

Determination of stability constants of tauro- and glyco-conjugated bile salts with the negatively charged sulfobutylether- β -cyclodextrin: comparison of affinity capillary electrophoresis and isothermal titration calorimetry and thermodynamic analysis of the interaction

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Abstract The aim of the present work was to investigate the interaction between bile salts present in the intestine of man, dog and rat with the negatively charged cyclodextrin (CD), sulfobutylether- β -cyclodextrin (SBE β CD). The interactions between bile salts and CDs are of importance for the release of CD-complexed drugs upon oral administration. This makes a good understanding of this particular interaction important for rational drug formulation. SBE β CD is a modified CD, which has attracted particular interest in formulation science. It is unique in the sense that it carries approximately seven negatively charged side chains, which can potentially interact electrostatically with the guest molecule. Bile salts are negatively charged at physiological pH, and the concomitant repulsion from SBE β CD could potentially reduce their affinity for this CD and hence their

ability to expel drugs delivered as SBE β CD complexes. However, this study has demonstrated that the interaction, between a bile salts and SBE β CD is only slightly weaker than the corresponding interactions with natural β CD. Significant differences between the thermodynamics of bile salt complexes with respectively HP β CD and SBE β CD were found, when comparing the same degree of substitution. This underscores the importance of the substituents on the interactions of modified CDs with bile salts.

Keywords Isothermal titration calorimetric · ACE · Bile salts · Complexation · Cyclodextrin · Beta-cyclodextrins · Simulations

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Introduction

Cyclodextrins (CDs) are macrocyclic oligosugars commonly composed of 6, 7 or 8 glucosidic molecules, α -, β - and γ -CDs, respectively [1–4]. The outer hydrophilic surface and the inner hydrophobic cavity make the CDs capable of forming inclusion complexes with molecules of appropriate shape and size. Although not exclusively, the formed ligand–CD complexes are often of 1:1 stoichiometry [1]. A variety of chemically modified CDs have been synthesised in order to optimise physicochemical and toxicological properties as compared to the native CDs. The inclusion of guest molecules into CDs and their derivatives facilitates many applications such as solubilisation [5–10], targeting of drugs [11], and chromatographic separations [12, 13].

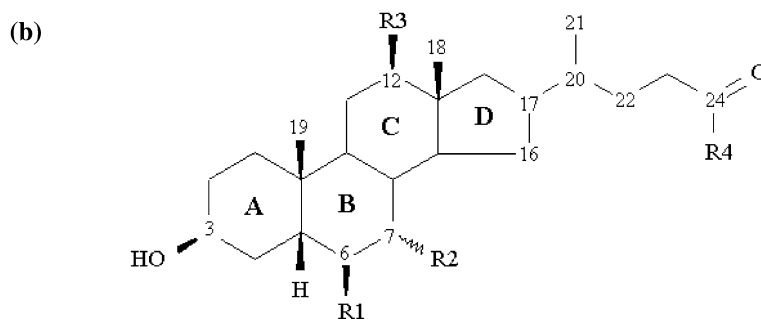
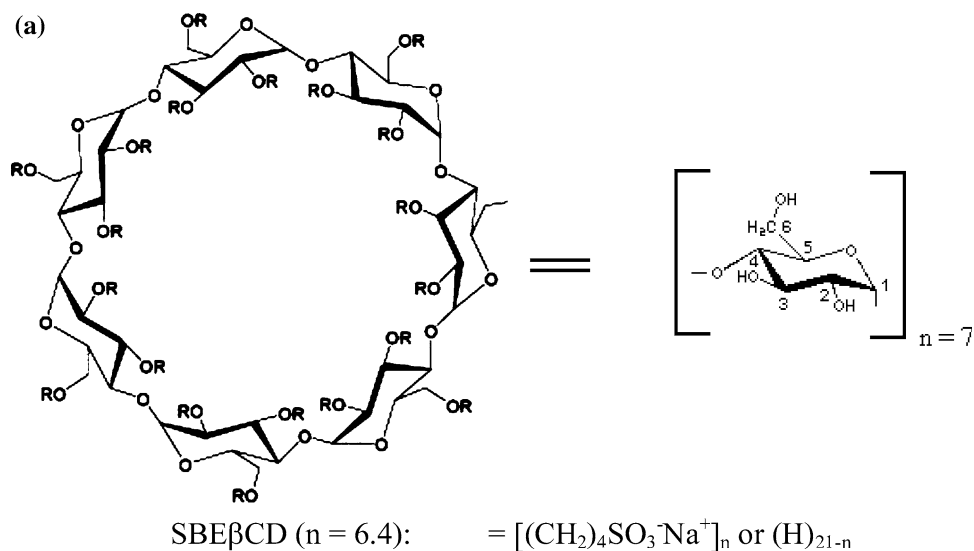
Commonly, the modified β CDs, methyl-, 2-hydroxypropyl- (HP β CD) and sulfobutylether- β -cyclodextrin (SBE β CD), are

used in commercial pharmaceutical products [14, 15]. The driving forces for the formation of CD inclusion compounds are mainly hydrophobic and van der Waals interactions [16, 17], but for the negatively charged SBE β CD Coulombic interactions have also been demonstrated to contribute significantly [18]. Generally, SBE β CD has been demonstrated to form stronger inclusion complexes than HP β CD [19–28], although some exceptions are published [29, 30]. The differences between the two modified CDs are, however, specially pronounced for positively charged molecules [19, 24, 27]. Many drugs are bases and, hence, positively charged at low pH, which makes SBE β CD particularly interesting in drug discovery and

development as it can induce a charge–charge interaction increasing the complexation efficiency significantly [10].

