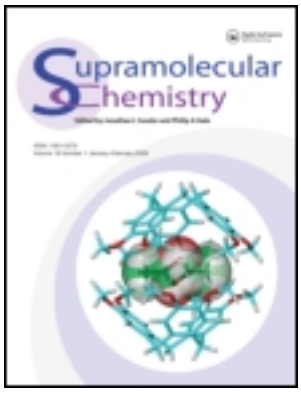

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Cooperative heterodimer formation between per-guadinylated and carboxylated or phosphorylated cyclodextrins in DMSO and DMSO–water studied by NMR spectroscopy and microcalorimetry

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The anionic cyclodextrins (CDs), heptakis[6-(3-thiopropionate)-6-deoxy]-β-CD, heptakis[(6-(thio-ethanoate)-6-deoxy]-β-CD and partially phosphorylated 6-(aminoethylphosphate)-6-deoxy-β-CD as triethylammonium salts, **bpsp.NHEt$_3$**, **bpse.NHEt$_3$** and **bphos.NHEt$_3$**, respectively, interacted with the positively charged picrate salt of heptakis[6-(guanidino)-6-deoxy]-β-CD, **bguan.picrate**, to form heterodimers as shown by NMR spectroscopic and microcalorimetric isothermal titration calorimetry (ITC) titrations in solution. Association constants, K_a (M^{-1}) in DMSO-d_6 and in DMSO-d_6–H_2O (80/20, v/v) were: **bguan.picrate/bpsp.NHEt$_3$**, 6.4×10^5 and 5.9×10^4; **bguan.picrate/bpse.NHEt$_3$**, 4.2×10^4 and 1.2×10^4; ITC data in DMSO agreed with those above: **bguan.picrate/bpsp.NHEt$_3$**, $K_a = 4.7 \pm 0.1 \times 10^5\,M^{-1}$, $\Delta H = -83.97\,kJ\,mol^{-1}$, $\Delta S = -173.19\,J\,mol^{-1}\,K^{-1}$; **bguan.picrate/bpse.NHEt$_3$**, $K_a = 2.4 \pm 0.2 \times 10^5\,M^{-1}$, $\Delta H = -88.49\,kJ\,mol^{-1}$ and $\Delta S = -182.27\,J\,mol^{-1}\,K^{-1}$. Heterodimers are highly stable in DMSO and less stable in DMSO–H_2O. Multivalency in the interactions is manifested by positive cooperativity, negative enthalpy of formation (ΔH) and sizeable negative entropy (ΔS), in support of the development of well-ordered supramolecular structures in solution.

Keywords: heterodimer; guadinylated; carboxylated; cyclodextrin; cooperativity

Introduction

Non-covalent interactions that lie at the heart of molecular recognition processes in systems either biological or synthetically designed, cover a wide range of energies. Coulombic and dipole–dipole non-covalent interactions can be either attractive or repulsive depending on the sign of charges and the mutual disposition of dipoles (*1*); however, all forces are distance dependent. Ion-pair binding complemented with other non-covalent associations can very effectively stabilise the interacting molecules, whereas the solvent plays a key role in the overall stabilisation. By selecting the geometry and functionalities present in components, artificial self-assembling species with cavities can be formed by the reversible non-covalent interaction of two or more subunits. The new entities will possess a well-defined structure in solution and be capable of binding other molecules. Thus, a vast array of functional supramolecular architectures (capsules, tweezers, clips, etc.) were created (*1–6*).

The guanidinium group, present not only in proteins as the end group of the amino acid arginine, but also in many artificial recognition systems (*1*, *7*, *8*), has both anion binding and hydrogen bond donating properties and plays a key role in ionic recognition and association processes. The guanidinium group is protonated at neutral pH (pK_a of guanidine, 13.5; pK_a of arginine, 12.5) and due to its flat, Y-shaped geometry forms strong electrostatic and directed H-bonds with carboxylate and phosphate groups (*1*). If many guanidinium groups are present on a core molecule, the multivalency of interactions is expected to strongly enhance the binding to multianionic substrates (*9*).

Cyclodextrins (CDs) are water-soluble cyclic oligosaccharides that have the shape of a cup and form inclusion complexes with organic molecules (*8*) that enter their hydrophobic cavity. The presence of guanidino groups on the primary (narrow) side of CDs (Scheme 1(a)), i.e. in hexakis-, heptakis- and octakis(6-guanidino-6-deoxy)-CDs (*10*), endows them with unique properties: (i) they include anionic guests strongly and preferentially in their cavity (*10*); (ii) consequently, they can efficiently capture nucleotides (*11*) and (iii) they strongly bind DNA which in their presence forms nanoparticles (*10*). The ability to include nucleotides has been previously demonstrated with amino-substituted CDs (*12–16*) as well as mono- or bis-guanidino-CDs (*15*, *17*), with the latter presenting an enthalpic advantage over the amino CDs in stabilisation of binding.

