COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Cell penetrable peptoid carrier vehicles: synthesis and evaluation†

Ilaria Peretto,[a] Rosario M. Sanchez-Martin,[b] Xui-hong Wang,[b] John Ellard,[b] Stifun Mittoo[b] and Mark Bradley*[b]

[a] *NiKem Research S.r.l., Via Zambelletti, 25, 20021 Baranzate di Bollate (MI), Italy*

[b] *Department of Chemistry, University of Southampton, Highfield, Southampton, UK SO17 1BJ. E-mail: mb14@soton.ac.uk; Fax: +44 (0)23 80596766; Tel: +44 (0)23 80593598*

Received (in Cambridge, UK) 6th June 2003, Accepted 25th July 2003
First published as an Advance Article on the web 11th August 2003

Using a highly efficient solid-phase route a series of fluorescein conjugated peptoid oligomers were synthesised and observed to display remarkable cell penetrating properties, offering the possibility of highly efficient cellular targeting.

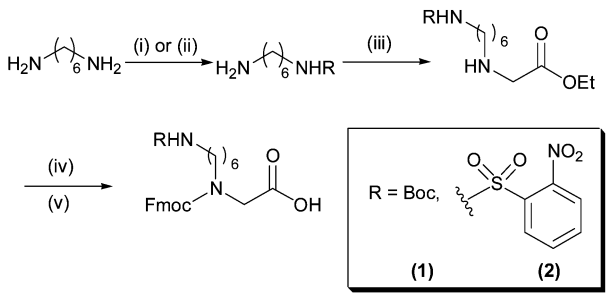
Cellular access to foreign compounds, be they probes of a diagnostic nature or potential drug candidates, is generally severely limited by the solubility properties of the material in question. The properties must be such that the substances are sufficiently polar to dissolve in biological fluids yet not so polar that they are unable to cross the relatively non-polar cell bilayer. It has been demonstrated that cellular penetration may be accomplished by the HIV Tat peptide which has been successfully used as a delivery vehicle into cells, this ability being attributed to the relatively short, but highly basic Tat (49–57) sequence (RKKRRQRRR).[1] Reports detailing the use of structurally analogous cationic β-peptide or peptoid oligomers based on lysine or arginine residues of the Tat sequence have been reported and potentially represent an efficient means of translocation across cell membranes.[2–6] Equally, poly-L-lysine has been shown to effectively increase the uptake of small organic molecules[7] and arginine rich molecules have been developed to successfully aid the administration of otherwise poorly absorbed drugs.[8]

The aims of this study were the efficient synthesis and evaluation of a number of peptoid based, polycationic, cell penetrable moieties. A solid phase route was developed to enable firstly efficient synthesis, without having to resort to the more classical methods of peptoid synthesis, and secondly to enable subsequent derivatisation of the peptoid thus allowing cellular access. Efforts were concentrated on peptoid derivatives of lysine because cellular penetration has been reported to be independent of the stereochemistry of polycationic polymers tested and peptoids are known to possess a greater stability to proteolytic degradation than peptides.[9]

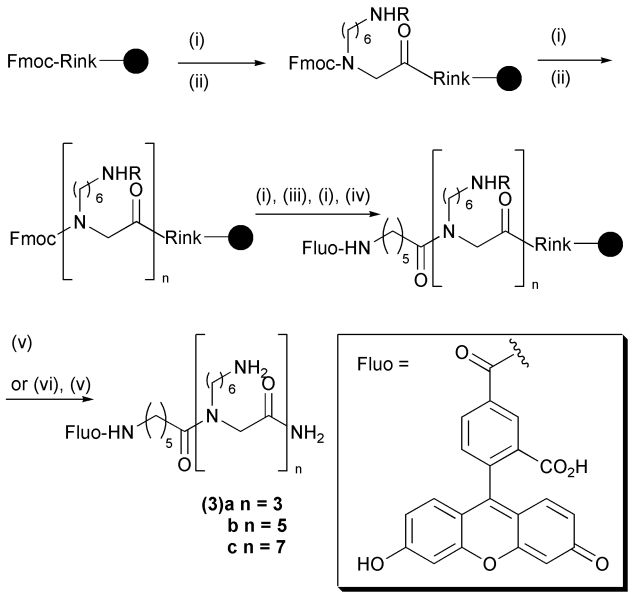
Cell penetrable peptoids were therefore synthesised and conjugated to fluorescein and their cellular uptake assayed on 2 cell lines. The peptoids were constructed on solid phase using monomer units **1** and **2**. These were prepared by reacting ethyl bromoacetate with mono-Boc or mono-2-nitrobenzenesulfonyl protected 1,6-hexanediamine (Scheme 1). Saponification of the resultant ester followed by *N*-Fmoc protection with Fmoc-succinimide made available large quantities of monomers **1** and **2** for solid phase oligomer synthesis.