Upon oral administration, xenobiotics complexed with CD must be displaced from the complex to be available for absorption, as CDs are only absorbed from the gastrointestinal tract to a very small extent [2]. In the small intestine this displacement is thought to depend upon bile salts [31–33], which may bind competitively to the CD. For SBE β CD, it may be speculated whether the negative charge may repel the negatively charged conjugated bile salts (see Fig. 1) present in the intestine. If indeed so, this would lead to a slower or lower release of the drug from the

Fig. 1 Schematic structure **a** SBE β CD and **b** the glyco- and tauro-conjugated bile salts; α indicates a steric orientation below the plan and β above



Bile acid	Abbreviation	R ₂ (C-6)	R ₃ (C-7)	R ₄ (C-12)	R ₅ (C-24)
Glycocholic acid	(GC)	H	OH (α)	OH	NHCH ₂ COO ⁻
Glycochenodeoxycholic acid	(GCDC)	H	OH (α)	H	NHCH ₂ COO ⁻
Glycodeoxycholic acid	(GDC)	H	H	OH	NHCH ₂ COO ⁻
Taurocholic acid	(TC)	H	OH (α)	OH	NHCH ₂ CH ₂ SO ₃ ⁻
Taurochenodeoxycholic acid	(TCDC)	H	OH (α)	H	NHCH ₂ CH ₂ SO ₃ ⁻
Taurodeoxycholic acid	(TDC)	H	H	OH	NHCH ₂ CH ₂ SO ₃ ⁻
Tauro- β -hyocholic acid	(T- β -HC)	OH	OH (β)	H	NHCH ₂ CH ₂ SO ₃ ⁻

CD complex. Therefore, knowledge about the interaction between SBE β CD and bile salts are of special interest for rational drug formulation design using these modified CDs.

Studies investigating the interaction between bile salts and CDs have mainly been exploited by nuclear magnetic resonance (NMR) [34–37], calorimetry [16, 33, 38–41] or affinity capillary electrophoresis (ACE) [42–44]. The highly negatively charged SBE β CD poses a special challenge in ACE experiments due to the need for correction for changes in ionic strength and temperature and the effect it has on electrophoretic as well as complexation behavior as a function of SBE β CD concentration. Østergaard et al. [45] have in a recent study developed a method to correct for the ionic strength effects for SBE β CD, but the results have not been evaluated by a method less affected by differences in ionic strength. In this work, this correction protocol is tested by comparisons of data from respectively ACE and isothermal calorimetry (ITC). The overall purpose of this study is to investigate possible effects of the negatively charged side chains on SBE β CD on the stability and structure of its complexes with bile salts by the use of a combination of calorimetric, electrophoretic and molecular dynamics (MD) simulation methods.

Experimental procedures

Materials

The sodium salts of the bile acids were purchased from various sources. Taurocholate (TC; CAS no. 83830-80-2) was purchased from Fluka (Buchs, Switzerland). Tauro- β -muricholate (T β MC; CAS no. 25696-60-0) was purchased from Makaira Ltd (London, GB). Taurochenodeoxycholate (TCDC, CAS no. 516-35-8), taurodeoxycholate (TDC; CAS no. 516-50-7), glycocholate (GC; CAS no. 475-31-0), glycodeoxycholate (GDC; CAS no. 16409-34-0), glycochenodeoxycholate (GCDC; CAS no. 640-79-9) and deuterium oxide (99.95 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All compounds were used as provided. SBE β CD was purchased from CyDex (Lenexa, USA). Sodium phosphate buffer used in the CE experiments, 0.050 M, pH 7.00, was obtained from Agilent (Palo Alto, USA). Ultra pure water for the ACE experiments was obtained from Agilent whereas water for the ITC experiments was from a Millipore purification system (Billerica, MA, USA). All other chemicals were of analytical grade and used as received.

Characterisation of SBE β CD by NMR

The NMR experiments were carried out at 25 °C on a Bruker Avance-600 NMR (Bruker Biospin, Rheinstetten, Germany) spectrometer operating at 14.1 T and equipped

with a cryogenically cooled probe: 32 scans were acquired for the 1D ^1H experiments, 5,120 scans were acquired for the attached proton test (APT) experiments, while for the two dimensional HSQC and HMBC experiments, 32 and 64 scans, respectively, were acquired for each of the 128 t_1 increments. The SBE β CD samples were run at 40 mM in D_2O .