The question of whether per-guanidino CDs would form stable supramolecular heterodimers with anionic,

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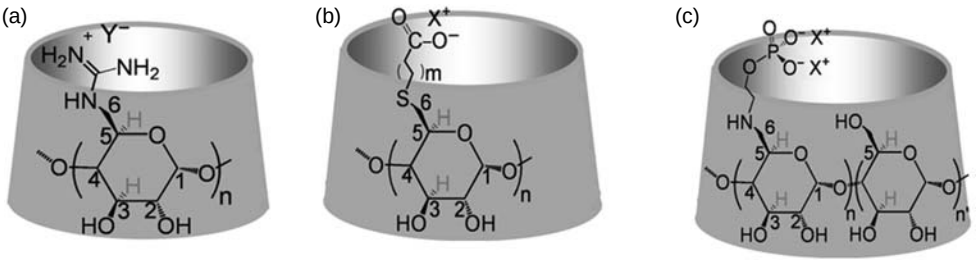


Scheme 1. (a) Per(6-guanidino-6-deoxy)-CDs; (b) per(6-carboxylate-6-deoxy)-βCDs and (c) 6-phosphate-6-deoxy-βCD.

specifically per-carboxylate-substituted CDs (Scheme 1(b), **bpse** and **bpsp**) and phosphate-substituted CDs (Scheme 1(c), **bphos**) has led to the present study based on results from NMR spectroscopy and isothermal titration calorimetry (ITC).

Specifically, the question of stabilisation via multivalency is addressed as well as its correlation with the structural features of the interacting CDs. The solvents of choice were the polar aprotic dimethyl sulphoxide (DMSO, $\varepsilon = 47.2$) and the mixture DMSO–H_2O (80/20, v/v). Water was avoided, since its large dielectric constant $\varepsilon = 80$ (*18*) is expected to decrease the strength of association between ions, the Coulombic attraction being proportional to the interacting charges but inversely proportional to ε. Water also competes for hydrogen bonding with the interacting host–guest system. Furthermore, in DMSO-d_6 and DMSO-d_6/water (but not in D_2O or H_2O/10% D_2O), the chemical shift changes of the exchangeable protons of the guanidinium groups could be monitored during titration runs by ^{1}H NMR spectroscopy and thus their interactions with the carboxyl groups would be directly measured.

Results and discussion

Preparation of charged CDs

Negatively charged CDs, heptakis[6-(3-thiopropionate)-6-deoxy]-β-CD (**bpsp**) and heptakis[(6-(2-thioethanoate)-6-deoxy]-β-CD (**bpse**) (Scheme 1(b)) were prepared as their sodium salts, according to the literature (*19*). They were then converted to the corresponding water-insoluble acids and subsequently to triethylammonium salts, **bpsp.NHEt$_3$** and **bpse.NHEt$_3$**, respectively, *in situ* (Figures S1 and S2 of the Supplementary material, available online). These triethylammonium salts were water and DMSO soluble. The positively charged CD, heptakis(6-guanidino-6-deoxy)-β-CD hydrochloride (**bguan.HCl**) (*10*), was the selected interacting partner. Per-6-guanidino-CDs have a p$K_a \cong 12$ (*20*); therefore, they are fully protonated at neutral pH, unlike the corresponding per-6-amino-CDs which are only partly protonated at neutral pH (*21*) owing to p$K_a \cong 8$. In order to facilitate solubility in DMSO, the product was converted to its picrate salt, **bguan.picrate** (Figure S3 of the Supplementary material, available online). [6-(2-Aminoethyl phosphate)-6-deoxy]-β-CD (**bphos.Na**), a 20% phosphorylated water-soluble CD derivative (Scheme 1(c)) was prepared in 30% yield after the reaction of heptakis[6-bromo-6-deoxy]-β-CD (water insoluble) with 2-aminoethyl dihydrogen phosphate at 80°C under pressure (10 atm N_2) in DMF (Figure S4 of the Supplementary material, available online). ^{31}P NMR spectra confirmed the presence of aminoethyl phosphate groups in the product, because two broad ^{31}P signals at frequencies different from that of the starting reagent were observed (Figure S5 of the Supplementary material, available online). The product was subsequently converted *in situ* in DMSO to **bphos.NHEt$_3$**. Enzymatic trials to obtain per-phosphorylated-βCD using either glycokinase or hexokinase, employing protocols for the conversion of α-D-glucose to α-D-glucose-6-phosphate (*22*, *23*), were not successful using natural βCD as the substrate. In spite of its partial phosphorylation, **bphos.NHEt$_3$** was included in the study in order to roughly estimate the binding of the phosphate groups with their guanidinium counterparts.

