# Efficient gene transfection with functionalised multicalixarenes†

**Ruth Lalor, Jessica L. DiGesso, Anja Mueller and Susan E. Matthews***

*Received (in Cambridge, UK) 7th August 2007, Accepted 5th September 2007*
*First published as an Advance Article on the web 14th September 2007*
**DOI: 10.1039/b712100h**

**Novel amino-functionalised multicalixarenes have been synthesised which show low cellular toxicity, effective DNA binding and, when featuring aliphatic amines, are efficient gene transfection agents.**

The efficient introduction of genetic material into cells is an area of considerable interest both to provide tools for biological research and for the development of gene-based therapeutics.[1,2] Synthetic vectors, in particular linear polymers[1] and dendrimers[2] incorporating a high level of surface positive charge, or cationic lipids[3] have proved useful in promoting gene transfection. However, these are often unable to afford transfection without the addition of either a buffering agent such as chloroquine to reduce DNA degradation in acidic lysosomes or an additional endosomolytic agent such as dioleoyl-L-α-phosphatidyethanolamine (DOPE).[4] One-component efficient gene transfection systems are thus an important target.

Calixarenes have recently been shown to have potential in a wide range of biological process and diseases including applications in drug delivery,[5,6] as ion channel mimics[7] and as anti-tuberculosis agents.[8] Single calixarenes,[9] featuring guanidinium functionality, have been demonstrated to promote DNA transfection and these studies indicate that architecture, size and lipophilicity are important in achieving transfection with less lipophilic derivatives requiring the addition of a DOPE adjuvent. Equally the interaction of amino-calixarene dimers[10] with DNA has recently also been reported although their transfection ability was not discussed.

The incorporation of calixarenes into dendrimeric or multicalixarene systems offers the potential of combining high levels of surface functionality, with a increased degree of pre-organisation, amphiphilic characteristics and a range of fixed three-dimensional architectures through the choice of calixarene conformation. Whilst multicalixarene systems have been known for some time,[11] apart from the excellent research from the groups of Böhmer, on the use of urea-functionalised multicalixarenes for self-assembly,[12] and of Beer, on the preparation of cation binders,[13] these systems have not incorporated surface functionality and have been principally of synthetic interest only.

In this communication, we present the synthesis of the first amino surface-functionalised multicalixarenes (Fig. 1), evaluation of their DNA-binding ability and toxicity and their exploitation as gene transfection vectors.

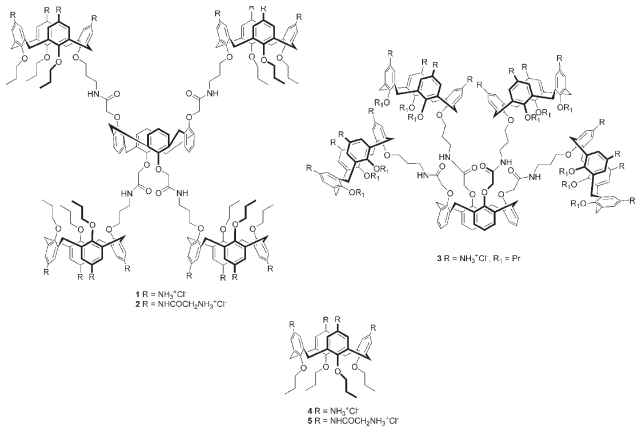
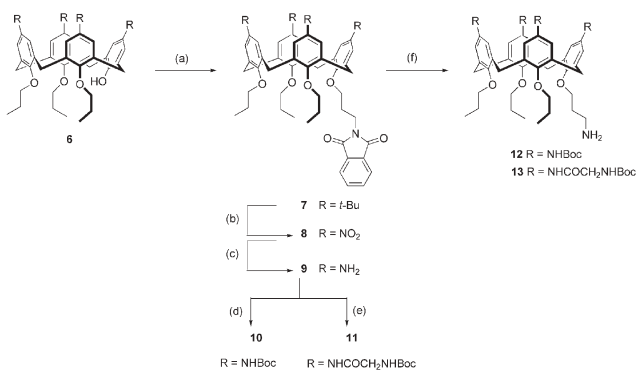