Capillary electrophoresis

As BGE a 50.0 mM phosphate buffer (pH 7.00, $I = 0.110$ M) was prepared by weighing adequate amounts of disodium hydrogen phosphate dodecahydrate and potassium dihydrogen phosphate, and dilution with water. SBE β CD stock solutions with a concentration of 1.00×10^{-2} M were prepared in 50 mM phosphate buffer (pH 7.00, $I = 0.110$ M) as well as in water. Three series of BGE solutions containing SBE β CD in the concentration range of $0\text{--}7.50 \times 10^{-3}$ M were prepared for the ACE experiments. BGE series 1 was prepared by suitable dilution of the 1.00×10^{-2} M SBE β CD stock solution in 50.0 mM phosphate with the 50 mM phosphate buffer.

The ACE experiments were executed using a Beckman PACE 5510 CE with a diode array detector [45]. Uncoated fused silica bubble cell capillaries (150 μm light path) of 37 cm \times 50 μm inner diameter, with a length of 30 cm to the detector (Agilent Technologies, Waldbronn, Germany) were used in the CE experiments. Conditioning of new capillaries was performed by flushing with 1 M NaOH and water for 30 min each. UV detection was performed at 200 nm and the capillary cassette temperature was set to 22 °C. The applied voltage was set to 10 kV in the normal polarity mode. Samples in buffer without SBE β CD, containing $5\text{--}8 \times 10^{-5}$ M bile salt and 3×10^{-4} % (v/v) formamide as a marker of the electroosmotic flow (EOF), were introduced by applying a pressure of 0.5 psi for 5 s. Each sample was run in triplicate. The capillary was flushed with 0.1 M NaOH, 0.05 M phosphate buffer, the back ground electrolyte (BGE), with or without SBE β CD for 1 min each between measurements.

Electrophoretic mobilities, μ , determined at constant voltage needs to be corrected for changes in temperature (Joule heating), viscosity and ionic strength to a selected reference state prior to the estimation of stability constants [46, 47]. The measured effective electrophoretic mobility, μ , was therefore recalculated to the reference temperature T_0 at 25 °C [41, 42] assuming the mobilities have the same temperature dependence as found for the conductivity of the BGE ($\alpha_{th} = 0.022 \text{ K}^{-1}$):

$$\mu_0 = \frac{\mu}{(1 + \alpha_{th}(T_{at} - T_0))} \quad (1)$$

The temperature corrected electrophoretic mobilities μ_0 were subsequently corrected for viscosity variations, due to

the presence of CD in the BGE and changes in ionic strength as previously described by Østergaard et al. [45].

The binding isotherms were constructed by plotting the corrected effective electrophoretic mobility μ' as a function of the CD concentration ($[CD]$) instead of μ versus $[CD]$ in order to correct for viscosity, ionic strength, and temperature changes. The binding parameters $K_{1:1}$ and μ_{BS-CD} were determined by non-linear regression analysis according:

$$\mu = \frac{\mu_{BS} + \mu_{BS-CD}K_{1:1}[CD]}{1 + K_{1:1}[CD]} \quad (2)$$

using SigmaPlot (version 9.0, Systat Software, Point Richmond, CA, USA).

Isothermal titration calorimetry

The energetics of the interaction between the bile salts and SBE β CD was evaluated on a VP-ITC from MicroCal (Northampton, MA, USA). The experiments were carried out at 25 °C in triplicate in 50 mM phosphate buffer, pH 7.0, and all solutions were degassed using a ThermoVac (MicroCal, Northampton, MA, USA) before the titration experiments. An aliquot of 1 mM bile salt was filled into the reaction cell, while the titration syringe was filled with a 10 mM SBE β CD solution. The binding experiment involved 27 sequential additions of small aliquots (10 μ l) of the SBE β CD solution into the reaction cell under continuous stirring (307 rpm). For T β TC, however, only 0.25 mM bile salt was in the reaction cell and 2.5 mM SBE β CD in the syringe, due to the very strong interaction between the two species. The heat of dilution was estimated from the heat change at high CD concentrations, where practically all bile salt had interacted, and subtracted from the raw data prior to non-linear regression analysis. The latter was performed with the ORIGIN software package (version 7.0). The fitted parameters were the binding constant ($K_{1:1}$), the change in standard binding enthalpy (ΔH^0) and the binding stoichiometry (n) and calculated by the one site model in ORIGIN. Knowledge of the binding constant and molar reaction enthalpy enabled calculation of the standard Gibbs free energy of binding (ΔG^0) and the change in standard entropy (ΔS^0) according to:

$$\Delta G^0 = -RT \ln K_S = \Delta H^0 - T\Delta S^0 \quad (3)$$

where R is the gas constant, and T is the absolute temperature.