In all the above cases, the anionic groups are connected to the βCD core via two or three bond linkers. On the contrary, the guanidino groups are grafted directly on the narrow CD periphery, thus are pre-organised and their spatial freedom is limited.

^{1}H NMR studies in DMSO-d$_6$ and DMSO-d$_6$–H_2O (80/20, v/v) solution

The ^{1}H NMR signals of the guanidinium group [—C6N*H*(=NH)NH_2.H^+] were monitored during titration of **bguan.picrate** with **bpsp.NHEt$_3$**, **bpse.NHEt$_3$** or **bphos.NHEt$_3$**, and the chemical shift changes were plotted against the equivalents of the titrants added (Figure 1). The graphs display a fast growing part, a clear change of slope near the points where 1 equivalent of titrant was added and

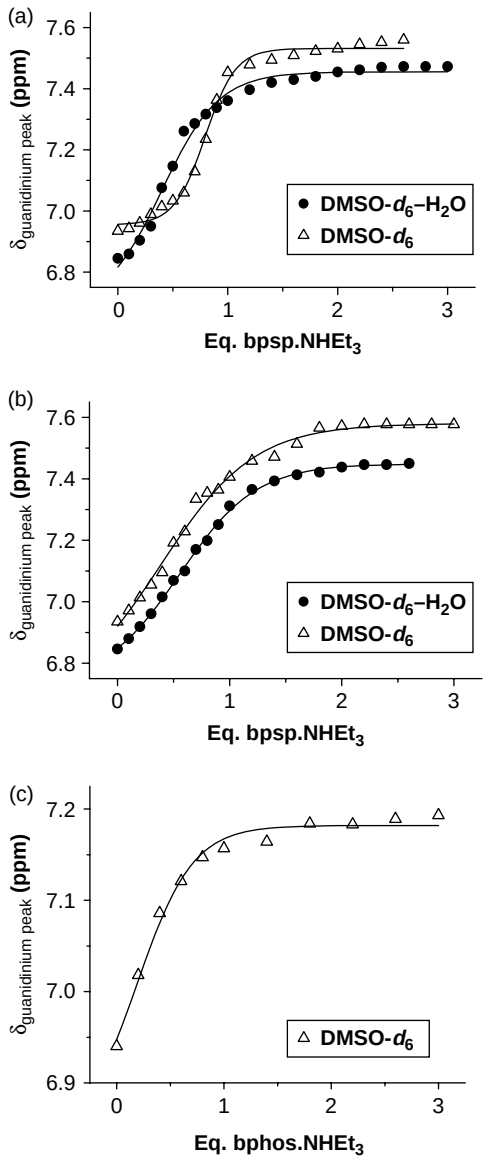


Figure 1. NMR titration data plots (chemical shift of guanidinium peak vs. equivalents of titrant). (a) **bguan.picrate/bpsp.NHEt$_3$**; (b) **bguan.picrate/bpse.NHEt$_3$** and (c) **bguan.picrate/bphos.NHEt$_3$**. Solid lines correspond to fitting according to Equation (4) (see Section 4).

subsequently a plateau, thus indicating 1:1 interaction between the positively and negatively charged CD partners. Blank experiments, i.e. self-titrations of each of the components, did not result in the change of the peaks; therefore, homodimers are not formed. It follows that the apparently strong binding is due to the formation of heterodimers via electrostatic and H-bonding interactions between guanidinium and carboxylate groups (*1*). The curves appear to be sigmoidal, most clearly in the plot of **bguan.picrate/bpsp.NHEt$_3$** in DMSO-d_6 (Figure 1(a)), indicating cooperative binding behaviour (*24*). Likewise, the titration plot for the same interacting pair obtained by recording the ^{13}C NMR chemical shift changes of the carbon atom C6 next to the guanidinium group also had an unambiguous sigmoidal shape.

In order to quantify these observations, the NMR data were fitted to a general 'Dose–Response' function (see Section 4, Equation (4)) that provides an estimation of h, the Hill coefficient that is interpreted as an index of cooperativity (*24*), for 1:1 interactions. The calculated h_i values, 3.13 ± 0.31 and 1.68 ± 0.20 (Table 1) for **bguan.picrate/bpsp.NHEt$_3$** indicate positive cooperativity for binding in both DMSO-d_6 and DMSO-d_6–H_2O, respectively. The pair **bguan.picrate/bpse.NHEt$_3$** having $h = 1.04 \pm 0.12$ and 1.28 ± 0.06, respectively in the two solvent systems, shows rather non-cooperative behaviour, whereas data of **bguan.picrate/bphos.NHEt$_3$**, cannot be used (and will not be used thereafter) to extract quantitative results.

