

## Protein-mediated dethreading of a biotin-functionalised pseudorotaxane†

Cite this: *Org. Biomol. Chem.*, 2014, **12**, 511

Stuart T. Caldwell,<sup>a</sup> Catherine Maclean,<sup>a</sup> Mathis Riehle,<sup>b</sup> Alan Cooper,<sup>a</sup> Margaret Nutley,<sup>a</sup> Gouher Rabani,<sup>a</sup> Brian Fitzpatrick,<sup>a</sup> Vincent M. Rotello,<sup>c</sup> Brian O. Smith,<sup>b</sup> Belal Khaled,<sup>d</sup> Patrice Woisel\*<sup>d</sup> and Graeme Cooke\*<sup>a</sup>

In this article, we describe the synthesis of new biotin-functionalised naphthalene derivatives **3** and **4** and their complexation behaviour with avidin and neutravidin using a range of analytical techniques. We have shown using 2-(4'-hydroxyazobenzene)benzoic acid displacement and ITC experiments, that compounds **3** and **4** have the propensity to form reasonably high-affinity bioconjugates with avidin and neutravidin. We have also demonstrated using <sup>1</sup>H NMR, UV-vis and fluorescence spectroscopy that the naphthalene moiety of **3** and **4** facilitates the formation of pseudorotaxane-like structures with **1** in water. We have then investigated the ability of avidin and neutravidin to modulate the complexation between **1** and **3** or **4**. UV-vis and fluorescence spectroscopy has shown that in both cases the addition of the protein disrupts complexation between the naphthalene moieties of **3** and **4** with **1**.

Received 7th August 2013,  
Accepted 14th November 2013

DOI: 10.1039/c3ob41612g

www.rsc.org/obc

## Introduction

Avidin<sup>1</sup> and the related biotin-binding proteins streptavidin<sup>2</sup> and neutravidin,<sup>3</sup> due to their ability to strongly bind to up to four units of biotin, have become important systems for developing diagnostics,<sup>4</sup> immunoassays,<sup>5</sup> protein purification techniques,<sup>6</sup> and surface-protein conjugates.<sup>7</sup> Chicken egg avidin together with bacterial-based streptavidin, possess a very high-affinity for biotin ( $K_a \approx 10^{15} \text{ M}^{-1}$ ). The two proteins have similar tetrameric quaternary structures and amino acid arrangement in their binding sites, however, their differing degrees of glycosylation plays an important role in controlling their physical properties. Avidin due to its glycosylated outer surface has a pI = 10, whereas the deglycosylated streptavidin has a pI = 6.8. Neutravidin like streptavidin is deglycosylated and has a similar pI to streptavidin (pI = 6.3), however, it possesses the highest degree of specificity of this family of biotin-binding proteins.<sup>8</sup>

Pseudorotaxane-based systems engineered from macrocyclic host units and appropriately functionalised guests offer exciting possibilities to reversibly modulate protein structure and function.<sup>9</sup> In this context, the host cyclobis(paraquat-*p*-phenylene) (CBPQT<sup>4+</sup>, **1**)<sup>10</sup> has the propensity to become an important system for the development of novel protein conjugates with appropriately functionalised electron rich guest units.<sup>11</sup> In particular, the ability to: synthesise this macrocycle using reliable and high yielding protocols;<sup>12</sup> modulate its recognition processes by chemical and electrochemical redox processes;<sup>13</sup> form reasonably strong complexes in aqueous solvents<sup>14</sup> and conveniently monitor complexation properties using UV-vis<sup>15,16</sup> and fluorescence spectroscopy facilitates the development of novel stimuli-responsive bioconjugates.

Previously, we have reported the fabrication of tuneable bioconjugates from **1**, biotin-functionalised axle **2** and avidin.<sup>11b</sup> However, the poor aqueous solubility of compound **2** required the investigations to be carried out in the presence of 30% ethanol limiting future biological applications of these systems. Furthermore, the ester link between the biotin and naphthalene units may be susceptible to cleavage by avidin, as this protein has previously been shown to possess hydrolytic activity.<sup>17</sup> Here, we report the synthesis of a new generation of naphthalene-functionalised biotin systems that have improved hydrolytic stability conferred by the amide linker group and in the case of oligomer **4**, significantly better water solubility than compounds **2** or **3**. We report the binding properties of **3** and **4** to avidin and neutravidin, and we have investigated the role the differing proteins have in controlling complexation of the naphthalene moieties of **3** and **4** with **1**.