For two of the complexes, SBE β CD:GDC and SBE β CD:GCDC, titrations were carried out at six temperatures in the interval 5–55 °C. This enabled calculation of the heat capacity, ΔC_p , which has previously been interpreted with respect to the extent of non-polar dehydration that takes place upon complexation [48, 49]. Precise values of ΔC_p were obtained by simultaneously fitting

the enthalpograms at all temperatures, assuming that ΔC_p is constant over the experimental temperature range and using the van't Hoff equation to link the temperature dependence of the binding constants to the enthalpy changes, as previously described [50].

Molecular dynamics simulations

Molecular dynamics simulations were carried out to study the behaviour of the SBE-chains and the complexation induced changes in water accessible surface area (ASA). 10 ns simulations of the non-complexed bile salts, GDC and GCDC were carried out in $34 \times 34 \times 34 \text{ \AA}^3$ water boxes, while a 25 ns simulation of SBE β CD and 30 ns simulations of its complexes with GDC and GCDC were carried out in water boxes measuring $40 \times 40 \times 40 \text{ \AA}^3$. The SBE β CD sample used in the experiments had on the average 6.4 SBE-chains on each CD molecule. Therefore, CDs with six substituents were used in the MD simulations. The systems were neutralised by adding six sodium ions (counter ions for the six negative charges on the to the SBE chains) to the simulation box. Simulations were done with NAMD version 2.7 [51] using the CHARMM27 force field [52]. Simulation details and parameters for the SBE chains have previously been described by Schönbeck et al. [53].

Calculations of polar and non-polar surface area of the free and complexed species were conducted in VMD [54] by averaging ASA of individual frames in the simulation intervals 2–10 ns for the free bile salts, 5–25 ns for the free SBE β CD and 5–30 ns for the two complexes.

Results and discussion

Though SBE β CD are frequently used for oral administration due to the CDs high solubilisation capacity [10], very little is known about the CDs thermodynamic behaviour, which will be investigated in the present work.

Degree and pattern of substitution of SBE β CD

The NMR analysis of the used SBE β CD suggested average DS of 0.91 and a position of the distribution of the on approximately 50, 40 and 10 % attached to O3, O2 and O6, respectively. A more detailed description can be found in the supplementary materials.

Binding stoichiometry

An electropherogram from the experiments can be seen in the supplementary information. Correlation coefficients, R^2 , were above 0.99, see Table 1, after fitting to the 1:1 model described in Eq. 2, indicating that the 1:1 complexation

Table 1 Stability constants, $K_{1:1}$ and complex mobilities, μ_{BS-CD} for inclusion complex formation of bile salts with SBE β CD determined in 0.050 M phosphate buffer pH 7.00 at 25 °C at constant V and variable I

Bile salt	$K_{1:1}$ (M^{-1})	μ_{BS-CD} ($cm^2 V^{-1} min^{-1}$)	R^2
TC ^a	$1.6 \times 10^3 \pm 3.8 \times 10^1$	$-1.78 \times 10^{-2} \pm 7 \times 10^{-5}$	0.999
GC	$1.6 \times 10^3 \pm 3.3 \times 10^1$	$-1.77 \times 10^{-2} \pm 6 \times 10^{-5}$	0.999
TDC	$4.1 \times 10^3 \pm 1.4 \times 10^2$	$-1.80 \times 10^{-2} \pm 8 \times 10^{-5}$	0.999
GDC	$4.3 \times 10^3 \pm 9.4 \times 10^1$	$-1.79 \times 10^{-2} \pm 5 \times 10^{-5}$	0.999
TCDC ^a	$7.6 \times 10^4 \pm 1.4 \times 10^3$	$-1.80 \times 10^{-2} \pm 3 \times 10^{-5}$	0.999
GCDC	$9.1 \times 10^4 \pm 7.3 \times 10^3$	$-1.78 \times 10^{-2} \pm 3 \times 10^{-5}$	0.999

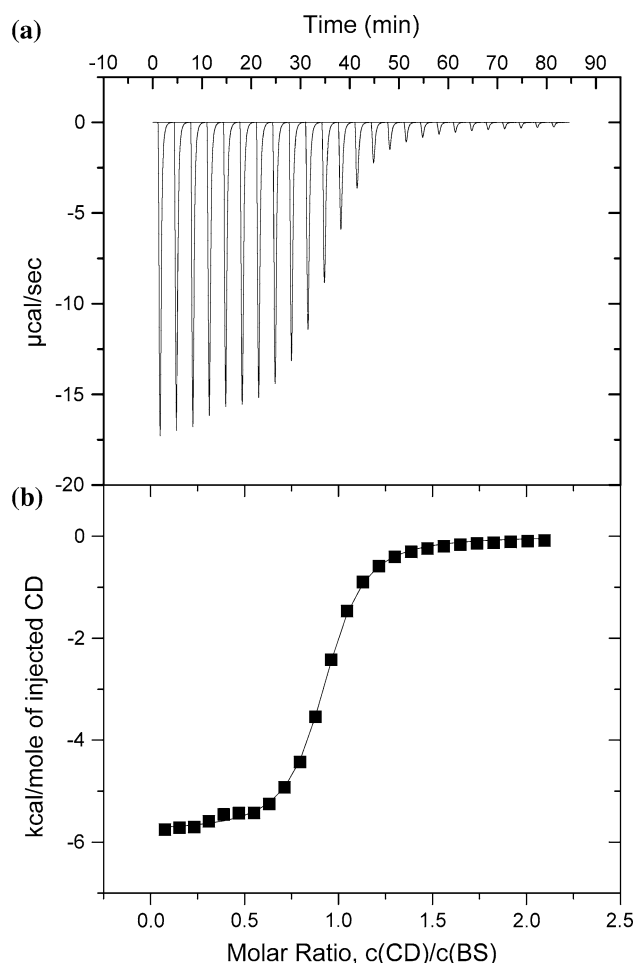
Data represents mean \pm SEM ($n = 3$)^a Data from (45)

