The role of hydrogen bonding of cyclodextrin-drug complexes probed by thermodiffusion

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Abstract

The temperature-gradient induced migration of biomolecules, known as thermophoresis or thermodiffusion, changes upon ligand binding. In recent years, this effect has been used to determine protein-ligand binding constants. The mechanism through which thermodiffusive properties change when complexes are formed, however, is not understood. An important contribution to thermodiffusive properties originates from the

thermal response of hydrogen bonds. Since there is a considerable difference between the degree of solvation of the protein-ligand complex and its isolated components, ligand-binding is accompanied by a significant change in hydration. The aim of the present work is therefore to investigate the role played by hydrogen bonding on the change in thermodiffusive behaviour upon ligand binding. As a model system we use cyclodextrins (CDs) and acetylsalicylic acid (ASA), where a quite significant change in hydration is expected, and where no conformational changes occur when a CD-ASA complex is formed in aqueous solution. Thermophoresis was investigated in a temperature range from 10 to 50°C by infrared thermal diffusion forced Rayleigh scattering (IR-TDFRS). NMR measurements were performed at 25°C to obtain information about the structure of the complexes. All CD-ASA complexes show a stronger affinity towards regions of lower temperature as compared to the free CDs. We found that the temperature sensitivity of thermophoresis correlates with the 1-octanol/water partition coefficient. This observation not only establishes the relation between thermodiffusion and the degree of hydrogen bonding, but also opens the possibility to relate thermodiffusive properties of complexes to their partition coefficient, which can not be determined otherwise. This concept is especially interesting for protein-ligand complexes where the protein undergoes a conformational change, different from the CD-ASA complexes, giving rise to additional changes in their hydrophilicity.

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Introduction

A measure to characterize the degree of hydrophilicity of a substance is the 1-octanol/water partition coefficient P (sometimes also denoted as K_{OW} and more commonly given as its logarithm $\log P$). It is used for transport models in several fields, including pharmacological research and environmental science. Although the microscopic meaning of the parameter is not clear, it strongly depends on the formation of a hydration layer, which also influences trans-

port properties[1] as well as the structure and function[2–4] of water soluble macromolecules. The degree of hydration of macromolecules, for example, significantly affects their migration induced by spatial gradients in the temperature, which transport mechanism is commonly referred to as thermophoresis or thermodiffusion. Thermophoresis is therefore an effective method to study macromolecular complex formation in cases where the hydration state of the complex is signifcantly different form that of the non-complexed macromolecule. The change of the degree of hydration of macromolecules and the resulting change in thermodiffusion upon complex formation has been utilized in MicroScale Thermophoresis (MST) to determine protein-ligand binding constants in dilute solutions, as well as the activity of biomolecules.[1] How such binding constants are affected by crowding, like for protein-ligand complex formation in living cells, is an active area of research.[5–7] Thermophoresis is a promising experimental technique to gain fundamental understanding on the role played by hydrogen bonding with the surrounding water in macromolecular complex formation, both in dilute systems and in crowded environments. It has not yet been employed in this area of research, since a quantitative understanding of how hydrogen bonding affects thermophoretic properties of macromolecules is yet to be developed. A fundamental understanding of the role played by hydrogen bonding in thermophoresis may also be used to tune the thermophoretic properties of drug-delivery complexes in order to enhance their tendency to migrate towards the warmer regions of inflammation, and thereby enhance their effectiveness.

As a first step towards a fundamental understanding of the role played by hydration in complex formation and the utility of thermophoresis as an experimental technique to probe the degree of hydration, we present experiments on a drug-delivery model system, consisting of several types of cyclodextrins (CDs) with different hydration properties that bind acetylsalicylic acid (ASA), also known as aspirin. CDs are ideal model systems for a systematic experimental study to investigate changes in the hydration layer due to complex formation, because, in contrast to proteins or other biomolecules, CDs are quite rigid so that there are no conformational changes upon complex formation, and CDs do not contain polymer-like

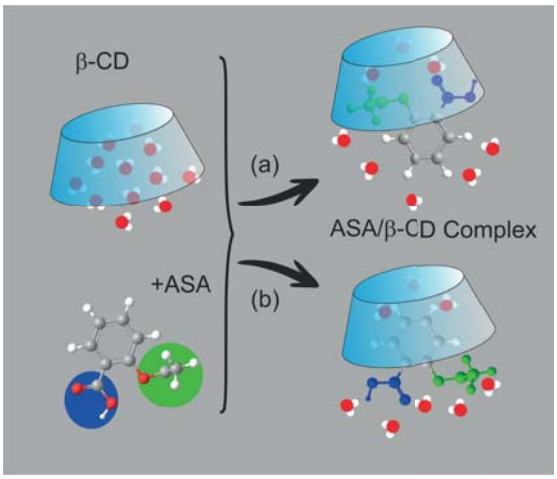

Figure 1: Schematic representation of a CD and CD-complexes with ASA. Depending on the conformation of the ASA in the CD, the hydration by the surrounding water changes. Two suggested configurations of the ASA/$\beta-$CD complex are shown: (a) This configuration has been obtained by circular dichroism measurements at pH=2 in H_2O[8] and (b) by NMR measurements in D_2O.[9]