<sup>a</sup>Glasgow Centre for Physical Organic Chemistry, WestCHEM, School of Chemistry, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK.  
E-mail: Graeme.Cooke@glasgow.ac.uk

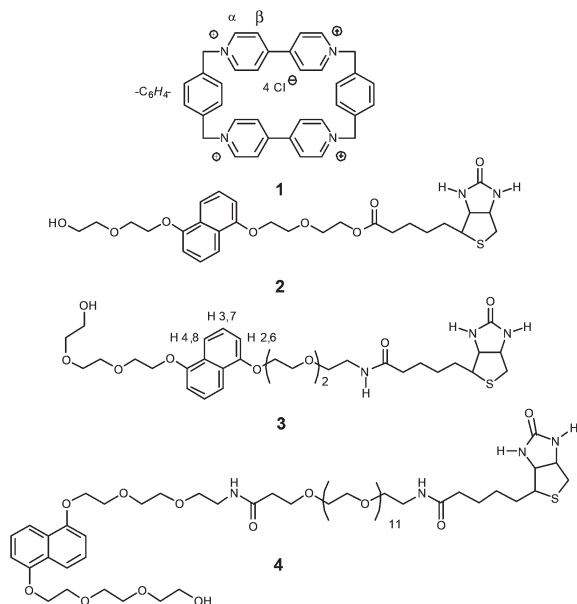
<sup>b</sup>College of MVLS, University of Glasgow, Glasgow, G12 8QQ, UK

<sup>c</sup>Department of Chemistry, University of Massachusetts at Amherst, Amherst, MA 01002, USA

<sup>d</sup>Unité Matériaux et Transformations (UMET), UMR-CNRS 8207, Université Lille Nord de France, ENSCL, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, 59655, France

† Electronic supplementary information (ESI) available: Synthesis of **3** and **4**. See DOI: 10.1039/c3ob41612g





## Results and discussion

### Avidin and neutravidin binding properties of 3 and 4

The synthesis of the new naphthalene derivatives 3 and 4 is outlined in the ESI†. Evidence to indicate that compounds 3 and 4 successfully bind to avidin and neutravidin was obtained by performing competition experiments between the complexes of 2-(4'-hydroxyazobenzene)benzoic acid (HABA) and avidin and neutravidin. The characteristic red-coloured complexes that arise when HABA binds to the biotin binding site on avidin,<sup>18</sup> streptavidin<sup>19</sup> and neutravidin,<sup>20</sup> allows its displacement to be conveniently monitored using the naked eye or UV-vis spectroscopy upon the addition of the more strongly binding guests 3 or 4 (see Fig. 1). Indeed, the addition of 3 or 4 to a solution of the avidin–HABA complex resulted in the formation of a new peak at  $\lambda \sim 350$  nm which is characteristic of free HABA and in the immediate disappearance of the

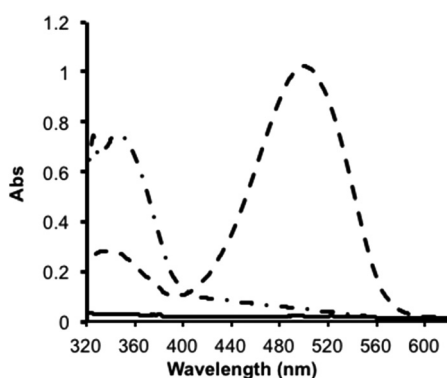


Fig. 1 UV-vis spectra of: (a) avidin ( $1 \times 10^{-5}$  M) (—); (b) upon the addition of 4 equivalents of HABA (---); (c) upon the addition of 4 equivalents of 4 (-.-). Spectra were recorded in water at 21 °C.

Table 1 Selected isothermal calorimetry data for the binding of 4 with avidin and neutravidin at 25 °C in water. *N* is the binding stoichiometry

	$K_a$ ( $M^{-1}$ )	<i>N</i>
Avidin	$2.0 \pm 0.3 \times 10^7$	$2.9 \pm 0.1$
Neutravidin	$4.7 \pm 1.1 \times 10^6$	$3.2 \pm 0.1$

absorption peak at  $\lambda \sim 500$  nm due to original avidin–HABA complex. Similar data were obtained when experiments were repeated with neutravidin.

