Synthesis and cellular uptake of cell delivering PNA–peptide conjugates†

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The synthesis and cellular uptake of fluorescently labelled PNA–peptide conjugates is described; Dde/Mmt protected PNA monomers, fully orthogonal to Fmoc chemistry, were used to develop a flexible strategy to give Peptide Nucleic Acids conjugated to *tri*- and *hepta*-arginine and the short basic $Tat_{48–57}$ peptide as examples of cellular penetrating peptides, thereby allowing efficient cellular delivery of PNA into cells.

Peptide Nucleic Acids (PNAs) are used extensively as DNA mimics[1] and have become one of the most important of DNA analogues due to their hybridisation abilities with complementary DNA sequences (following standard Watson–Crick base paring rules), together with their relatively simple chemical synthesis and their chemical and biological robustness. Indeed the binding affinities of PNA with DNA, under physiological conditions, are greater than for the corresponding DNA/DNA counterparts, mainly due to the absence of electrostatic repulsion forces present in DNA/DNA or DNA/RNA interactions.

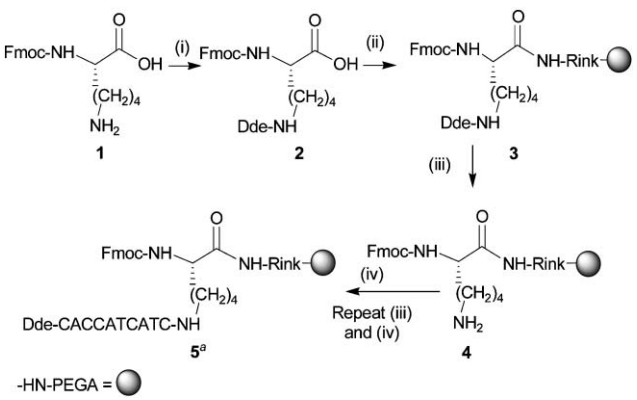
DNA delivery into cells, for a variety of applications such as gene therapy[2,3] and cellular modulation,[4] can be accomplished using a number of transfection agents which are typically cationic lipid based. These work by DNA compaction and neutralisation of the negatively charged DNA backbone and delivery into cells following the formation of lipoplexes. However, PNA lacks the formal charges found in DNA and thus classical transfection agents are often unable to deliver PNA into cells. A number of different approaches[5] have therefore been undertaken in order to improve their cellular uptake including, for example, the incorporation of multiple positive charges into the PNA backbone.[6] Other methods used for delivery have been the conjugation of the PNA to cellular penetrating peptides, which themselves are often of a polycationic nature, such as the Tat peptide from HIV or Anntenapedia peptide derivatives.[5,7–9]

However the combined and orthogonal synthesis of PNA–peptide conjugates has been far from ideal due to the combination of sets of monomer which were not truly orthogonal and which rely on using traditional solid phase chemistry with the coupling of amino acids followed by PNA monomers or *vice versa*[6] or ligation through disulfide bonds.[8,10] Here we report the flexible synthesis of fluorescently labelled PNA conjugates to arginine-rich peptides[7] and the $Tat_{48–57}$ decapeptide[11] based on a branched lysine core which was derivatised with two orthogonal protecting groups, Fmoc and Dde. This allowed a highly flexible approach to synthesis, with the peptide or PNA strands being prepared at will, with full control of termini labelling of either the PNA and/or the peptide.

To prepare these conjugates Fmoc-Lys(Dde)-OH[12] was coupled to amino-Rink-PEGA resin (Scheme 1)[13] and the Dde group was cleanly and selectively deprotected at pH 6 with hydroxylamine hydrochloride/imidazole (1/0.75 equiv.) in NMP/DMF (5/1) to give rise to **4**.[14] This was coupled to a series of Dde/Mmt protected PNA monomers which were then rapidly and cleanly extended to give the 10 mer PNA construct **5** with complete retention of the Fmoc protecting group.

Resin **5** was then used for the construction of four different PNA conjugates in a parallel fashion. One of the pools (control sample) was thus labelled on the terminus of the PNA strand with fluorescein prior to cleavage from the resin. The other three samples were used in standard Fmoc chemistry protocols to give *tri*-arginine, *hepta*-arginine and the short basic $Tat_{48–57}$ decapeptide (Scheme 2),[11] before labelling the end of the PNA sequence with 5(6)-carboxyfluorescein (FAM).