units which can fold and unfold. Furthermore, in contrast to protein-ligand complexes, the CD/ASA systems have the advantage of being uncharged, made up of identical units, and stable in water without addition of buffer. The change in thermophoresis properties due to changes in charge and the influence of added buffer that would complicate the interpretation of thermophoresis data are therefore absent in the CD/ASA systems.

Cyclodextrins (CDs) are cyclic oligosaccharides, which have been developed as drug delivery systems.[9–12] They have a toroidal shape with a hydrophobic cavity which serves as a container for guest molecules, enhancing solubility and controlling speed of uptake of drugs.[10] One of the known guest molecules with an affinity to reside within the cavity of CDs is acetylsalicylic acid (ASA),[8,13–18] which will be used in the present study. The complex formation by inclusion of this guest molecule in its cavity is mostly enthalpy-driven and is known to be related to hydration effects.[19–21] The complex formation as sketched in Figure 1 is accompanied by the dehydration of the CD's cavity and the guest molecule upon formation of hydrophobic contacts between the guest molecule and hydrophobic sites within the cavity.[22] The hydration of the complex could reveal whether the remaining contact area with

the water is more hydrophobic or hydrophilic (sketches (a) and (b) of fig.1, respectively).

There are several types of CDs with varying degree of hydration. The CDs used in the present study are displayed in Figure 2. The difference between the two ASA configurations is discussed in more detail in the section on NMR measurements. The α-, β- and γ-CDs have 6, 7 and 8 glycopyranose units, respectively, and are not methylated. The other two CDs are methyl-β-CDs. In one of them 55% of the hydroxyl groups are methylated randomly (m-β-rand), in the other the hydroxyl groups in position 2 and 6 are methylated resulting in a methylation degree of 67% (m-β-def, where "def" stands for "defined"). We also investigated mixtures of m-β-def and β-CD to vary the degree of methylation. The conformation of ASA within the cavity of these CDs is determined by means of NMR.

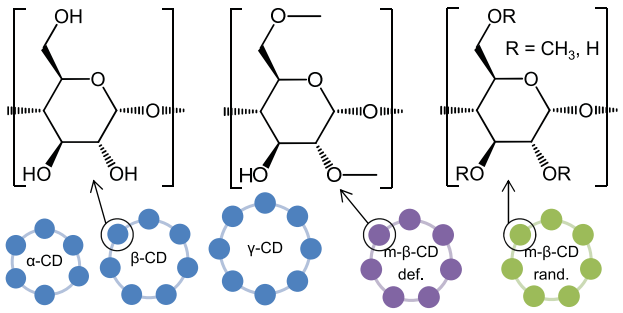

Figure 2: Investigated CDs: α-, β- and γ-CD are unmethylated (blue), in one methyl-β-CD 55% of the hydroxyl groups are randomly methylated (green) and in one the hydroxyl groups in position 2 and 6 are methylated (violet).

In a binary fluid mixture, thermophoretic mass transport as induced by temperature gradients contributes with $-c(1-c)D_{\mathrm{T}}\,\vec{\nabla}T$ to the mass flux $\vec{j}$, where D_{T} is the thermodiffusion coefficient and c the concentration given as a fraction (mass, mole or volume fraction, corresponding to the unit connected with the concentration gradient). In this work, concentrations are given in weight fractions and all experiments are performed in conditions where $c \ll 1$. When $D_{\mathrm{T}} > 0$, mass transport occurs from high to low temperature. In addition, there is a mass flux due to spatial gradients in the concentration c, equal to $-D\vec{\nabla}c$, where

D is Fick's mass-diffusion coefficient. The total mass flux is thus given by,

$$\vec{j} = -\rho D \vec{\nabla} c - \rho c \left(1 - c\right) D_{\mathrm{T}} \vec{\nabla} T \ . \tag{1}$$

For a time independent temperature gradient, a steady state is reached when the mass fluxes induced by the Fickian- and thermodiffusion contributions balance each other. The ratio of the resulting concentration gradient and the applied temperature gradient is characterized by the Soret coefficient $S_{\mathrm{T}} = D_{\mathrm{T}}/D$. A larger Soret coefficient implies a larger concentration gradient for a given temperature gradient. For the system of CD and ASA in water, equation 1 cannot describe the flux of every component in the system. It is, however, not possible to differentiate experimentally between the signal of CD and ASA in solution, due to the similar diffusion times of the components. Therefore, we treat the system as quasi-binary mixture and only one S_{T} is observed, which contains contributions of ASA, CD and CD/ASA complex. Knowing the complex fraction and the contribution of ASA and CD, S_{T} of the complex can be calculated (see SI section 2).