The good solubility of compound 4 in water has allowed the investigation of the binding of this compound to avidin and neutravidin using isothermal titration microcalorimetry (ITC) (see Table 1 and ESI†). In all cases the ITC data indicate exothermic binding of 4 to the target proteins, with larger observed binding affinities ( $K_a$ ) for avidin compared to neutravidin. The protein binding affinities for 4 ( $K_a$  of the order of  $10^7$   $M^{-1}$ ) are much lower than previously observed for avidin and biotin, presumably due to the inevitable constraints imposed by the long chain attached to the biotin moiety of 4. Bearing in mind the uncertainties regarding absolute concentrations and purities of the proteins, the apparent binding stoichiometries (*N*) determined by ITC (Table 1) are consistent with the four binding sites present in the tetrameric proteins and suggests that 4 may only bind to three of the four possible binding sites of these proteins.<sup>21</sup> In line with related investigations involving biotin species featuring poly(ethylene glycol) spacer moieties, we attribute the oligomeric nature of 4 being partly responsible for preventing this unit binding to all four of the available binding sites.<sup>22</sup> Thus, the data are consistent with the HABA measurements, demonstrating that compound 4 has a good affinity for avidin and neutravidin in aqueous environments.

Non-covalent interactions (*e.g.* donor–acceptor interactions) can have a profound influence on the fluorescence properties of dialkoxynaphthalene units.<sup>14e</sup> Thus, we have investigated the fluorescence properties of 3 and 4 both free in solution and bound to avidin and neutravidin. In all cases, the biotin-mediated binding of 3 or 4 to the proteins had negligible affect on the fluorescence intensity of the naphthalene moiety, suggesting that this unit does not undergo significant interactions with these proteins (Fig. 2). This observation is in accordance with previously reported fluorophore-appended biotin/avidin conjugates featuring ethylene glycol linker units.<sup>23</sup>

### Complexation properties of 3 and 4 with 1 in water

We have investigated the complexation of 1 with 3 and 4 using NMR spectroscopy in  $D_2O$ . The  $^1H$  NMR of a 1 : 1 admixture of 1-3 and 1-4 were in accordance with previously reported data for complexes of this type,<sup>12,14g</sup> and revealed significant shifts for the  $H_\alpha$ ,  $H_\beta$  and  $-C_6H_4-$  protons of 1 and the  $H_{(4,8)}$  protons of the naphthalene moiety of 3 or 4 upon complexation (Fig. 3 and ESI†). Thus the NMR data are in accordance with the formation of pseudorotaxane-like architectures in aqueous



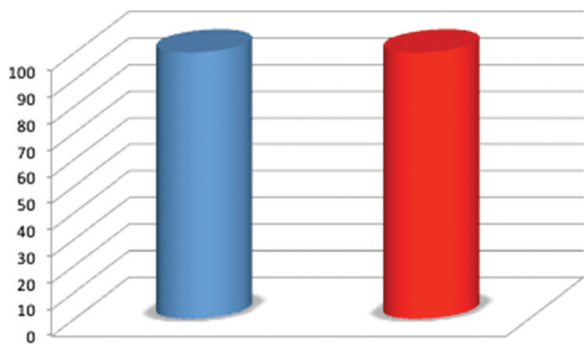


Fig. 2 Representation of the change in the normalised fluorescence emission intensity at 330 nm for compound **4** ( $\sim 1 \times 10^{-5}$  M) (blue cylinder) and upon complexation with avidin (red cylinder). Spectra were recorded in water at 21 °C. Excitation wavelength = 295 nm.

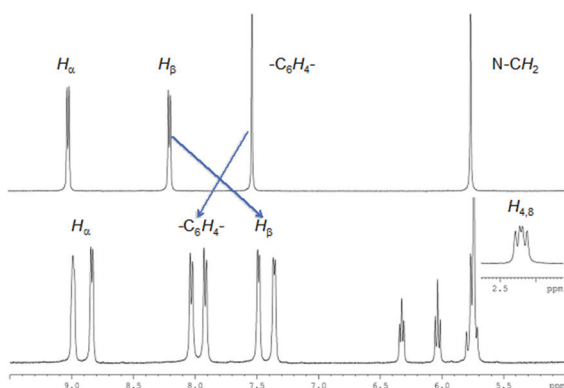


Fig. 3 Partial  $^1\text{H}$  NMR spectra of **1** (top spectrum) and upon the addition of one equivalent of **3** (bottom spectrum). Recorded in  $\text{D}_2\text{O}$  at 25 °C.