**Fig. 1** Calixarenes and multicalixarenes studied.

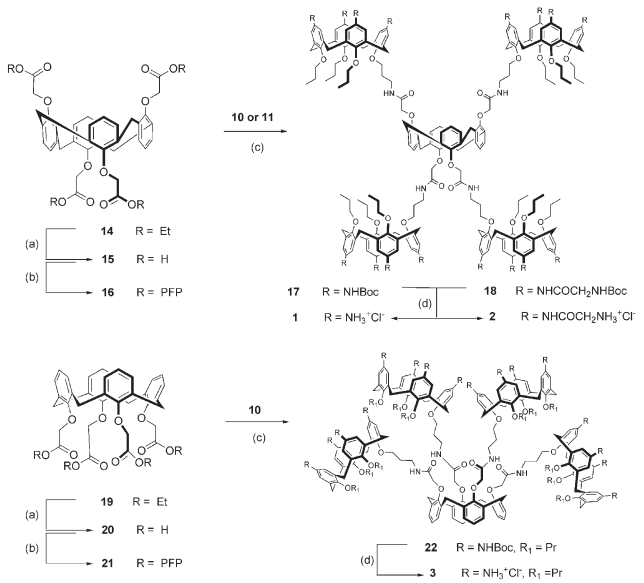
A multi-step convergent synthetic route was designed for the multicalixarenes (MCs) studied. The introduction of functionality to the surface relied on the key bifunctional intermediate **9**. This was prepared from the known tripropyl derivative[14] through introduction of a masked amine as the phthalimide[15] **7** and subsequent *ipso* nitration and reduction at the calixarene upper rim (Scheme 1). Compound **9** was then either protected as the orthogonal Boc derivative **10** to allow for the formation of multicalixarenes featuring aromatic amines for DNA binding or treated with a Boc-protected glycine derivative for the introduction of surface aliphatic amines to yield **11**. Coupling of these functionalised outer surfaces to the central core was achieved through initial deprotection (**12**, **13**) and subsequent reaction with activated pentafluorophenol esters **16** and **21** of the known




**Scheme 1** Synthesis of functionalised outer surface. *Reagents and conditions*: (a) *n*-3-(bromopropyl)phthalimide, NaH, DMF; (b), $HNO_3$, TFA, DCM, 0 °C; (c) $SnCl_2$, EtOH, reflux; (d) $Boc_2O$, DCM; (e) BocGlyONSu, DCM; (f) hydrazine hydrate, EtOH.

*School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, UK. E-mail: susan.matthews@uea.ac.uk; Fax: 44 1603 592003; Tel: 44 1603 593986*

† Electronic supplementary information (ESI) available: Full experimental details for synthesis of multicalixarenes and biological studies. See DOI: 10.1039/b712100h




**Scheme 2** Synthesis of functionalised multicalixarenes. *Reagents and conditions*: (a) $NMe_4OH$, THF, reflux; (b) PFP, DCC, EtOAc; (c) Hünig's base, DMAP, DCM; (d) HCl (g), DCM.

carboxylic acid derivatives **14**[16] and **19**[17] providing the protected multicalixarenes in yields of between 39 and 78% (Scheme 2) These were then readily deprotected to reveal surface amines, in near quantitative yields, by treatment with HCl. The two comparative single calixarenes **4**,[18] **5**,[19] as their hydrochloride salts, were prepared according to literature procedures.

The DNA-binding ability of calixarenes **1–5** was initially evaluated through gel electrophoresis studies[20] using a 3000 Base-Pair plasmid (either pGEM-EYFP or pEGFP-N1). Screening at a high ligand concentration of 2 mM indicated that the MCs **1–3** were able to bind DNA through charge neutralisation and thus the plasmid DNA was unable to pass through the gel in response to applied charge (Fig. 2(a)). In contrast, the single amino calixarene **4** was unable to bind the plasmid DNA at this concentration. In an effort to understand the role of cooperativity

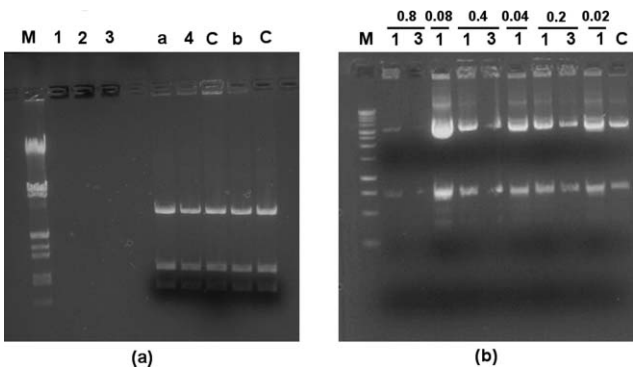



**Fig. 2** Gel electrophoresis studies were performed on a 1% agarose gel at 130 V. (a) High concentration (2 mM) pGEM-EYFP plasmid. (b) Dilution study (concentrations shown in mM) pEGFP-N1. M is DNA ladder, C is a DNA control with no calixarene present. Single calixarenes, not featuring amino functionality, have been included as additional controls: (a) *p*-$^t$Bu calix[4]arene tetrapropylether,[23] (b) calix[4]arene tetrapropylether.[24]