Conjugates **9–12** were obtained following Fmoc deprotection and acidic cleavage (TFA/TIS/DCM: 90/5/5) from the solid support, with all acid-labile side chain protecting groups (peptide and PNA) cleaved concomitantly.[15] Fig. 1 shows the HPLC[16] trace of the crude PNA–peptide conjugate **12** showing the degree of purity and homogeneity of the final compound and the efficiency of synthesis.



Scheme 1 Synthesis of a 10 mer PNA using Dde/Mmt protected PNA monomers.[14] *Reagents and conditions*: (i) Dde-OH (2 equiv., 26 mM), TFA (0.1 equiv.) in EtOH, reflux, 2 days;[12] (ii) H_2N-Rink-PEGA (1 equiv.), PyBop (5 equiv., 0.1 M), DIPEA (11 equiv.) in DMF, 3 h; (iii) 20% $NH_2OH.HCl$/imidazole (1/0.75 equiv.), in NMP/DMF (5/1), 1 h;[14] (iv) Dde-PNA-OH (5.5 equiv., 0.11 M), PyBop (5 equiv.), NEM (11 equiv.) in DMF, 3 h. Adenine and cytosine are Mmt protected.

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b5/b503777h/

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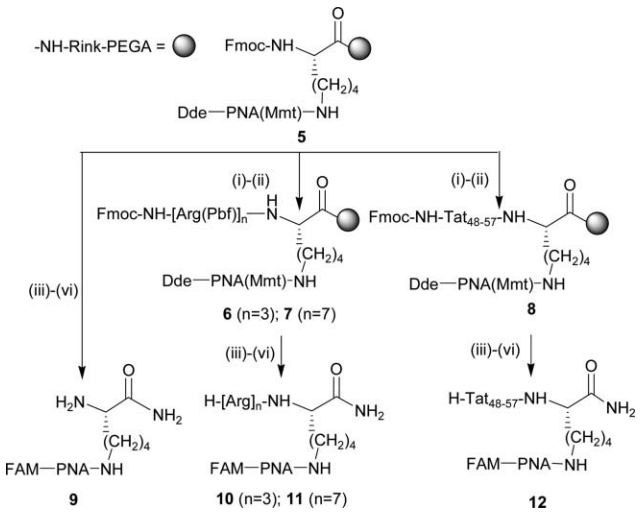

Scheme 2 Fluorescein labelled PNA–peptide conjugates synthesis. *Reagents and conditions*: (i) 20% piperidine in DMF (2 × 5 min); (ii) Fmoc-AA-OH (5.5 equiv.), PyBop (5 equiv.), DIPEA (11 equiv.), HOBt (5.5 equiv.) in DMF, 3 h; Repeat (i) and (ii) as necessary. (iii) $NH_2OH.HCl$/imidazole, in DMF/NMP, 1 h;[11] (iv) FAM-OH (5.5 equiv.), PyBop (5 equiv.), DIPEA (11 equiv.) in DMF, 3 h; (v) 20% piperidine in DMF; (vi) TFA/TIS/CH_2Cl_2 (90/5/5), 1.5 h. PNA sequence = -CACCATCATC. $Tat_{48–57}$ = -HN-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-CO- (in **8** side chains protected with acid labile protecting groups, Arg(Pbf), Lys(Boc) and Gln(Trt)); FAM = 5(6)-carboxyfluorescein.

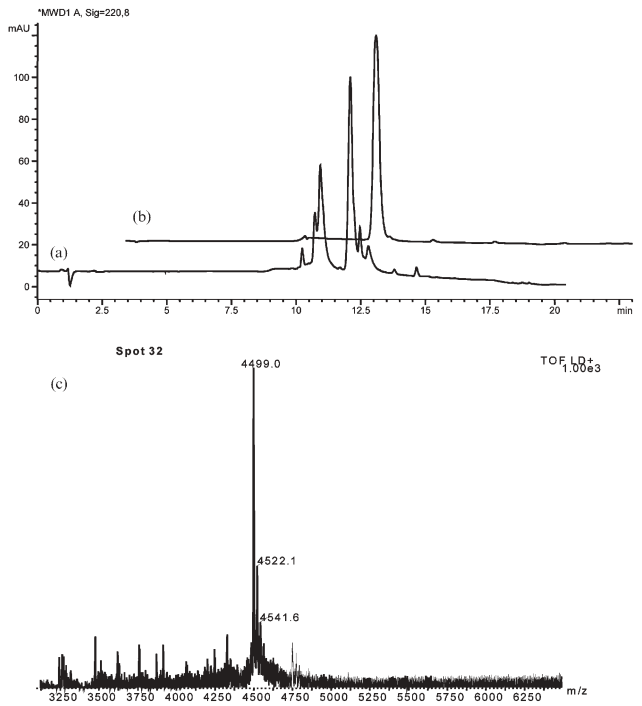

Fig. 1 HPLC[16] analysis of **12** (a) crude and (b) purified (λ = 220 nm). (c) MALDI-TOF MS.