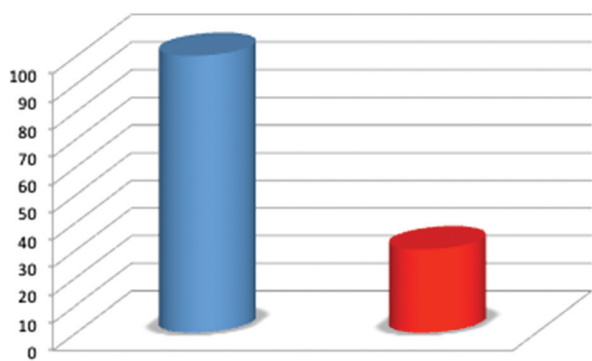


Fig. 4 Representation of the change in the normalised fluorescence emission intensity at 330 nm for compound **3** ( $\sim 1 \times 10^{-5}$  M) (blue cylinder) and upon the addition of one equivalent of **1** (red cylinder). Spectra were recorded in water at 21 °C. Excitation wavelength = 295 nm.

conditions. In both cases, the formation of the admixture resulted in the formation of a purple-coloured solution ( $\lambda = 520$  nm) and a significant decrease in the fluorescence intensity of the naphthalene moiety that are consistent with complex formation (Fig. 4).<sup>16</sup> ITC experiments provided a  $K_a$  of

$2.6 \pm 0.1 \times 10^5 \text{ M}^{-1}$  for complex **1·4**, which is approximately two orders of magnitude lower than that observed for **4** binding to the biotin-binding proteins.

### Investigation of the avidin/neutravidin-mediated complexation between **3** or **4** with **1**

With complexation verified between **3** and **4** with avidin and neutravidin and pseudorotaxane formation demonstrated between the naphthalene-based systems and **1** in aqueous solution, we next investigated whether pseudorotaxane-like bioconjugates could be fabricated. To investigate these processes we have adopted two strategies. Firstly, we have investigated the addition of avidin and neutravidin to pre-formed pseudorotaxanes of **1** with **3** and **4**. To complement this study, we have also investigated the complexation behaviour of the avidin/neutravidin complexes of **3** and **4** with **1**.

The addition of avidin to a purple coloured solution of **1·3** or **1·4** immediately resulted in the decolourisation of the solution suggesting the disruption of the pseudorotaxane architecture.<sup>24</sup> UV-vis spectroscopy experiments revealed a total disappearance of the absorption at 520 nm indicating that cyclophane **1** is no longer bound to the naphthalene moiety of **3** to any significant extent (Fig. 5a). In addition, fluorescence spectroscopy was also used to monitor changes in the emission spectrum of **1·3** upon the addition of avidin. A marked increase in fluorescence intensity for the naphthalene moiety was observed. When the same UV-vis experiment was repeated

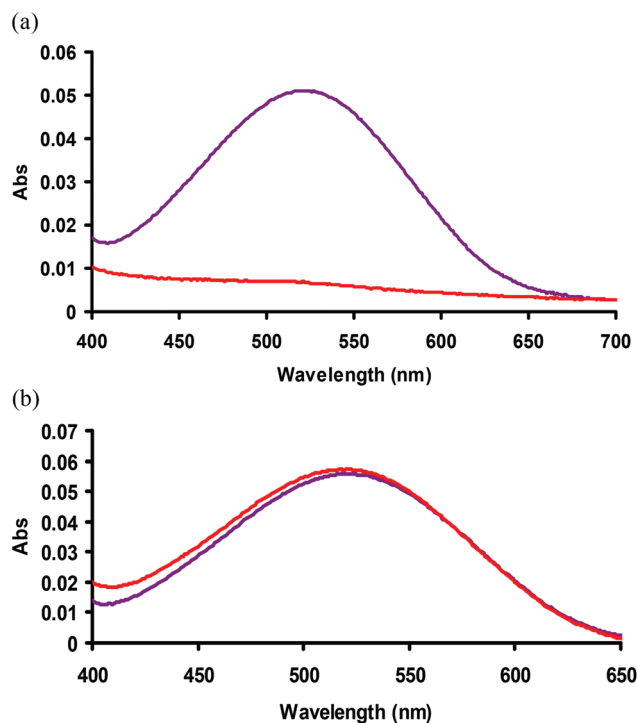
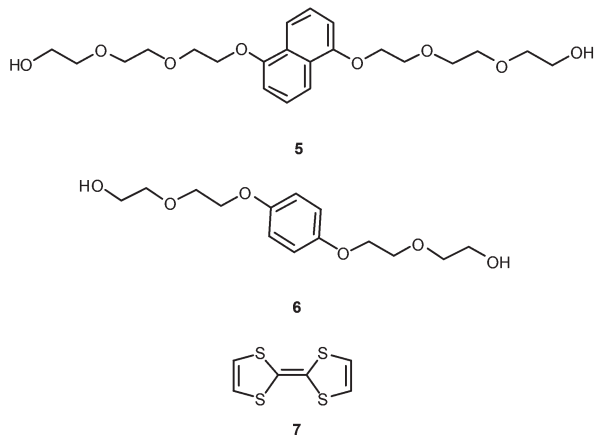