in DNA binding we further evaluated the binding of **1–3** over a range of concentrations (0.2–1.0 mM) compared to an amine equivalent concentration of the control single calixarene **5**. On reducing the concentration of vector, some discrimination is seen between the different architectures of the MCs (Fig. 2(b)) with the asymmetrically functionalised **3** showing stronger binding than the symmetrical functionalised **1**. Functionalisation with glycine MC **2** results in enhanced binding at lower concentrations compared to the conformationally analogous MC **1** (see ESI,† Fig. 5). Interestingly, the glycyl amino derivative **5** possesses significant DNA binding ability, comparable with that of MCs **1** and **3**, but less than that of **2**, however, previous studies[21] indicate that, unless self assembly of the ligands occurs, vectors featuring less that 6–8 positive charges are unable to mediate transfection successfully. These results indicate that there may be a degree of cooperativity in the binding of DNA between the functionalised calixarenes in multicalixarenes, which is not available with the single calixarenes, and, additionally, that the binding is affected by the multicalixarene architecture and the nature of the amine and is more efficient when the charged groups are provided on a monodirectional surface.

The toxicity of the multicalixarenes was determined in a range of cell lines including Chinese Hamster Ovary (CHO), Human Embryonic Kidney (HEK) and a Human monocytic cell line (THP-1) using a cell viability MTS assay over 24–72 h. Representative data for the MTS assay in HEK cells, after 48 h, is shown in Fig. 3 (equivalent data was obtained in CHO and THP-1 cells). All the multicalixarenes **1–3** showed a comparable lack of cell toxicity to both the PBS control and β-cyclodextrin over the full pharmaceutically relevant concentration range and are thus suitable for use as *in vitro* vectors. It is interesting to note that the single calixarene **4** showed significant toxicity, at high concentrations, compared with controls and MCs **1–3** in both HEK and CHO cells and this may indicate a different cell uptake process for the single and multicalixarenes, a process we are currently investigating.

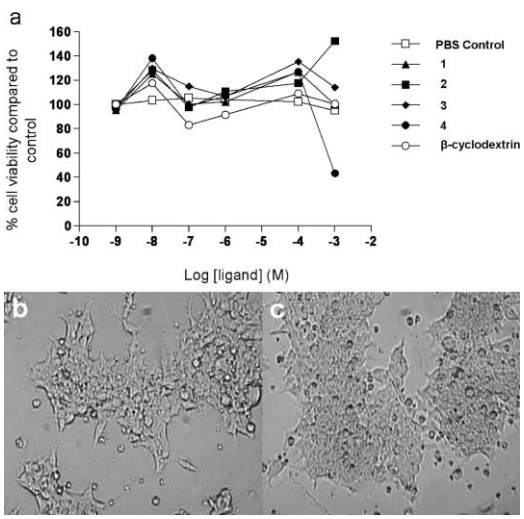



**Fig. 3** (a) Representative cell viability assays (HEK cells tested for proliferation after 48 h using an MTS assay) for MCs **1–3** and **4**. (b) HEK cells after 48 h incubation with PBS. (c) HEK cells after 48 h incubation with MC **3**. Images were acquired using a digital camera mounted on a microscope.

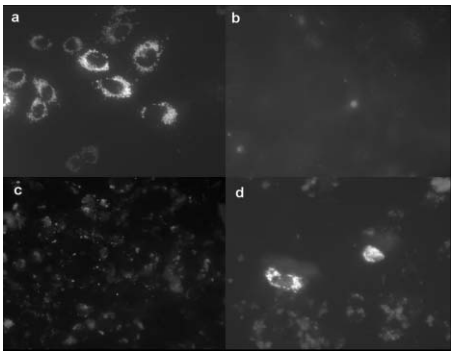



**Fig. 4** Transfection of a plasmid expressing a fluorescent protein that accumulates in mitochondria (pDs2-mito) in CHO cells after 48 h. (a) Positive control (cells transfected with commercial FuGene®); (b) negative control (PBS buffer); (c) transfection with **3**; (d) transfection with **2**. Images acquired using a Zeiss Axiovision 2 microscope.