model adequately described the binding data obtained by CE. However, as reported previously [55], a high correlation coefficient is not sufficient to demonstrate that the 1:1 model is the best to describe the data. Linear x-reciprocal plots [55–57] and physically unrealistic binding parameters when a 1:2 model was applied to the ACE data (negative $K_{1:2}$ and positive 1:2 complex mobilities) supported that 1:1 binding is dominating at the investigated concentration range.

The stoichiometric ratios (n value) observed from the fitting curve in the ITC experiments was within the range of 0.9–1.1 at the investigated concentration. This clearly indicate that the majority of the inclusion complexes had a 1:1 stoichiometry of bile salt and SBE β CD, which is in accordance with the stoichiometry from the ACE experiments and previously reported stoichiometries for the interaction between bile salts and β CD [39, 40, 42, 58–60] and other modified β CDs [35, 37, 38, 40, 42, 53, 61]. Figure 2 shows a typical enthalpogram for a complex with a high stability constant from which the stoichiometry was estimated.

Stability constants

The stability constants determined by ACE and ITC are presented in Table 1 and 2, respectively. The data showed a high degree of agreement of the results determined by ACE and ITC, demonstrating that the correction methods for the influence of differences in ion strength and viscosity in the ACE experiments previously defined by Østergaard et al. [45] ensured accurate measurements. This means that the ACE method efficiently and with very little material consumption could be used for screening cyclodextrins in a preformulation setup to define the most suited cyclodextrin for the compound instead of conducting the more classical phase-solubility profiles with a much higher compound expenditure.

**Fig. 2** Calorimetric titration of GCDC with SBE β CD in 50 mM phosphate buffer (pH 7.0) at 25 °C. **a** Raw data for sequential 10 ml injections of γ CD solution (10 mM) into the bile salt solution (1 mM). **b** Heats of reaction as obtained from the integration of the calorimetric traces

Stability constants for two bile salts–SBE β CD complexes have previously been reported in the literature [45], while the majority of the relevant bile salts for human, dog and rat remain unexplored. The stability constants for all investigated bile salts were lower than the those reported for binding to natural β CDs [33, 34, 39, 58, 59]. This trend is in accordance with what has been found for HP β CDs, where the substitution also had a negative effect on the stability of bile salt complexes [61]. Specifically, HP β CD with a DS of 0.54 [62] showed comparable stability to the SBE β CD complexes for two different bile salts. However, when comparing the current stability constants for SBE β CD complexes to the results reported by Schönbeck et al. [61] for HP β CD with a similar degree of substitution (DS = 1.0), it is clear that SBE β CD produces more stable inclusions. The repulsive effects between the negatively charged bile salt and the negatively charged sulfobutylether group therefore seem to affect the interaction to a very

Table 2 Stability constant ($K_{1:1}$), standard free energy (ΔG^0), enthalpy (ΔH^0) and entropy changes (ΔS^0) of 1:1 inclusion complexes of bile salts with SBE β CD

Bile salt	$K_{1:1}$ (M^{-1})	ΔG^0 ($kJ\ mol^{-1}$)	ΔH^0 ($kJ\ mol^{-1}$)	ΔS^0 ($J\ K^{-1}\ mol^{-1}$)	ΔC_p ($J\ K^{-1}\ mol^{-1}$)
TC	1.460 \pm 40	-18.0 \pm 0.1	-12.9 \pm 0.4	17.2 \pm 1.5	-
GC	1.520 \pm 17	-18.1 \pm 0.0	-13.4 \pm 0.1	15.9 \pm 0.6	-
TDC	3.970 \pm 26	-20.5 \pm 0.0	-12.0 \pm 0.05	28.6 \pm 0.2	-
GDC	4.697 \pm 513	-20.9 \pm 0.3	-12.0 \pm 0.7	30.1 \pm 3.1	-
	<i>4160</i>	<i>-20.66</i>	<i>-11.68</i>	<i>30.11</i>	<i>-584</i>
TCDC	78.033 \pm 4.002	-27.9 \pm 0.1	-23.9 \pm 0.1	13.4 \pm 0.7	-
GCDC	92.800 \pm 4.513	-28.3 \pm 0.1	-24.0 \pm 0.4	14.6 \pm 1.6	-
	<i>112,978</i>	<i>-28.84</i>	<i>-22.24</i>	<i>22.14</i>	<i>-669</i>
T β MC	144.000 \pm 10.440	-29.4 \pm 0.2	-26.6 \pm 0.5	9.3 \pm 1.8	-