Fitting of NMR titration data and calculation of binding constants

In order to extract binding constants fittings to Equation (1) that describes binding for 1:1 systems in fast exchange in NMR spectroscopy (*25*, *26*) and to its simplified form 2, according to the Eadie–Hofstee approximation (*24*), $[(\Delta\delta_i/\Delta\delta_{max})C \rightarrow 0]$ were attempted, where $\Delta\delta_i$ is the observed difference of the chemical shifts, $\Delta\delta_{max}$ is the maximum difference of the chemical shifts, X is the concentration of the titrant added, C is the concentration of the component held constant and K and K_D ($K = (K_D)^{-1}$) are the association and the dissociation constants, respectively.

$$[X] = (\Delta\delta_i/\Delta\delta_{max})[C + (K\cdot(1 - [\Delta\delta_i/\Delta\delta_{max}])^{-1}] \quad (1)$$

or

$$\Delta\delta_i = -K_D\cdot(\Delta\delta_i/[X]) + \Delta\delta_{max}. \quad (2)$$

Fitting of the data to either equation was unsuccessful; however, the data were satisfactorily fitted (Figure 2) to the linear Hill Equation (3) (*24*), where $Y = \Delta\delta_i/\Delta\delta_{max}$:

$$\log[Y/(1 - Y)] = -\log K_D + h_{ii}\log X. \quad (3)$$

The results, although with considerable errors, give an estimation of the binding constants, listed in Table 1. The highest h_{ii} and K_a values are observed for **bguan.picrate/bpsp.NHEt$_3$** in DMSO. The coefficients h_{ii} calculated from Equation (3) are greater than 1 in all cases, confirming positive cooperativity in the supramolecular assembly of the pairs to a stoichiometric capsule. This behaviour could be explained by considering a model where the first guanidinium–carboxylate binding event at glucopyranose unit A of **bguan.picrate** is followed by sequential binding at adjacent units (B, C...), facilitated by local confor-

Table 1. Fitting results of NMR titration data.

System	Stoichiometry	Hill coefficient h_i[a]	Hill coefficient h_{ii}[b]	K_a (M^{-1}) (R^2)[c]
bguan.picrate/bpsp.NHEt$_3$				
DMSO-d_6	1:1	3.13	2.21	6.4×10^5 (0.982)
DMSO-d_6–H_2O	1:1	1.68	1.77	5.9×10^4 (0.978)
bguan.picrate/bpse.NHEt$_3$				
DMSO-d_6	1:1	1.04	1.72	4.2×10^4 (0.993)
DMSO-d_6–H_2O	1:1	1.28	1.56	1.2×10^4 (0.994)

[a] Obtained from Equation (4) (see Section 4).
[b] Obtained from Equation (3).
[c] Goodness of fit.

mational changes of the carboxylate linkers of **bpse** or **bpsp** that result in accelerating the binding rate until this 'zipping' process is completed. Increase in the carboxylate-CD concentration during the titration of **bguan.picrate** favours this 'dimerisation by zipping' mechanism over other oligomerisation or polymerisation processes, thus giving a sigmoidal binding curve. It follows that the binding Equation (1), that assumes a linear relationship between the binding rate and titrant concentration, cannot be satisfactorily applied to fit the data.

ITC in DMSO solution

ITC is frequently used to calculate thermodynamic parameters and binding constants in CD systems (*8*). The sample placed in a cell with adiabatic walls is titrated automatically with a titrant's solution. The heat change recorded during the titrations, compared to a reference sample, is translated to an enthalpy change vs. titrant's molar ratio plot. For the present supramolecular systems, data plots obtained in mixtures of DMSO–H_2O (80/20, v/v) gave non-reproducible results due to large background noise, evidently owing to strong DMSO–water exothermic association which is very much dependent on the exact composition of the mixture (*27*). Thus, ITC data were obtained only in pure DMSO solutions and were analysed by fitting to the function used for 1:1 interacting systems built in the Microcal Origin software. The fitting results are displayed in Figure 3. The fitting results are summarised in Table 2.