The peptoids were assembled on polystyrene aminomethyl resin functionalised with the Rink amide linker. In each of the coupling steps the Fmoc-protected monomer, **1** or **2**, and bromo-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBrOP) were added to the resin in a two-fold molar excess (0.08 M) (PyBrOP was necessary to enable efficent coupling of the secondary amine). Deprotection of the Fmoc group (20% piperidine in DMF) and repeated coupling gave oligomers of the required length. A fluorescein group was then covalently attached to the *N*-terminus of each oligomer, separated by a six carbon (aminohexanoic acid) spacer unit (Scheme 2) to enable efficient coupling.

Resin bound peptoids **3** were cleaved from the solid support by treatment with a mixturre of TFA : TIS (95 : 5) for 3 h. The crude materials were precipitated in cold diethyl ether and purified by semi-preparative RP-HPLC (> 95% purity).[10] The use of the sulfonamide during the construction of peptoids **3** (Scheme 2) allowed selective deprotection (2-mercaptoethanol)



Scheme 1 Synthesis of monomer units **1** and **2**. *Reagents and conditions*: i, Boc_2O (0.13 eq.), dioxane, 24 h, 91%; ii, 2-nitrobenzenesulfonyl chloride (0.13 eq), dioxane, 24 h, 87%; iii, ethyl bromoacetate (1 eq.), Et_3N (3 eq.), THF, 24 h (R = Boc, 55%; R = $O_2NC_6H_4SO_2$, 42%); iv, NaOH (1 eq.), CH_3OH : H_2O : dioxane (3 : 1 : 8); v, Fmoc-OSu (1 eq.), H_2O : CH_3CN (1 : 1), pH = 8.5–9.0, 45 min (R = Boc, 40%; R = $O_2NC_6H_4SO_2$, 60% over two steps).



Scheme 2 Synthesis of peptoid oligomers **3** (*n* = 3, 5, 7). *Reagents and conditions*: i, 20% piperidine/DMF; ii, **1** or **2** (2 eq.), PyBrOP (2 eq.); *N*,*N*-diisopropylethylamine (DIPEA) (4 eq.), CH_2Cl_2; iii, *N*-Fmoc-aminohexanoic acid (2 eq.), PyBrOP (2 eq.); DIPEA (4 eq.), CH_2Cl_2; iv, 5(6)-carboxyfluorescein (3 eq.), 1-hydroxybenzotriazole (HOBt) (3 eq.), *N*,*N*′-diisopropylcarbodiimide (DIC) (3 eq.), DMF : CH_2Cl_2 (1 : 1); v, TFA : triisopropylsilane (TIS) (95 : 5); vi, 2-mercaptoethanol (0.3 M)/DBU (0.3 M) in DMF (3 × 45 min).

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b3/b306438g/

DOI: 10.1039/b306438g

and subsequent functionalisation of specific amine groups while still attached to the solid phase.

The ability of the peptoid oligomers to enter cells was assayed by fluorescence microscopy and flow cytometry on HEK293T (human embryonic kidney) and B16F10 (mouse melanoma) cells. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 4 mM glutamine and the labelled oligomers were tested in parallel (in triplicate), using free fluorescein as a control.

The peptoids **3a,b,c** (n = 3, 5, 7) were incubated with the HEK293T cells at 37 °C for 3 h at concentrations of 0.1, 1, 10 and 100 μM. Results indicated that the order of uptake of the peptoids was dependent on the number of monomer units of the oligomer. Higher oligomers yielded greater cellular penetration (**3c** (n = 7) > **3b** (n = 5) > **3a** (n = 3)) as evidenced by a greater population of fluorescently labelled cells. Quantification by laser scanning cytometry (Table 1 and Fig. 1) showed oligomer **3a** (n = 3) at 10 μM resulted in 99% of the cell population being labelled after 4 h and this labelling was equalled by **3c** (n = 7) at an equivalent concentration. Further examination of the peptoids by fluorescence microscopy revealed that cellular uptake was concentration dependent, fewer cells exhibiting fluorescence at lower concentrations, although in all cases cell permeability was evident. A similar result was observed with the B16F10 cell line, though it was noticed that cellular penetration with peptoid **3a** (n = 3) was only observed at concentrations as high as 50 μM. These findings are complementary to reports by other groups who have also indicated a greater uptake rate the larger the oligomer.[2,5] In all cases, free fluorescein as control showed no marked cellular uptake stipulating that the carrier molecules were playing a vital transportation role.

Table 1 FACS analysis on HEK293T cells with oligomer **3**

Oligomer **3**[a]	% Incorporation
n = 3	99
n = 5	93[b]
n = 5	97
n = 7	99

[a] c = 10 μM, 37 °C, 4 h. [b] c = 10 μM, 20 °C, 4 h.

