due course.

Acknowledgements

This work was supported by the CNRS, Univ. Bordeaux, the Conseil Regional d'Aquitaine (Project #20091102003), ANR (TRANSPEP ANR-07-PCVI-0018, LabEx chemistry of Complex systems), and the RTRA International Center for Frontier Research in Chemistry. A Predoctoral fellowship from DGA and Conseil Régional d'Aquitaine (to S.A.) is gratefully acknowledged.

Keywords: cell-penetrating peptides · disulfide-mediated dimerization · foldamers · gene delivery · transfection

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 11133–11137
Angew. Chem. **2015**, *127*, 11285–11289

[1] a) M. A. Mintzer, E. E. Simanek, *Chem. Rev.* **2009**, *109*, 259–302; b) J. Hoyer, I. Neundorf, *Acc. Chem. Res.* **2012**, *45*, 1048–1056.

[2] a) D. M. Copolovici, K. Langel, E. Eriste, Ü. Langel, *ACS Nano* **2014**, *8*, 1972–1994; b) E. G. Stanzl, B. M. Trantow, J. R. Vargas, P. A. Wender, *Acc. Chem. Res.* **2013**, *46*, 2944–2954.

[3] a) P. Järver, Ü. Langel, *Drug Discovery Today* **2004**, *9*, 395–402; b) B. R. Meade, S. F. Dowdy, *Adv. Drug Delivery Rev.* **2007**, *59*, 134–140; c) P. Boisguérin, S. Deshayes, M. J. Gait, L. O'Donovan, C. Godfrey, C. A. Betts, M. J. A. Wood, B. Lebleu, *Adv. Drug Delivery Rev.* **2015**, *87*, 62–67; d) Z. Siprashvili, F. A. Scholl, S. F. Oliver, A. Adams, C. H. Contag, P. A. Wender, P. A. Khavari, *Hum. Gene Ther.* **2003**, *14*, 1225–1233.

[4] a) P. Midoux, A. Kichler, V. Boutin, J.-C. Maurizot, M. Monsigny, *Bioconjugate Chem.* **1998**, *9*, 260–267; b) J. E. Murphy, T. Uno, J. D. Hamer, F. E. Cohen, V. Dwarki, R. N. Zuckermann, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1517–1522; c) K. Rittner, A. Benavente, A. Bompard-Sorlet, F. Heitz, G. Divita, R. Brasseur, E. Jacobs, *Mol. Ther.* **2002**, *5*, 104–114; d) W. Li, F. Nicol, F. C. Szoka, Jr., *Adv. Drug Deliv. Rev.* **2004**, *56*, 967–985; e) C. Cordier, F. Boutimah, M. Bourdeloux, F. Dupuy, E. Met, P. Alberti, F. Loll, G. Chassaing, F. Burlina, T. E. Saison-Behmoaras, *PLoS ONE* **2014**, *9*, e104999; f) M. Li, S. Schlesiger, S. K. Knauer, C. Schmuck, *Angew. Chem. Int. Ed.* **2015**, *54*, 2941–2944; *Angew. Chem.* **2015**, *127*, 2984–2987.

[5] A. K. Varkouhi, M. Scholte, G. Storm, H. J. Haisma, *J. Controlled Release* **2011**, *151*, 220–228.

[6] a) C. Pichon, M. B. Roufaï, M. Monsigny, P. Midoux, *Nucleic Acids Res.* **2000**, *28*, 504–512; b) A. Kichler, A. J. Mason, B. Bechinger, *Biochim. Biophys. Acta Biomembr.* **2006**, *1758*, 301–307; c) P. Midoux, C. Pichon, J.-J. Yaouanc, P.-A. Jaffrès, *Br. J. Pharmacol.* **2009**, *157*, 166–178.

[7] a) S. H. Gellman, *Acc. Chem. Res.* **1998**, *31*, 173–180; b) G. Guichard, I. Huc, *Chem. Commun.* **2011**, *47*, 5933–5941.

[8] a) N. Umezawa, M. A. Gelman, M. C. Haigis, R. T. Raines, S. H. Gellman, *J. Am. Chem. Soc.* **2002**, *124*, 368–369; b) Y. Demizu, M. Oba, K. Okitsu, H. Yamashita, T. Misawa, M. Tanaka, M. Kurihara, S. H. Gellman, *Org. Biomol. Chem.* **2015**, *13*, 5617–5620; c) E. R. Gillies, F. Deiss, C. Staedel, J.-M. Schmitter, I. Huc, *Angew. Chem. Int. Ed.* **2007**, *46*, 4081–4084; *Angew. Chem.* **2007**, *119*, 4159–4162; d) J. Iriondo-Alberdi, K. Laxmi-Reddy, B. Bouguerne, C. Staedel, I. Huc, *ChemBioChem* **2010**, *11*, 1679–1685; e) C. Rosés, D. Carbajo, G. Sanclimens, J. Farrera-Sinfreu, A. Blancafort, G. Oliveras, A. D. Cirac, E. Bardají, T. Puig, M. Planas, L. Feliu, F. Albericio, M. Royo, *Tetrahedron* **2012**, *68*, 4406–4412.