The values of K_a obtained from ITC are similar to the ones calculated from NMR data (Table 1) and the stoichiometry was confirmed 1:1, showing that both methods reflect the same types of interactions, i.e. charge–charge, dipole–dipole and H-bonding, in pure DMSO. The processes are exothermic and the ΔG values are high. A sizeable negative entropy contribution in both cases signifies the formation of well-ordered supramolecular structures. Thus, NMR and calorimetric titration results both indicate the formation of heterodimers between

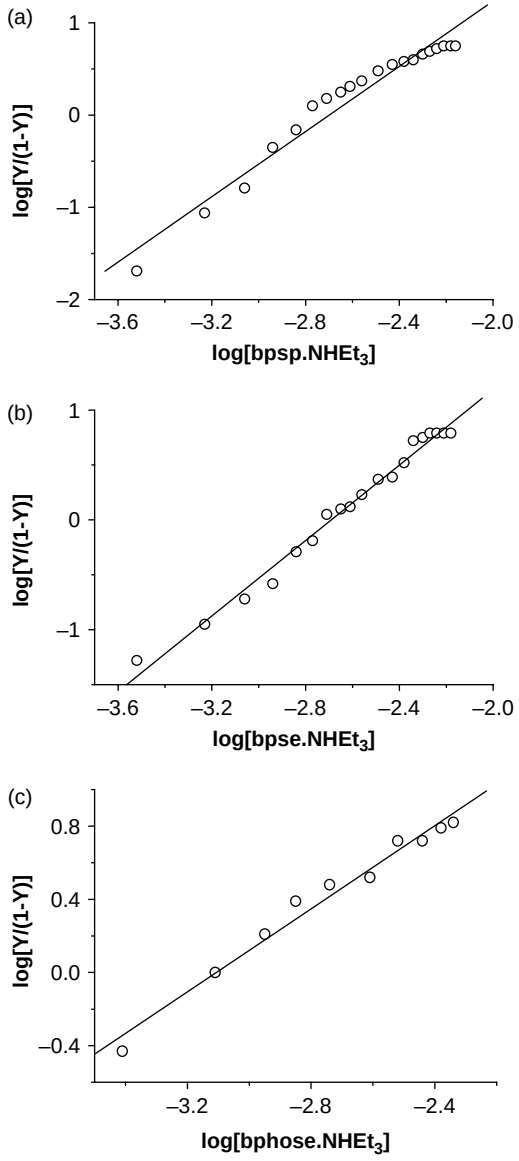


Figure 2. Fitting of the NMR data to the linear Equation (3): (a) **bguan.picrate/bpsp.NHEt$_3$** in DMSO-d_6–H_2O, (b) **bguan.picrate/bpse.NHEt$_3$** in DMSO-d_6 and (c) **bguan.picrate/bphos.NHEt$_3$**.