Fig. 5 UV-vis spectra of: (a) complex formed between **3** and **1** (purple line) ( $\sim 1 \times 10^{-4}$  M) and upon the addition of one equivalent of avidin (red line) and (b) the spectra obtained when **5** was used instead of **3** upon addition of avidin. Spectra were recorded in water at 21 °C.



with non-biotin functionalised naphthalene derivative **5**,<sup>25</sup> no change in the colour or intensity of the absorption at 520 nm was observed indicating that the presence of the biotin moiety was required to induce the avidin-mediated disruption of complexation and its associated colour change (Fig. 5b). Similar UV-vis and fluorescence data was also observed when neutravidin was added to **1-3** and **1-4**.



Further evidence for the avidin-induced dethreading process of **1-3** was obtained by adding either guest **6** or **7** after the protein-mediated decomplexation process. Previous experiments have shown that in aqueous conditions guest **6** forms a lower affinity complex with macrocycle **1** than naphthalene-based guests such as **3**.<sup>14g</sup> Therefore compound **6** could be a good system for investigating whether free **1** is available in solution following the addition of the protein to **1-3**. The addition of excess **6** following the addition of avidin to **1-3** resulted in an orange/red coloured solution consistent with the formation of **1-6**. Furthermore, when experiments were repeated with guest **7**, an emerald-green solution was formed due to an absorption in the UV-vis spectrum at ~850 nm. This observation is consistent with complexation between **1** and **7** (Fig. 6).<sup>26</sup>

Finally, we have investigated whether pseudorotaxane-like bioconjugates could be prepared from pre-complexed **4** and

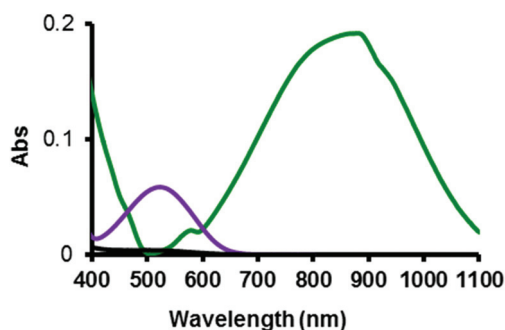


Fig. 6 UV-vis spectra of: (a) the complex formed between **3** and **1** (purple line) ( $\sim 1 \times 10^{-4}$  M); (b) upon the addition one equivalent of avidin (black line) and (c) upon the addition of excess **7** to the cuvette (green line). Spectra were recorded in water at 21 °C.

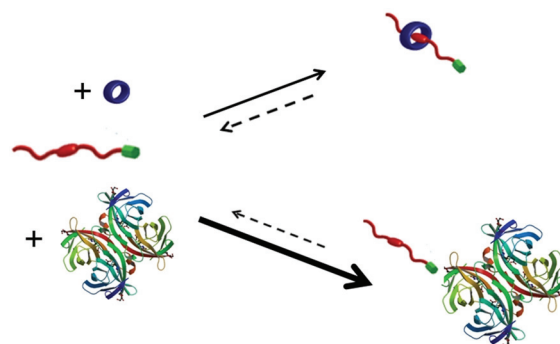


Fig. 7 Schematic diagram showing the avidin-mediated decomplexation of pseudorotaxane **1-3** or **1-4**.<sup>28</sup>

avidin or neutravidin upon the addition of **1**. The addition of aliquots of **1** to a solution containing either avidin and neutravidin and **4** led to a negligible change in colour, suggesting that despite their differing pI values, binding does not occur between these species to any significant extent under the conditions investigated, and suggests that steric factors may be responsible.<sup>27</sup>