The cellular internalisation of these PNA conjugates at a concentration of 500 nM was determined by flow cytometry using HEK293T cells.[17] Fig. 2 shows FACS analysis histograms of both untreated cells (Fig. 2a) and cells incubated with *hepta*-arginine conjugate **11** (Fig. 2b). Fluorescence intensity displayed by cells treated with **11** was on average 30-fold higher than for untreated cells, demonstrating the high efficiency of the conjugates as carrier systems of PNA oligomers. The relative fluorescence intensities displayed by cells incubated with all the conjugates **9–12** are shown in Fig. 2c. The highest levels of uptake were shown by conjugates **11** and **12** while conjugate **10**, having the shortest polycationic chain (*tri*-arginine), displayed the poorest uptake. As expected, the lack of any peptide delivery conjugate resulted in no uptake (control PNA **9**). The efficiencies of uptake by both the polycationic conjugate **11** and the Tat conjugate **12** were comparable, suggesting a similar mechanism of cellular uptake.

In conclusion, the synthesis of branched PNA–peptide conjugates and their intracellular delivery have been demonstrated. The strategy presented exploits the newly found orthogonality between Dde and Fmoc chemistries to allow fluorescent, branched PNA conjugates to be prepared using parallel synthesis, and allowed a set of fluorescent PNA-conjugated compounds to be synthesised and tested on HEK293T cells. Conjugates bearing a *hepta*-arginine peptide **11** and the $Tat_{48–57}$ decapeptide **12** were taken up by cells with high efficiency and show that the Dde/Fmoc approach to PNA and peptide synthesis is a powerful alternative to currently used methods, allowing high flexibility in order to modify at will either branch of the conjugates. In addition, since the fluorophore is on the PNA (rather than the peptide) we have unequivocally demonstrated the delivery of PNA into cells.

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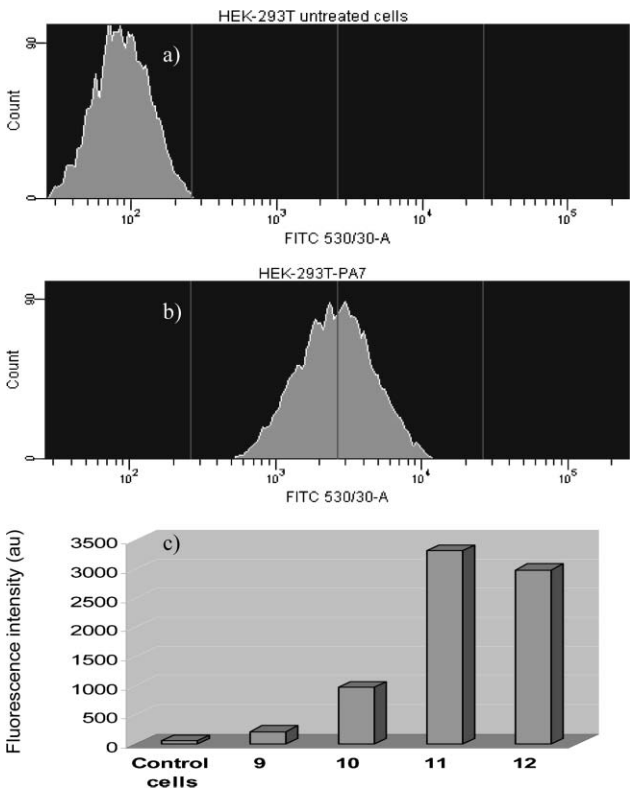

Fig. 2 FACS analysis of both untreated cells (a) and cells incubated with **11** (b) and relative fluorescence intensities (c).