[9] a) G. Guichard, A. Violette, G. Chassaing, E. Miclet, *Magn. Reson. Chem.* **2008**, *46*, 918–924; b) L. Fischer, P. Claudon, N. Pendem, E. Miclet, C. Didierjean, E. Ennifar, G. Guichard, *Angew. Chem. Int. Ed.* **2010**, *49*, 1067–1070; *Angew. Chem.* **2010**, *122*, 1085–1088; c) J. Fremaux, L. Fischer, T. Arbogast, B. Kauffmann, G. Guichard, *Angew. Chem. Int. Ed.* **2011**, *50*, 11382–11385; *Angew. Chem.* **2011**, *123*, 11584–11587; d) N. Pendem, Y. R. Nelli, C. Douat, L. Fischer, M. Laguerre, E. Ennifar, B. Kauffmann, G. Guichard, *Angew. Chem. Int. Ed.* **2013**, *52*, 4147–4151; *Angew. Chem.* **2013**, *125*, 4241–4245; e) N. Pendem, C. Douat, P. Claudon, M. Laguerre, S. Castano, B. Desbat, D. Cavagnat, E. Ennifar, B. Kauffmann, G. Guichard, *J. Am. Chem. Soc.* **2013**, *135*, 4884–4892.

[10] a) A. Violette, S. Fournel, K. Lamour, O. Chaloin, B. Frisch, J.-P. Briand, H. Monteil, G. Guichard, *Chem. Biol.* **2006**, *13*, 531–538; b) P. Claudon, A. Violette, K. Lamour, M. Decossas, S. Fournel, B. Heurtault, J. Godet, Y. Mély, B. Jamart-Grégoire, M.-C. Averlant-Petit, J.-P. Briand, G. Duportail, H. Monteil, G. Guichard, *Angew. Chem. Int. Ed.* **2010**, *49*, 333–336; *Angew. Chem.* **2010**, *122*, 343–346.

[11] Y. R. Nelli, C. Douat-Casassus, P. Claudon, B. Kauffmann, C. Didierjean, G. Guichard, *Tetrahedron* **2012**, *68*, 4492–4500.

[12] A. Kichler, C. Leborgne, J. Marz, O. Danos, B. Bechinger, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1564–1568.

[13] a) J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, P. L. Felgner, *J. Biol. Chem.* **1994**, *269*, 2550–2561; b) A. Kwok, G. A. Eggimann, J.-L. Reymond, T. Darbre, F. Hollfelder, *ACS Nano* **2013**, *7*, 4668–4682; c) T. Yu, X. Liu, A. L. Bolcato-Bellemin, Y. Wang, C. Liu, P. Erbacher, F. Qu, P. Rocchi, J. P. Behr, L. Peng, *Angew. Chem. Int. Ed.* **2012**, *51*, 8478–8484; *Angew. Chem.* **2012**, *124*, 8606–8612.

[14] a) A. S. Ulrich, W. Tichelaar, G. Förster, O. Zschörnig, S. Weinkauf, H. W. Meyer, *Biophys. J.* **1999**, *77*, 829–841; b) C. Pichon, B. Guérin, M. Réfrégiers, C. Gonçalves, P. Vigny, P. Midoux, *J. Gene Med.* **2002**, *4*, 548–559.

[15] H. L. Åmand, B. Nordén, K. Fant, *Biochem. Biophys. Res. Commun.* **2012**, *418*, 469–474.

[16] F. G. Ottaviano, E. D. Handy, J. Loscalzo, *Circ. J.* **2008**, *72*, 1–16.

[17] a) G. Saito, J. A. Swanson, K.-D. Lee, *Adv. Drug Delivery Rev.* **2003**, *55*, 199–215; b) S. Bauhuber, C. Hozsa, M. Breunig, A. Göpferich, *Adv. Mater.* **2009**, *21*, 3286–3306; c) R. Cheng, F. Feng, F. Meng, C. Deng, J. Feijen, Z. Zhong, *J. Controlled Release* **2011**, *152*, 2–12; d) S. Son, R. Namgung, J. Kim, K. Singha, W. J. Kim, *Acc. Chem. Res.* **2012**, *45*, 1100–1112.

[18] a) T. Blessing, J.-S. Remy, J.-P. Behr, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1427–1431; b) D. L. McKenzie, K. Y. Kwok, K. G. Rice, *J. Biol. Chem.* **2000**, *275*, 9970–9977; c) D. L. McKenzie, E. Smiley, K. Y. Kwok, K. G. Rice, *Bioconjugate Chem.* **2000**, *11*, 901–909.

[19] L. Wightman, R. Kircheis, V. Rössler, S. Carotta, R. Ruzicka, M. Kursa, E. Wagner, *J. Gene Med.* **2001**, *3*, 362 – 372.
[20] A. Kichler, C. Leborgne, O. Danos, B. Bechinger, *J. Mol. Med.* **2007**, *85*, 191 – 201.
[21] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297 – 7301.
[22] A. Kichler, C. Leborgne, E. Coeytaux, O. Danos, *J. Gene Med.* **2001**, *3*, 135 – 144.
[23] The pK_a values of both His^u side chains of **3** were determined by ^{1}H NMR spectroscopy in an aqueous environment. His$_4^u$ and His$_7^u$ exhibited similar p*K*a values of 6.80 and 6.93 respectively (see Figure S8 in the Supporting Information).

Received: May 29, 2015
Revised: July 4, 2015
Published online: August 5, 2015