## Conclusions

In conclusion, we have shown that compound **3** and **4** have the propensity to form reasonably high-affinity complexes with avidin and neutravidin and moderate affinity complexes with **1** in water. Furthermore, experiments show that, in a thermodynamic equilibrium situation and under similar concentration conditions, the proteins avidin or neutravidin will complete favourably with **1** for binding of **3** or **4**, and will thus shift the equilibrium from the pseudorotaxanes to the protein-biotin complexes, as illustrated in Fig. 7. This study paves the way for the elaboration of these systems into protein mediated molecular machines,<sup>29</sup> and our investigations in this area will be reported in due course.

## Acknowledgements

G.C. and B.F. thank the EPSRC for funding. C.M. thanks the University of Glasgow for the award of a Kelvin-Smith Scholarship. P.W. thanks the Agence Nationale de la Recherche (STRAPA project, ANR-12-BS08-0005). VR acknowledges CHE-1307021 from the National Science Foundation.

## Notes and references

- 1 N. M. Green, *Biochem. J.*, 1963, **89**, 585.
- 2 (a) L. Chalet and F. J. Wolf, *Arch. Biochem. Biophys.*, 1964, **106**, 1; (b) N. M. Green, *Methods Enzymol.*, 1990, **184**, 51.
- 3 Y. Hiller, E. A. Bayer and M. Wilchek, *Methods Enzymol.*, 1990, **184**, 68.