The ability of **1–5** to transfect plasmid DNA was investigated in CHO cells using pDs2-mito (Clontech) a plasmid leading to expression of a fluorescent protein that accumulates in mitochondria. The conditions used[22] were those for a standard transfection with FuGene® (Roche) a commercial positive control, and 1 μg DNA, and are not optimised for MCs. Effective transfection (10%), compared to FuGene (50%), was observed for **2**, the multicalixarene featuring aliphatic amines after 48 h (Fig. 4). However, interestingly, no transfection was observed with **1** and **3**, where the amine is an arylamine, which show good binding in electrophoresis studies, or with the single calixarene controls **4** and **5**. This variation in transfection ability may be a consequence of the degree of charge exhibited by the multicalixarenes in pH 7 buffer with only multicalixarene **2** being fully positively charged and binding DNA effectively or a result of reduced DNA release within the endosomal pathway in the case of multicalixarenes **1** and **3**.

The potential of functionalised multicalixarenes to effect gene transfection has been clearly shown and the low toxicity of these materials indicates that they are of great promise as delivery agents. We are currently investigating the effect of charge loading and scaffold architecture of the multicalixarenes on DNA transfection.

This work was supported by the Association for International Cancer Research (03-256) and the University of East Anglia. The authors acknowledge the EPSRC Mass Spectrometry Service, University of Swansea for providing characterisation.