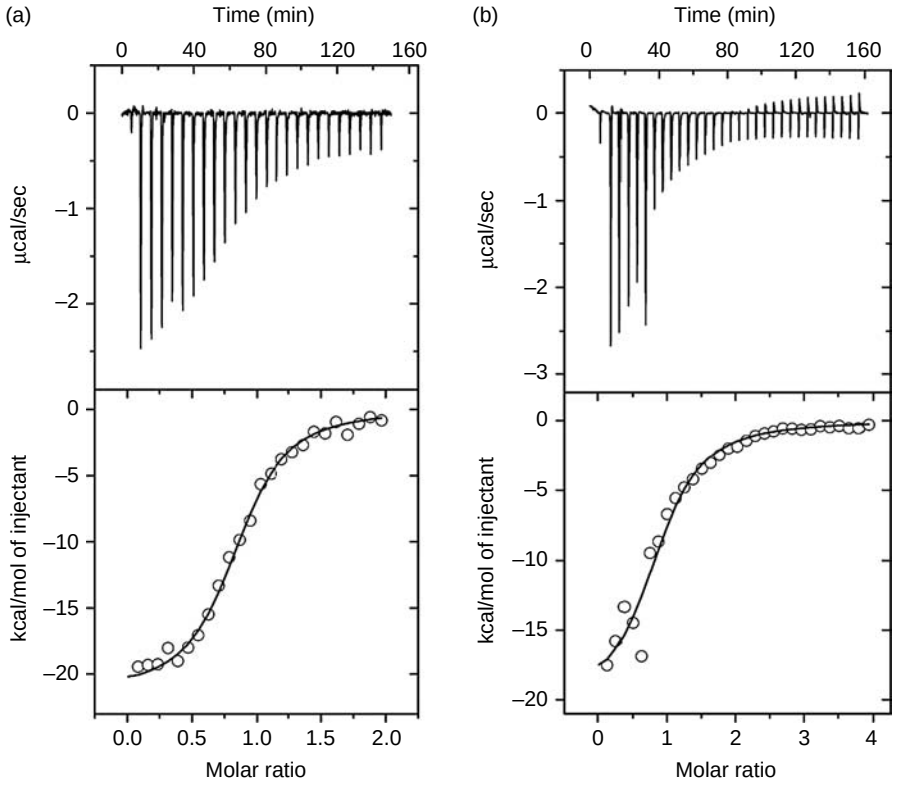


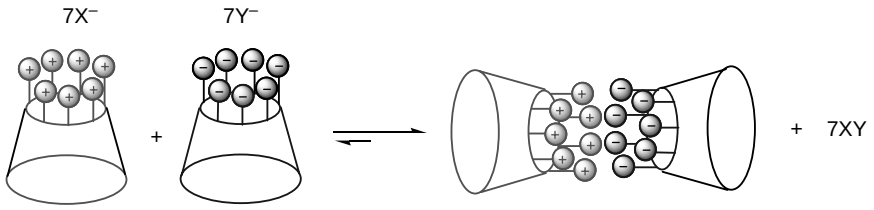
Figure 3. Microcalorimetric titrations in DMSO of (a) **bguan.picrate** with **bpsp.NHEt$_3$** and (b) **bguan.picrate** with **bpse.NHEt$_3$**. Top panels: detected heat signals; bottom panels: heat of reaction and fitting results (solid lines).

the fully cationic **bguan** and the fully carboxylated or the partially phosphorylated CDs, **bpsp**, **bpse** and **bphos**, in DMSO (NMR, ITC) and in DMSO–H_2O (80/20, v/v or % 50.4/49.6% mole) (NMR). There is strong binding between **bguan** and **bpsp** in DMSO ($\log K \sim 5$) which decreases in DMSO–H_2O ($\log K \sim 4$) or upon shortening of the chain connecting the carboxylate groups to the CD torus by one methylene group (**bpse**). Introduction of water lessens the H-bonding-directed ion pairing, whereas the optimal spatial arrangement of the interacting groups is also important, as expected. The present ΔH values of $\cong 12$ kJ mol^{-1} per glucopyranose unit compare well with the literature calorimetric data obtained for model guanidinium–carboxylate systems in DMSO ($\Delta H = 15.06$ kJ mol^{-1}) (*28*), where planarity was structurally imposed in the two interacting charged molecules. In the same study (*28*), it was additionally demonstrated that when the counterion of the guanidinium species was changed from chloride to tetraphenyl borate and then to acetate, the binding constant almost doubled (*28*). The latter observation can be rationalised if one considers that soft–soft ion pairs interact more favourably than soft–hard ones, according to Pearson's hard–soft (Lewis) acid–base principle (*29*). In the present interacting systems with soft counterions, picrate and triethylammonium, the anticipated favourable overall effect is reflected in the high binding constants. The ionic capsule between amidinium and carboxylate calyx[4]arenes was reported (*3*) to assemble in water with $K_a \cong 3.3 \times 10^{-4}$ M^{-1}, a value close to the ones reported for our CDs in DMSO–H_2O. Previous potentio-

Table 2. Binding constants and thermodynamic parameters obtained from ITC (DMSO, 25°C).

Thermodynamic parameters	**bguan.picrate/bpsp.NHEt$_3$**[a]	**bguan.picrate/bpse.NHEt$_3$**[b]
K_a (M^{-1})	$4.7 \pm 0.1 \times 10^5$	$2.4 \pm 0.2 \times 10^5$
N[c]	0.99 ± 0.06	0.93 ± 0.06
ΔH (kJ mol^{-1})	-83.97 ± 1.04	-82.44 ± 3.45
ΔS (J mol^{-1} K^{-1})	-173.19 ± 3.93	-182.27 ± 0.20
ΔG (kJ mol^{-1})	-32.36 ± 2.20	-28.10 ± 3.55

[a] $X^2 = 84{,}465$.
[b] $X^2 = 295{,}652$.
[c] Stoichiometry.



Scheme 2. Heterodimer formation between positively and negatively charged CDs.

metric titrations (*30*, *31*) between carboxy and amino CDs in water have afforded $\log K_a \sim 10$ (*30*) and 8.5 (*31*). The displayed cooperativity in the present binding cases could be anticipated to promote stronger binding and association constants higher than the measured $\sim 10^5$, given that the interacting charged groups reside in a rigid circle on the macrocycle of the CDs. Apparently, the ease of approach of the two oppositely charged macrocycles, the efficiency of solvation of the large counterions and the degree of organisation between the seven interacting ion couples are effective but not absolutely optimal in the narrow CD region. Our results also indicate that the binding would be much stronger if the perphosphorylated CDs were available for testing.

Conclusions

Heterodimers are efficiently and cooperatively formed between a guanidino CD and two carboxylated as well as one partially phosphorylated CDs, a result of Coulomb and H-bonding interactions. The stoichiometry is 1:1 in all cases, and the association is characterised by high stability in DMSO, which weakens in DMSO–H_2O, positive cooperativity indicating multivalency effects, negative enthalpy of formation, ΔH and sizeable negative entropy, ΔS, indicating formation of very ordered supramolecular structures (Scheme 2).