Values are given as mean \pm range from three different experiments in 50 mM phosphate buffer (pH 7.0) at 25 °C (mean \pm SD, $n = 3$). Values in italic are obtained from global fits to a new series of titrations conducted at six different temperatures in the interval 5–55 °C

limited extent if at all. Assuming that the bile salts are oriented similarly as in complexes with β CD, methyl- and HP β CD [41, 61–63], the charged glycine or taurine chain on the bile salts protrudes from the narrow rim of the CD where only 10 % of the sulfobutyl chains are attached, as discussed in the supplementary information. Additionally, the negative charge on the SBE β CD is placed on a highly flexible butyl chain, which in total seems to be oriented in a way that ensures sufficient separation of the charges to support a good interaction between the two species, as also previously reported by Zia et al. [28, 64]. This explanation is confirmed by the MD simulations and is illustrated in Fig. 3.

In accordance with previous observations for neutral modified β CDs, the affinity of the tauro-conjugated bile salts for SBE β CD was slightly lower than its glycol-conjugated counterparts [53, 61], a difference suggested to emerge as the conjugation of the bile salts is situated in or at the proximity of the extended CD cavity potentially leading to interactions. The stability constants for TCDC, GCDC and T β MC are significantly higher than for TC, GC, TDC and GDC, which is consistent with earlier reports concerning m β CD and HP β CD [42, 53]. The hydroxyl group at C12 is highly important for the structural positioning of the bile salt within the modified CDs due to steric hindrances, hence explaining the distinct negative impact on the stability constant of this group. Adding a charge to the modification of the CDs has no significant implication on this as it happens within the CD cavity.

Enthalpy and entropy changes

Compensatory relationships for the changes in enthalpy and entropy have been reported for a series of guest molecules and CDs [3, 65, 66], including bile salts [41, 53, 61]. This compensation may be illustrated in plots of $T\Delta S^0$

versus ΔH^0 , as shown in Fig. 4, where linear relationships are indicative of a common underlying mode of interaction. This type of analysis for β CD (41) and modified β CDs [53, 61] suggested that the weakly bound bile salts (the cholates and deoxycholates) fall into one group with a mode of interaction qualitatively different from the strongly bound bile salts. For SBE β CD a similar pattern was observed and apparently the charges on SBE β CD do not affect the mode of interaction. Figure 4 suggests a relative large intercept ($T\Delta S_0^0$), which is in accordance with reported values for simple modified CDs possessing a flexible hydrophilic sidearm [3]. It has been suggested that the slope (α) and the intercept ($T\Delta S_0^0$) of the entropy–enthalpy compensation plot can be related to the degree of conformational change and the extent of desolvation induced upon complexation, respectively [3, 65–67]. Assuming that this correlation exists, the data in Fig. 4 suggests extensive desolvation effect in the interaction between SBE β CD and the bile salts.

The interaction with SBE β CD is favoured by both enthalpic and entropic contributions for all seven bile salts. ΔH^0 is the dominant contribution to ΔG^0 , with values in the -13 to -27 kJ/mol range. In general, it was found that the net affinity was essentially proportional to the magnitude of the (negative) enthalpy change. Comparison of the data in Table 2 with analogous results for HP β CD with a degree of substitution of 0.6 [53, 61] shows some, but not major, differences in ΔH^0 and ΔS^0 nor their relative contribution to the total ΔG^0 when values for a specific bile salt are considered. If, however, the comparison is made towards the highly substituted HP β CD having a DS of 1.02 [61], differences are observed for the relative contribution of ΔH^0 and ΔS^0 for TC, GC, TDC and GDC whereas the similarity is kept for TCDC and GCDC. These differences probably reflect modifications of the mode of interaction at high densities of substituents on the CD. The