- 4 For a recent examples see: H. Schettters, *Biomol. Eng.*, 1999, **16**, 73; H. Sakahara and T. Saga, *Adv. Drug Delivery Rev.*, 1999, **37**, 89; J. P. Bingham, S. Bian, Z. Y. Tan, Z. Takacs and E. Moczydlowski, *Bioconjugate Chem.*, 2006, **17**, 689.
- 5 For a recent example see: R. C. Morton and E. P. Diamandis, *Anal. Chem.*, 1990, **62**, 1841.
- 6 For a recent example see: T. Nguyen, N. S. Joshi and M. B. Francis, *Bioconjugate Chem.*, 2006, **17**, 869.
- 7 For a recent example see: K. L. Christman, M. V. Requa, V. D. Enriquez-Rios, S. C. Ward, K. A. Bradley, K. L. Turner and H. D. Maynard, *Langmuir*, 2006, **22**, 7444.
- 8 (a) T. T. Nguyen, K. L. Sly and J. C. Conboy, *Anal. Chem.*, 2012, **84**, 201; (b) Polysciences, Inc., Technical data sheet 779; (c) Pierce Biotechnology. Instructions for neutravidin. Product no. 31000.
- 9 For representative examples of (pseudo)rotaxane-like molecules interacting with proteins see: (a) A. G. Cheetham, M. G. Hutchings, T. D. W. Claridge and H. L. Anderson, *Angew. Chem., Int. Ed.*, 2006, **45**, 1596; (b) T. Ooya and N. Yui, *Chem.-Eur. J.*, 2006, **12**, 6730; (c) J. Zhu and D. B. Smithrud, *Org. Biomol. Chem.*, 2007, **5**, 2992; (d) A. Nelson, J. M. Belitsky, S. Vidal, C. S. Joiner, L. G. Baum and J. F. Stoddart, *J. Am. Chem. Soc.*, 2004, **126**, 11914; (e) T. Higashi, F. Hirayama, S. Misumi, K. Motoyama, H. Arima and K. Uekama, *Chem. Pharm. Bull.*, 2009, **57**, 541; (f) A. Ito, T. Ooya and N. Yui, *J. Inclusion Phenom. Mol. Recognit. Chem.*, 2007, **57**, 233; (g) T. Higashi, F. Hirayama, H. Arima and K. Uekama, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 1871; (h) T. Ikunaga, H. Ikeda and A. Ueno, *Chem.-Eur. J.*, 1999, **5**, 2698; (i) J. Geng, F. Biedermann, J. M. Zayed, F. Tian and O. A. Scherman, *Macromolecules*, 2011, **44**, 4276; (j) F. Biedermann, U. Rauwald, J. M. Zayed and O. A. Scherman, *Chem. Sci.*, 2011, **2**, 279; (k) S. Ghosh and L. Isaacs, *J. Am. Chem. Soc.*, 2010, **132**, 4445; (l) J. M. Chinai, A. B. Taylor, L. M. Ryno, N. D. Hargreaves, C. A. Morris, P. J. Hart and A. R. Urbach, *J. Am. Chem. Soc.*, 2011, **133**, 8810; (m) K. Kato, R. Goto, K. Katoh and M. Shibakami, *Biosci., Biotechnol., Biochem.*, 2005, **69**, 646; (n) X. J. Loh, M. H. Tsai, J. D. Barrio, E. A. Appel, T.-C. Lee and O. A. Scherman, *Polym. Chem.*, 2012, **3**, 3180; (o) A. Fernandes, A. Viterisi, F. Coutrot, S. Potoc, D. A. Leigh, V. Aucagne and S. Papoy, *Angew. Chem., Int. Ed.*, 2009, **48**, 6443; (p) K. Patel, S. Angelos, W. R. Dichtel, A. Coskun, Y.-W. Yang, J. I. Zink and J. F. Stoddart, *J. Am. Chem. Soc.*, 2008, **130**, 2382; (q) F. Porta, G. E. M. Lamers, J. I. Zink and A. Kros, *Phys. Chem. Chem. Phys.*, 2011, **13**, 9982; (r) V. Dvornikovs, B. E. House, M. Kaetzel, J. R. Dedman and D. B. Smithrud, *J. Am. Chem. Soc.*, 2003, **125**, 8290; (s) X. Bao, I. Isaacsohn, A. F. Drew and D. B. Smithrud, *J. Am. Chem. Soc.*, 2006, **128**, 12229.
- 10 B. Odell, M. V. Reddington, A. M. Z. Slawin, N. Spencer, J. F. Stoddart and D. J. Williams, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 1547.
- 11 (a) E. Katz, L. Sheeney-Haj-Ichia and I. Willner, *Angew. Chem., Int. Ed.*, 2004, **43**, 3292; (b) S. T. Caldwell, G. Cooke, A. Cooper, M. Nutley, G. Rabani, V. Rotello, B. O. Smith and P. Woisel, *Chem. Commun.*, 2008, 2650.
- 12 M. Asakawa, W. Dehaen, G. L'Abbé, S. Menzer, J. Nouwen, F. M. Raymo, J. F. Stoddart and D. J. Williams, *J. Org. Chem.*, 1996, **61**, 9591.
- 13 For representative examples see: (a) P. L. Anelli, P. R. Ashton, R. Ballardini, V. Balzani, M. Delgado, M. T. Gandolfi, T. T. Goodnow, A. E. Kaifer, D. Philp, M. Pietraszkiewicz, L. Prodi, M. V. Reddington, M. V. Slawin, A. M. Z. Spencer, J. F. Stoddart, C. Vicent and D. J. Williams, *J. Am. Chem. Soc.*, 1992, **114**, 193; (b) O. A. Matthews, F. M. Raymo, J. F. Stoddart, A. J. P. White and D. J. Williams, *New J. Chem.*, 1998, **22**, 1131; (c) R. Ballardini, V. Balzani, M. T. Gandolfi, L. Prodi, M. Venturi, D. Philp, H. G. Ricketts and J. F. Stoddart, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1301; (d) M. Asakawa, S. Iqbal, J. F. Stoddart and N. D. Tinker, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 976; (e) J. Louisy, F. Delattre, J. Lyskawa, A. Malfait, C. E. Maclean, L. Sambe, N. Zhu, G. Cooke and P. Woisel, *Chem. Commun.*, 2011, **47**, 6819; (f) L. Scarpantonio, A. Tron, C. Destribats, P. Godard and N. D. McClenaghan, *Chem. Commun.*, 2012, **48**, 3981.
- 14 For examples of previous reports of pseudorotaxane formation of **1** in aqueous conditions see: (a) R. Ballardini, V. Balzani, M. T. Gandolfi, L. Prodi, M. Venturi, D. Philp, H. G. Ricketts and J. F. Stoddart, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1301; (b) P. R. Ashton, R. Ballardini, V. Balzani, S. E. Boyd, A. Credi, M. T. Gandolfi, M. Gómez-López, S. Iqbal, D. Philp, J. A. Preece, L. Prodi, H. G. Ricketts, J. F. Stoddart, M. S. Tolley, M. Venturi, A. J. P. White and D. J. Williams, *Chem.-Eur. J.*, 1997, **3**, 152; (c) S. Chia, J. Cao, J. F. Stoddart and J. I. Zink, *Angew. Chem., Int. Ed.*, 2001, **40**, 2447; (d) R. Hernandez, H.-R. Tseng, J. W. Wong, J. F. Stoddart and J. I. Zink, *J. Am. Chem. Soc.*, 2004, **126**, 3370; (e) M. Venturi, S. Dumas, V. Balzani, J. Cao and J. F. Stoddart, *New J. Chem.*, 2004, **28**, 1032; (f) R. L. Lilienthal and D. K. Smith, *Anal. Chem.*, 1995, **67**, 3733; (g) M. Bria, G. Cooke, A. Cooper, J. F. Garety, S. G. Hewage, M. Nutley, G. Rabani and P. Woisel, *Tetrahedron Lett.*, 2007, **48**, 301.
- 15 A recent computational study has indicated that the associated colour change following complexation of TTF by macrocycle **1** does not necessarily indicate pseudorotaxane formation. B. Tejerina, C. M. Gothard and B. A. Grzybowski, *Chem.-Eur. J.*, 2012, **18**, 5606.
- 16 A. Credi, M. Montalti, V. Balzani, S. J. Langford, F. M. Raymo and J. F. Stoddart, *New J. Chem.*, 1998, 1061.
- 17 T. Huberman, Y. Eisenberg-Domovich, G. Gitlin, T. Kulik, E. A. Bayer, M. Wilchek and O. Livnah, *J. Biol. Chem.*, 2001, **276**, 32031.
- 18 S. Repo, T. A. Paldanius, V. P. Hytönen, T. K. M. Nyholm, K. K. Halling, J. Huuskonen, O. T. Pentikäinen, K. Rissanen, J. P. Siotte, T. T. Airenne, T. A. Salminen, M. S. Kulomaa and M. S. Johnson, *Chem. Biol.*, 2006, **13**, 1029.