Experimental

Synthesis

General

Heptakis(6-bromo-6-deoxy)-β-CD (*32*), heptakis(6-azido-6-deoxy)-β-CD (*10*, *33*), heptakis (6-amino-6-deoxy)-β-CD (*10*, *33*) and heptakis(6-guanidino-6-deoxy)-β-CD hydrochloride (**bguan.HCl**) (*10*) were prepared using literature procedures. Likewise, heptakis[6-(3-thiopropionate-6-deoxy]-β-CD, sodium salt (**bpsp.Na**, 71%) and heptakis[6-thio-ethanoate-6-deoxy)-β-CD, sodium salt (**bpse.Na**, 89%) were synthesised according to the published procedures (*19*).

The salt ***bguan.picrate***

It was received after the addition of solid **bguan.HCl** to an aqueous solution of picric acid. The copious precipitate that was formed was filtered and recrystallised from hot water (98%). ^{1}H NMR (DMSO-d_6, 298 K, 500 MHz): δ 8.61 (s, 14H, picr), 7.23 (br s, 7H, C_6NH), 6.94 (br s, 21H, (C(=NH)NH_2), 6.20 (br d, $J = 6$ Hz, 7H, OH3), 6.03 (br s, 7H, OH2), 4.89 (br s, 7H, H1), 3.82–3.80 (br d, 7H, H5), 3.71–3.69 (br d, 7H, H3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 162.10 (C(=NH)NH), 158.46 (picr), 141.87 (picr), 126.69 (picr), 102.98 (C_1), 82.56 (C_4), 73.20 (C_5), 72.68 (C_2), 70.51 (C_3), 42.50 (C_6, obscured by residual DMSO signal). Anal. calcd for $C_{49}H_{91}O_{28}N_{21}.7C_6H_3N_3O_7.7H_2O$: C, 34.7; H, 4.0; N, 18.7%. Found: C, 35.2; H, 4.3; N, 17.5.

The salt ***bpsp.NHEt$_3$***

It was prepared *in situ* in a DMSO-d_6 solution using the acid **bpspH** (Anal. calcd for $C_{63}H_{98}O_{42}S_7.NaCl.7H_2O$: C, 39.1; H, 5.8%. Found: C, 38.7; H, 6.0) received from a Sephadex column after washing **bpsp.Na** with water acidified with HCl to pH 2.3, and seven equivalents of triethylamine: ^{1}H NMR (DMSO-d_6, 298 K, 500 MHz): δ 5.84 (br s, 14H, OH2, OH3), 4.86 (d, $J = 2$ Hz, 7H, H1), 3.79 (br t, $J = 7$ Hz, 7H, H5), 3.61 (t, $J = 9$ Hz, 7H, H3), 3.38 (t, $J = 9$ Hz, 7H, H4), 3.32 (dd, $J = 2, 9$ Hz, 7H, H2), 3.06 (d, $J = 12$ Hz, 7H, H6), 2.87 (dd, $J = 5, 12$ Hz, 7H, H6′), 2.72 (t, $J = 7$ Hz, 14H, H7, H7′), 2.55 (q, $J = 7$ Hz, 42H, $^+$NH$(CH_2)_3$), 2.44 (dd, $J = 7$ Hz, $J = 13$ Hz, 14H, H8, H8′), 0.98 (t, $J = 7$ Hz, 63H, $(CH_3)_3$). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 173.58 (C=O), 101.83 (C1), 84.07 (C4), 72.26 (C3), 72.08 (C2), 70.76 (C5), 45.64 (CH_2, Et), 35.42 (SCH_2), 33.04 (C6), 28.23 (—O=C*C*H_2), 10.24 (CH_3, Et).

The salt ***bpseNHEt$_3$***

It was prepared from **bpseH** (Anal. calcd for $C_{56}H_{84}O_{42}S_7.7NaCl.14H_2O$: C, 29.1; H, 4.9%. Found: C, 28.9; H, 4.4) in the same manner: ^{1}H NMR (DMSO-d_6, 298 K, 500 MHz): δ 5.79 (br s, 14H, OH2, OH3), 4.85 (d, $J = 3$ Hz, 7H, H1), 3.81 (br t, 7H, H5), 3.60 (t, $J = 9$ Hz,

7H, H3), 3.43 (d, $J = 9$ Hz, 7H, H4), 3.32 (dd, $J = 3, 9$ Hz, 7H, H2), 3.23 (br s, 14H, H7, H7′), 3.06 (d, $J = 13$ Hz, 7H, H6), 2.88 (dd, $J = 5, 13$ Hz, 7H, H6′), 2.59 [br q, $J = 7$ Hz, 42H, $^{+}NH(CH_2)_3$], 0.99 [t, $J = 7$ Hz, 63H, $(CH_3)_3$]. ^{3}C NMR (DMSO-d_6, 125 MHz): δ 172.30 (C═O), 102.06 (C1), 84.12 (br, C4), 72.56 (C3), 72.28 (C2), 70.68 (C5), 45.43 (CH_2, Et), 36.47 (SCH_2), 33.47 (C6), 9.74 (CH_3, Et).