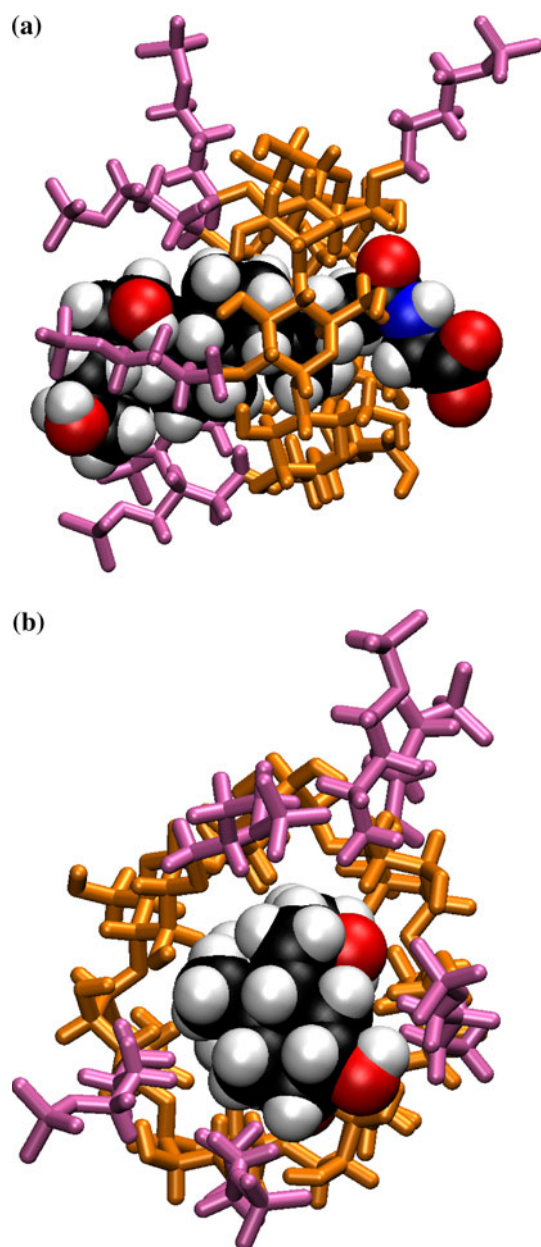


Fig. 3 Snapshot taken from MD simulation of SBE β CD complexed with GCDC seen from **a** the side and **b** top—the secondary opening of the CD. The natural β CD and the sulfobutyl substituents are colored orange and pink, respectively

thermodynamic effects of methyl and hydroxypropyl substituents have been interpreted in terms of buried surface area [63]. The substituents form an extended hydrophobic cavity, as shown in Fig. 4, which upon complexation causes a larger number of ordered water molecules to be released from non-polar surfaces of both bile salt and substituents. This gives a large and positive contribution to both ΔH^0 and ΔS^0 , leading to the entropy–enthalpy compensation observed in Fig. 4, in which the complexes with highly substituted CDs are found in the upper right corner.

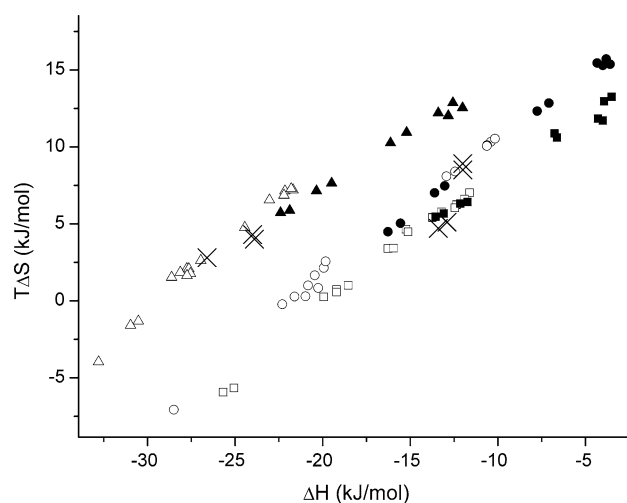


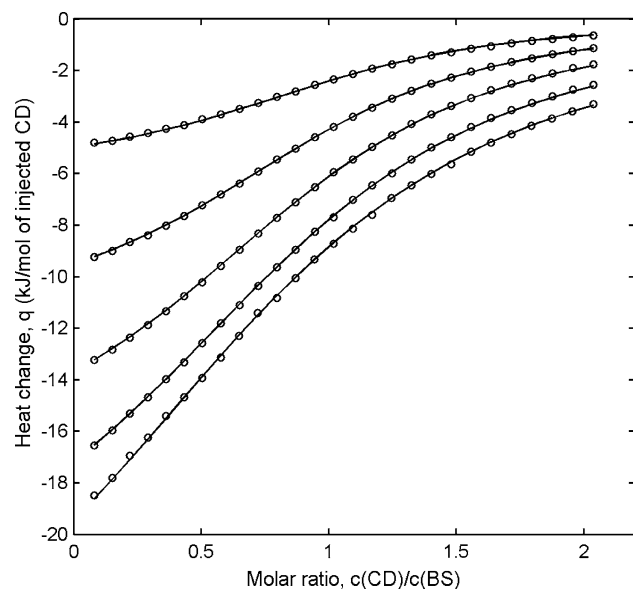
Fig. 4 The complexes of the BSs with SBE β CD (X) lie on the three lines that appear in the entropy–enthalpy compensation plot for the interaction of BSs with natural and methylated (*open symbols* [50, 55]) and hydroxypropylated (*closed symbols* [66]) β CDs. Squares denote complexes with TC and GC, circles with TDC and GDC and triangles with TCDC and GCDC