## Notes and references

1 D. W. Pack, A. S. Hoffman, S. Pun and P. S. Stayton, *Nat. Rev. Drug Discovery*, 2005, **4**, 581; T. Merdan, J. Kopeček and T. Kissel, *Adv. Drug Delivery Rev.*, 2002, **54**, 715.
2 M. Guillot-Nieckowski, S. Eisler and F. Diederich, *New J. Chem.*, 2007, **31**, 1111; U. Boas and P. M. H. Heegaard, *Chem. Soc. Rev.*, 2004, **33**, 43; C. Dufes, I. F. Uchegbu and A. G. Schatzlein, *Adv. Drug Delivery Rev.*, 2005, **57**, 2177; M. Guillot, S. Eisler, K. Weller, H. P. Merkle, J.-L. Gallani and F. Diederich, *Org. Biomol. Chem.*, 2006, **4**, 766.
3 P. L. Fegner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringol and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413; J. Zabner, *Adv. Drug Delivery Rev.*, 1997, **27**, 17.
4 J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin and P. L. Felgner, *J. Biol. Chem.*, 1994, **269**, 2550; F. Liu, J. Yang, L. Huang and D. Liu, *Pharm. Res.*, 1996, **13**, 1642.
5 P. Shahgaldian, E. Da Silva and A. W. Coleman, *J. Inclusion Phenom. Macrocycl. Chem.*, 2003, **46**, 175; E. Da Silva, P. Shahgaldian and A. W. Coleman, *Int. J. Pharm.*, 2004, **273**, 57; J. Gualbert, P. Shahgaldian and A. W. Coleman, *Int. J. Pharm.*, 2003, **257**, 69; P. Shahgaldian, L. Quattrocchi, J. Gualbert, A. W. Coleman and P. Goreloff, *Eur. J. Pharm. Biopharm.*, 2003, **55**, 107; E. Da Silva, A. N. Lazar and A. W. Coleman, *J. Drug Delivery Sci. Technol.*, 2004, **14**, 3.
6 W. Z. Yang and M. M. de Villiers, *Eur. J. Pharm. Biopharm.*, 2004, **58**, 629; W. Z. Yang and M. M. de Villiers, *AAPS J.*, 2005, **7**, 241; W. Z. Yang and M. M. de Villiers, *J. Pharm. Pharmacol.*, 2004, **56**, 703; S. A. Fernandes, L. F. Cabeca, A. J. Marsaioli and E. De Paula, *J. Inclusion Phenom. Macrocycl. Chem.*, 2007, **57**, 395.
7 A. J. Wright, S. E. Matthews, W. B. Fischer and P. D. Beer, *Chem.–Eur. J.*, 2001, **7**, 3474; Y. Tanake, Y. Kobuke and M. Sokabe, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 693; J. de Mendoza, F. Cuevas, P. Prados, E. S. Meadows and G. W. Gokel, *Angew. Chem., Int. Ed.*, 1998, **37**, 1534; J. L. Seganish, P. V. Santacroce, K. J. Salimian, J. C. Fettinger, P. Zavalij and J. T. Davis, *Angew. Chem., Int. Ed.*, 2006, **45**, 3334; P. J. Cragg, M. C. Allen and J. W. Steed, *Chem. Commun.*, 1999, 553.
8 J. W. Cornforth, P. D. Hart, G. A. Nicholls, R. J. W. Rees and J. A. Stock, *Br. J. Pharmacol. Chemother.*, 1955, **10**, 73; P. D. Hart, J. A. Armstrong and E. Brodaty, *Infect. Immun.*, 1996, **64**, 1491; M. J. Colston, H. C. Hailes, E. Stavropoulos, A. C. Herve, G. Herve, K. J. Goodworth, A. M. Hill, P. Jenner, P. D. Hart and R. E. Tascon, *Infect. Immun.*, 2004, **72**, 6318.
9 M. Dudic, A. Colombo, F. Sansone, A. Casnati, G. Donofrio and R. Ungaro, *Tetrahedron*, 2004, **60**, 11613; F. Sansone, M. Dudic, G. Donofrio, C. Rivetti, L. Baldini, A. Casnati, S. Cellai and R. Ungaro, *J. Am. Chem. Soc.*, 2006, **128**, 14528; L. Baldini, A. Casnati, F. Sansone and R. Ungaro, *Chem. Soc. Rev.*, 2007, **36**, 254.
10 R. Zadmard and T. Schrader, *Angew. Chem., Int. Ed.*, 2006, **45**, 2703.
11 For a review see: (*a*) L. Baklouti, N. Cheriaa, M. Mahouachi, R. Abidi, J. S. Kim, Y. Kim and J. Vicens, *J. Inclusion Phenom. Macrocycl. Chem.*, 2006, **54**, 1; O. Haba, K. Haga, M. Ueda, O. Morikawa and H. Konishi, *Chem. Mater.*, 1999, **11**, 427; Y. Yamakawa, M. Ueda, R. Nagahata, K. Takeuchi and M. Asai, *J. Chem. Soc., Perkin Trans. 1*, 1998, 4135; P. Lhotak and S. Shinkai, *Tetrahedron*, 1995, **51**, 7681; O. Mogck, P. Parzuchowski, M. Nissinen, V. Böhmer, G. Rokicki and K. Rissanen, *Tetrahedron*, 1998, **54**, 10053.
12 Y. Rudzevich, V. Rudzevich, C. Moon, I. Schnell, K. Fischer and V. Böhmer, *J. Am. Chem. Soc.*, 2005, **127**, 14168; Y. Rudkevich, K. Fischer, M. Schmidt and V. Böhmer, *Org. Biomol. Chem.*, 2005, **3**, 3916.
13 F. Szemes and P. D. Beer, *Chem. Commun.*, 2002, 1228.
14 K. Iwamato, K. Araki and S. Shinkai, *Tetrahedron*, 1991, **47**, 4325.
15 S. E. Matthews, P. Parzuchowski, A. Garcia-Carrera, C. Grüttner, J.-F. Dozol and V. Böhmer, *Chem. Commun.*, 2001, 417; S. Barboso, A. Garcia-Carrera, S. E. Matthews, F. Arnaud-Neu, V. Böhmer, J.-F. Dozol, H. Rouquette and M.-J. Schwing-Weill, *J. Chem. Soc., Perkin Trans. 2*, 1999, 719.
16 B. Genorio, J. Kobe, G. Giester and I. Leban, *Acta. Crystallogr., Sect. C*, 2003, **59**, 221.
17 K. Iwamoto and S. Shinkai, *J. Org. Chem.*, 1992, **57**, 7066.
18 F. Sansone, E. Chierici, A. Casnati and R. Ungaro, *Org. Biomol. Chem.*, 2003, **1**, 1802.
19 G. M. K. Consoli, F. Cunsolo, C. Geraci and V. Sgarlata, *Org. Lett.*, 2004, **6**, 4163.
20 A. Mueller, E. Kelly and P. G. Strange, *Blood*, 2002, **99**, 785.
21 M. S. Wadhwa, W. T. Collard, R. C. Adami, D. L. McKenzie and K. G. Rice, *Bioconjugate Chem.*, 1997, **8**, 81; C. Plank, M. X. Tang, A. R. Wolfe and F. C. Szoka, *Hum. Gene Ther.*, 1999, **10**, 319; D. V. Schaeffer, N. A. Fieldman, N. Dan and D. A. Lauffenburger, *Biotechnol. Bioeng.*, 2000, **67**, 598.
22 A. Mueller and P. G. Strange, *FEBS lett.*, 2004, **570**, 126.
23 K. Iwamoto, K. Araki and S. Shinkai, *J. Org. Chem.*, 1991, **56**, 4955.
24 L. C. Groenen, B. H. M. Ruël, A. Casnati, P. Timmerman, W. Verboom, S. Harkema, A. Pochini, R. Ungaro and D. N. Reinhoudt, *Tetrahedron Lett.*, 1991, **32**, 2675.