- 19 P. C. Weber, J. J. Wendoloski, M. W. Pantoliano and F. R. Salemme, *J. Am. Chem. Soc.*, 1992, **114**, 3197.
- 20 C. A. Lackey, N. Murthy, O. W. Press, D. A. Tirrell, A. S. Hoffman and P. S. Stayton, *Bioconjugate Chem.*, 1999, **10**, 401.
- 21 The inevitable uncertainties in absolute concentrations of active binding species prevent a more unambiguous statement of binding stoichiometry.
- 22 S. Ke, J. C. Wright and G. S. Kwon, *Bioconjugate Chem.*, 2007, **18**, 2109.
- 23 H. J. Gruber, M. Marek, H. Schindler and K. Kaiser, *Bioconjugate Chem.*, 1997, **8**, 552.
- 24 For an example of biotin–avidin-mediated decomplexation see: B. C. Satishkumar, L. O. Brown, Y. Gao, C.-C. Wang, H.-L. Wang and S. K. Doorn, *Nat. Nano*, 2007, **2**, 560.
- 25 P. R. Ashton, J. Huff, S. Menzer, I. W. Parsons, J. A. Preece, J. F. Stoddart, M. S. Tolley, A. J. P. White and D. J. Williams, *Chem.–J. Eur.*, 1996, **2**, 31.
- 26 (a) D. Philp, A. M. Z. Slawin, N. Spencer, J. F. Stoddart and D. J. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1991, 1584; (b) W. Devonport, M. A. Blower, M. R. Bryce and L. M. Goldenberg, *J. Org. Chem.*, 1997, **62**, 885; (c) P. R. Ashton, V. Balzani, J. Becher, A. Credi, M. C. T. Fyfe, G. Mattersteig, S. Menzer, M. B. Nielsen, F. M. Raymo, J. F. Stoddart, M. Venturi and D. J. Williams, *J. Am. Chem. Soc.*, 1999, **121**, 3951.
- 27 A very pale pink colour was observed when >10 equivalents of macrocycle **1** was added to the solution of the neutral-vidin-3 complex indicating that a low-affinity complex may form.
- 28 Avidin structure image adapted from the RCSB PDB (<http://www.rcsb.org>) of PDB ID: 2AVI. O. Livnah, E. A. Bayer, M. Wilchek and J. L. Sussman, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 5076.
- 29 E. R. Kay, D. A. Leigh and F. Zerbetto, *Angew. Chem., Int. Ed.*, 2007, **46**, 72.