The density and hydrophobicity of substituents on SBE β CD are expected to result in a significant dehydration of non-polar surface upon complexation. This is supported by the MD simulations from which it is determined that around 500 \AA^2 of non-polar surface is buried (see Table 3). This is around 150 \AA^2 more than for the analogous complexes with natural β CD [53]. ΔC_p was determined for GDC and GCDC by the use of a global fit, and an example of such global fit is shown in Fig. 5. The large and negative ΔC_p 's, derived from this analysis (-600 to $-700 \text{ J mol}^{-1} \text{ K}^{-1}$, Table 2) are about twice as large (numerically) as those for complexes of natural β CD, which again points towards dehydration of a significant hydrophobic surface area. A rough estimate of the hydrophobic surface area dehydrated by the substituents may be calculated from the experimental values of ΔC_p , assuming that each \AA^2 of dehydrated hydrophobic surface area contributes with approximately $-2 \text{ J K}^{-1} \text{ mol}^{-1}$ to ΔC_p [53], i.e. 150 \AA^2 , which is in agreement with the values obtained from the MD simulations. Further, the magnitude of the ΔC_p 's for SBE β CD are similar in magnitude to the ΔC_p 's observed for complexation of bile salts with highly substituted HP β CDs, i.e. with a degree of substitution of approximately 1.0 [53]. This indicates that the overall behaviour of the sulfobutyl chains is similar to the behaviour of hydroxypropyl chains. The hydrophobic surface seems predominantly to be buried within the complex as the substituents interact with the parts of the bile salt that protrudes from the wider rim of the CD. However, although the calculated ΔASA values and the experimentally determined ΔC_p 's are similar to what is observed for HP β CD with the same number of substituents ($DS \approx 1$), the rest of the thermodynamic functions, K , ΔH^0

Table 3 Water accessible surface area (ASA) in Å² of the molecular species obtained from the MD simulations using a spherical probe of radius 1.4 Å

	Free species		In complex with SBEβCD		Change ^a in complex	
	ASA _{Pol}	ASA _{Non}	ASA _{Pol}	ASA _{Non}	ΔASA _{Pol}	ΔASA _{Non}
GDC	217	442	1,120	813	-116	-500
GCDC	220	438	1,127	811	-112	-498
SBEβCD	871	1,890	–	–	–	–

^a The change in ASA was calculated by subtracting the sum of ASAs for the free species from ASA obtained for the complex



Parameter	Best fit	95%	Correlation Matrix		
	value	Error			
K25 (M ⁻¹)	4160	37	1	0.76	0.80
ΔH (kJ/mol)	-11.68	0.05		1	0.57
ΔC _p (J/mol/K)	-584	4			1

Fig. 5 Titrations of GDC with SBEβCD conducted at 10 degree intervals from 15 °C (*upper*) to 55 °C (*lower*). All five titrations were fitted simultaneously using K and ΔH^0 at 25 °C and ΔC_p as global fitting parameters and c as local fitting parameters. A titration was conducted at 5 °C but was not included in the fit since the complexation enthalpies at this temperature are very small and overshadowed by other heat effects

and ΔS^0 , resembles those of HPβCDs with considerably fewer substituents (DS ≈ 0.6). These differences may be attributed to the different character of the two types of substituents. The more favourable interaction for the sulfbutyl than the hydroxypropyl substitution could reflect a more optimal structural placement of the hydrophilic/hydrophobic groups for the interaction, i.e. at the end or the middle of the substitution chain, respectively.

ΔC_p for GCDC was more negative than for GDC, which indicates a larger contribution of dehydration to the

process. As demonstrated earlier by Holm et al. [41, 62] using NMR, GDC and GCDC are positioned differently within the CD complex, where GCDC have the entire D- and part of the C-ring of the steroid skeleton within CD, whereas for GDC only the D-ring in the CD due to the steric hindrance of the hydroxyl group on C12 in the bile salt. This difference in physical orientation seems to be reflected in the difference in ΔC_p , as also suggested by the ASA calculations. The deeper inclusion and concomitant larger dehydration of GCDC is supported by the entropy–enthalpy compensation plot in which the intercept obtained for the group of GCDC complexes is larger than what is obtained for the GDC complexes.

In conclusion, this study has presented 1:1 stability constants for the binding of seven biologically relevant glyco- and tauro-conjugated bile salts to SBEβCD. A good agreement between the stability constants obtained by ACE and ITC is observed suggesting that the two techniques are equally effective for obtaining stability constants. The size of the obtained stability constants were in the same range as those previously reported for the interaction of the same bile salts with less substituted HPβCDs, but significantly higher than those reported for HPβCD with a similar DS. Also ΔH^0 and ΔS^0 were similar to those of less substituted HPβCDs. In contrast to this, the observed ΔC_p s were in the same range as those reported for HPβCD with a similar degree of substitution, indicating that the sulfbutyl substituents behave similarly to HP-substituents and results in significant dehydration of hydrophobic surface upon complexation as also indicated by the ASA data extracted from the MD simulations. These results demonstrate the importance of the substituents on the thermodynamic parameters for the interaction with a host compound.

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