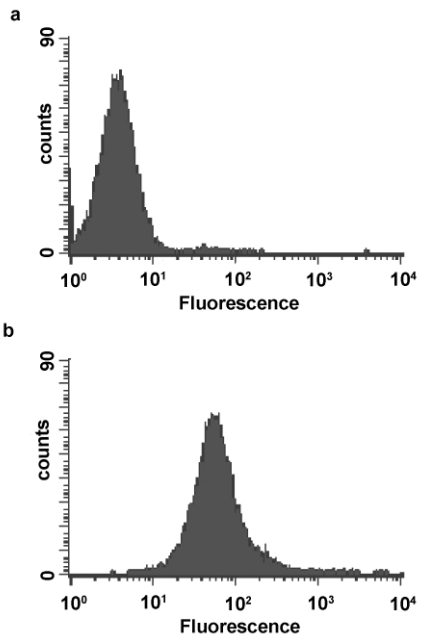


Fig. 1 FACS analysis of HEK293T cells: (a) untreated cells, (b) incubation of cells with peptoid **3** (n = 7).

For both cell types, treatment of the cells with 0.5% sodium azide for 30 min prior to exposure to the peptoids (10 μM) resulted in complete inhibition of uptake, indicating that the transport mechanism of these cationic homopolymers is energy dependent. In addition incubation of **3** (n = 3, 5, 7, at c = 10 μM) at varying temperatures (4, 20 and 37 °C) revealed that lowering the temperature greatly affected the rate of uptake. At 4 °C no internalisation was observed, whereas at 20 °C only slightly fewer cells showed green fluorescence compared to 37 °C where virtually all the cells were fluorescently labelled (Table 1). These temperature-dependent results may offer an insight into the mechanism of uptake, suggesting possible endocytosis pathways which have been implicated in the transport of small organic molecules covalently bound to structurally similar poly-L-lysine[7,11] though other transport pathways cannot be completely dismissed.

Finally none of the peptoids **3** (n = 3, 5, 7) assayed were found to be toxic at any of the concentrations tested, as verified by a MTT[12] toxicity and a trypan blue assay[13] to ascertain cell viability.

A versatile and efficient solid phase approach has been developed for the synthesis of these cell permeable peptoid oligomers. A series of fluorescein conjugates were constructed and fluorescence microscopy and FACS analysis indicated that these materials possessed the ability to successfully penetrate the membrane of cells with internal localisation observed in the cytosol and the nucleus, with concentration in the nucleoli. Of the peptoid oligomers tested **3c** (n = 7) exhibited maximum internalisation (> 99% of cells labelled) and enabled efficient cellular access.

This work was supported by a grant from the MRC (ROPA).

Notes and references

1 H. Nagahara, A. M. Vocero-Akbani, E. L. Snyder, A. Ho, D. G. Latham, N. A. Lissy, M. Becker-Hapak, S. A. Ezhevsky and S. F. Dowdy, *Nature Medicine*, 1998, **4**, 1449–1452.
2 C. Garcia-Echeverria and S. Ruetz, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 247–251.
3 D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman and J. B. Rothbard, *J. Peptide Res.*, 2000, **56**, 318–325.
4 N. Umezawa, M. A. Gelman, M. C. Haigis, R. T. Raines and S. H. Gellman, *J. Am. Chem. Soc.*, 2002, **124**, 368–369.
5 M. Rueping, Y. Mahajan, M. Sauer and D. Seebach, *ChemBioChem*, 2002, **3**, 257–259.
6 P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman and J. B. Rothbard, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 13003–13008.
7 H. J. P. Ryser and W.-C. Shen, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 3867–3870.
8 J. B. Rothbard, S. Garlington, Q. Lin, T. Kirschberg, E. Kreider, P. L. McGrane, P. A. Wender and P. A. Khavari, *Nature Medicine*, 2000, **6**, 1253–1257.
9 S. M. Miller, R. J. Simon, S. Ng, R. N. Zuckermann, J. M. Kerr and W. H. Moos, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2657–2662.
10 Semi-preparative HPLC purifications were carried out on a Hewlett Packard HP-1100 system equipped with a Phenomenex Luna C18 reverse phase column (150 mm × 10 mm, 5 μm). Solvents used were: A: 0.1% TFA in H_2O and B: 0.04% TFA in CH_3CN, gradient 0% B to 65% B over 20 min, then 100% B for 5 min. The column effluent was monitored using a detector wavelength of 220 nm.
11 H. J. P. Ryser, I. Drummond and W. C. Shen, *J. Cell. Physiol.*, 1982, **113**, 167–178.
12 T. Mossman, *J. Immunol. Methods*, 1983, **65**, 55–63.
13 H. J. Phillips and J. E. Terryberry, *Exp. Cell Res.*, 1957, **13**, 341–347.