*6-(2-Aminoethylphosphate)-6-deoxy-β-CD, sodium salt (**bphosNa**)*

It was obtained after the reaction of heptakis(6-bromo-6-deoxy)-β-CD (200 mg, 0.126 mmol) with an aqueous solution (3 ml) of 2-aminoethyl dihydrogen phosphate (177.7 mg, 1.26 mmol, 10 eq.) and triethylamine (0.35 ml, 2.52 mmol, 20 eq.) in DMF (6 ml). The mixture was placed into a Parr autoclave at 80°C and N_2 (10 atm) for 1 week. The product was received after dialysis using a benzoylated cellulose membrane (MWCO 1200) (30% yield). NMR and MS data proved a 20% substitution. Mp 220–230°C; ^{1}H NMR (D_2O, 298 K, 500 MHz): δ 5.10 (br s, 7H, H1), 4.25–3.50 (m, 42H, H3, H5, H8, H2, H4, H6), 3.35 (br s, 7H, H7), 2.95 (br s, 7H, H6′). ^{13}C (D_2O, 298 K): δ (broad signals) 102.1 (C1), 81.7 (C4), 73.0 (C3), 72.0 (C5), 71.5 (C2), 60.8 (br, C6, C8), 59.4 (C7), 44.2 (C_6NH). ^{31}P NMR (D_2O, 298 K, 202 MHz, pH = 6.0): δ 3.71. MALDI-TOF-MS *m/z*: 1153.6 (100%, βCD.H_2O), 1258.4 (54%, $C_{44}O_{38}H_{77}NP$), 1382.4 (9%, $C_{46}O_{41}H_{84}N_2P_2$).

NMR titrations

The NMR titrations and self-titrations (molar ratio method) were recorded on a Bruker Avance DRX 500 MHz spectrometer at 298 K. A solution of **bguan.picrate** (3.0 mM, 500 μl) in DMSO-d_6 or DMSO-d_6–H_2O (80/20, v/v), respectively, was titrated with either **bpsp.NHEt$_3$** or **bpse.NHEt$_3$** solution (30 mM) of the same composition. Additions of 5 μl were carried out until molar ratio 1:1 and continued with 10 μl aliquots of the titrants' solutions until molar ratio 1:3. A **bguan.picrate** solution (2.0 mM, 500 μl) in DMSO-d_6 was titrated with a **bphos.NHEt$_3$** solution (20 mM). The additions were carried out in 10 μl aliquots until molar ratio 1:1 and in 20 μl aliquots until molar ratio 1:3. All titrants' solutions were fixed in the same concentration of **bguan.picrate** as the solutions were being titrated.

NMR data fitting

The NMR data were analysed using the 'Dose–Response' built-in function (4) below in Origin® 5.0 and GraphPad Prism® 1.0, used for 1:1 interactions where A_1 and A_2 are the bottom and top asymptotes, respectively, h is the Hill constant and x_0 is the value which refers to the point halfway between the bottom and the top of the curve,

$$y = A_1 + [(A_2 - A_1)/(1 + 10^{(x_0 - x)\cdot h})]. \quad (4)$$

Microcalorimetry

The ITC measurements were carried out using a MCS-ITC unit by MicroCal Inc., Northampton, MA, USA. The reaction cell with a 1.4 ml volume was filled with **bguan.picrate** solution in DMSO (0.03 mM). A 250-μl titration syringe was filled with the titrants, **bpsp.NHEt$_3$** (0.3 mM) and **bpse.NHEt$_3$** (0.6 mM), at 298 K. Injection experiments of 8 μl each were programmed and executed automatically. A time delay of 300 s was set between the injections and the contents of the sample were stirred throughout the experiment at 400 rpm to ensure thorough mixing. All solutions were degassed under vacuum for 15 min immediately before measuring. Enthalpies of dilution of host and of guest compounds were determined in separate experiments and subtracted from the corresponding experimental host–guest curves, before fitting of the latter. The final calorimetric data were analysed using the ORIGIN® 5.0 software with embedded calorimetric fitting routines.

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