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Notes and references

1 P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, 1991, **254**, 1497.
2 G. Brooks, *Gene Ther.*, 2002, 1.
3 K. Ciftci and P. Trovitch, *Adv. Drug Delivery Rev.*, 2000, **43**, 57.
4 X. Jiao, R. Y.-H. Wang, Z. Feng, H. J. Alter, W. Shih and J. Wai-Kuo, *Hepatology*, 2003, **37**, 452.
5 U. Koppelhus and P. E. Nielsen, *Adv. Drug Delivery Rev.*, 2003, **55**, 267.
6 A. Dragulescu-Andrasi, P. Zhou, G. He and D. H. Ly, *Chem. Commun.*, 2005, 244; P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner and D. H. Ly, *J. Am. Chem. Soc.*, 2003, **125**, 6878.
7 S. Futaki, I. Nakase, T. Suzuki, Z. Youjun and Y. Sugiura, *Biochemistry*, 2002, **41**, 7925; S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda and Y. Sugiura, *J. Biol. Chem.*, 2001, **276**, 5836.
8 U. Koppelhus, S. K. Awasthi, V. Zachar, H. U. Holst, P. E. Ebbesen and P. E. Nielsen, *Antisense Nucleic Acid Drug Dev.*, 2002, **12**, 51.
9 L. Good, S. K. Awasthi, R. Dryselius, O. Larsson and P. E. Nielsen, *Nat. Biotechnol.*, 2001, **19**, 360; G. Cutrona, E. M. Carpaneto, M. Ulivi, S. Roncella, O. Landt, M. Ferrarini and L. C. Boffa, *Nat. Biotechnol.*, 2000, **18**, 300; M. Pooga, U. Soomets, M. Hallbrink, M. Valkna, K. Saar, K. Rezaei, U. Kahl, J. X. Hao, X. J. Xu, Z. Wiesenfeld-Hallin, T. Hokfelt, T. Bartfai and U. Langel, *Nat. Biotechnol.*, 1998, **16**, 857.
10 M. C. de Koning, D. V. Filippov, N. Meeuwenoord, M. Overhand, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 2002, **43**, 8173.
11 E. Vives, *J. Mol. Recognit.*, 2003, **16**, 265.
12 S. R. Chhabra, B. Hothi, D. J. Evans, P. D. White, B. W. Bycroft and C. Chan, *Tetrahedron Lett.*, 1998, **39**, 1603.
13 M. Meldal, *Tetrahedron Lett.*, 1992, **33**, 3077.
14 J. J. Diaz-Mochon, L. Bialy and M. Bradley, *Org. Lett.*, 2004, **6**, 1127.
15 Analysis by MALDI-TOF MS, following precipitation with ice-cold ether, centrifugation and purification by semipreparative HPLC[16] gave molecular ions which agreed, in all cases, with the proposed structures. MALDI-TOF MS Analysis: **9** $C_{132}H_{157}N_{57}O_{36}$, 3118 (average mass), mass found *m*/*z*: 3119.3 $[M + H]^+$; **10** $C_{150}H_{193}N_{69}O_{39}$, 3586.5 (average mass), mass found *m*/*z*: 3589.6 $[M + H]^+$; **11** $C_{174}H_{241}N_{85}O_{43}$, 4211.3 (average mass), mass found *m*/*z*: 4213.4 $[M + H]^+$; **12** $C_{187}H_{264}N_{88}O_{47}$, 4496.6 (average mass), mass found *m*/*z*: 4499.0 $[M + H]^+$.
16 HPLC analysis was carried out on an HP1100 equipped with a Phenomenex column (Prodigy 5 μm ODS(3); 150 mm × 2 mm). Mobile phases were HPLC grade, water containing 0.1% TFA and MeCN. Method: 100% H_2O for 5 min, then increasing from 0% to 60% MeCN over 10 min.
17 HEK-293T (human embryonic kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 10% fetal calf serum (FCS) and 100 units/ml penicillin/streptomycin until 80% confluency. Cells were suspended using trypsin/EDTA and counted. Cells were then seeded in 24 well plates at 4×10^4 cells per well and incubated overnight. Then cells were washed with warm PBS buffer and preincubated in 350 μl serum free medium (SFM) at 37 °C for 30 min. Compounds **9–12** were mixed with serum free medium at a final concentration of 500 nM. To each well different samples of **9–12** were added and incubated at 37 °C for 2 h. Each experiment was performed in triplicate. The internalisation of free fluorescein, under the same conditions, was tested simultaneously and untreated cells were used as negative control. After incubation, cells were washed twice with PBS, harvested with trypsin/EDTA, washed again and resuspended in 1% FCS in PBS buffer. To analyze the internalisation of fluorescein-labeled PNA conjugates, cell-associated fluorescence was determined by flow cytometry analysis using a FACSAria flow cytometer (Becton Dickinson). A total of 10 000 events per sample were analyzed. FITC (530/30 nm) band pass filters were used for fluorescence analysis of the cell suspensions. None of the conjugates **9–12** assayed was found to be toxic as verified by an MTT toxicity and a trypan blue assay to determine cell viability (see Supplementary Information).