Thermophoresis has been studied theoretically, experimentally and by computer simulations for many different systems such as low molecular weight mixtures, polymer solutions and colloidal suspensions.[23–40] So far, thermophoresis of low molecular weight mixtures is still not understood on a microscopic level. While for non-polar systems the Soret coefficient often shows a linear dependence on physical parameters e.g. mass and moment of inertia of the solute molecules,[37] the understanding of aqueous systems is complicated due to specific interactions. The strong temperature dependence of S_{T} in aqueous solution can generally be attributed to a chemical contribution[37] caused by hydrogen bond interactions, which decrease with increasing temperature.[41] Many studies have been performed for charged colloids[42–45] and biopolymers,[39] but also non-ionic aqueous solutions have been investigated.[46–51] For the latter type of systems the behavior is dominated by hydrogen bonding, where it has recently been suggested that S_{T} depends linearly on the difference of the number of donor

and acceptor sites of the solute molecule belonging to a homologous series.[30]

Iacopini and Piazza[52] suggested an empirical equation to describe the temperature dependence of the Soret coefficient,

$$S_{\mathrm{T}}(T) = S_{\mathrm{T}}^{\infty}\left[1 - \exp\left(\frac{T^* - T}{T_0}\right)\right] \tag{2}$$

with free parameters S_{T}^{∞}, T^* and T_0 that can be adjusted to fit experimental data. The temperature T_0 is a measure of the sensitivity of the Soret coefficient for changes of the ambient temperature. T^* is the temperature at which the Soret coefficient changes sign. For $T > T^*$ the Soret coefficient is positive, so that migration from high to low temperatures occurs, while there is a preference for higher temperatures when $T < T^*$. This behaviour was explained qualitatively by Wang *et al.*[53] as minimization of free energy: At low temperatures, the enthalpy contribution dominates, favouring pure water (with a hydrogen bond network undisturbed by solute) on the colder side. At moderate to high temperatures, entropy is increased by maximizing the number of water molecules (small molecules with high orientational and translational entropy) at the hot side. Later, Vigolo *et al.*[44] suggested a master curve, which indicates that $T^*/T_0 = \text{const.}$, at least for similar systems (see SI section 6 for details). It turns out that the empirical working equation (2) describes thermal diffusion of many types of macromolecules in dilute aqueous solutions quite well,[28,29,42] but deviations occur when micro-structural heterogeneities are present.[31] There is so far no detailed theory for the contribution of hydrogen bonding to the thermal diffusion coefficient, and therefore a microscopic interpretation of the meaning of the free parameters in eq.(2) is in general not available. For charged colloidal particles considering the thermoelectrophoresis[54,55] and the variation of the double-layer energy around the particles[26] gives a comprehensive explanation for the temperature dependence of the Soret coefficient.[56] However, at the present stage it is not possible to derive a microscopic theory of thermophoresis of systems dominated by hydrogen bonds. We will use an approach, which correlates the adjustable parameters with

an established empirical parameter describing the hydrophilicity of the system.

Experimental section

Sample preparation

Studied compounds were acetylsalicylic acid (ASA, Sigma-Aldrich, $\geq 99.0\%$), α-cyclodextrin (α-CD, Tokyo chemical industry, $> 98.0\%$), β-cyclodextrin (β-CD, Tokyo chemical industry, 99.0%), γ-cyclodextrin (γ-CD, Tokyo chemical industry, $> 98.0\%$), and two different types of methyl-β-cyclodextrin: one where randomly 55% of the hydroxyl groups were methylated (m-β-rand, Tokyo chemical industry) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (m-β-def, Sigma-Aldrich, $> 98.0\%$) where two out of three hydroxyl groups at defined positions are methylated. Solutions were prepared by using deionized water (Millpore). The concentration of CD was always $1.00wt\%$ and they were measured without ASA as well as with equimolar amounts of ASA (see SI section 7 for details). The pD/pH of our solutions without and with ASA is 6 ± 1 and 3.5 ± 0.5, respectively. The hydrolysis half life of ASA is 644 hr and 13 hr at 10 °C and 50 °C, respectively.[57] We carefully checked the experimental data by repeating the measurements at the low temperatures after the sample had been measured at 50 °C and never noticed a systematic change due to hydrolysis.

Additionally, we adjusted the degree of methylation by mixing β-CD and m-β-def in defined ratios: mix 23% and mix 53%, where 22.5 and 53.4% of the hydroxyl groups are methylated (see SI section 7 for details). All solutions were stirred for 40 minutes at room temperature, then filtered through a membrane filter (0.8/0.2 μm, PALL Acrodisc PF) before the measurement.

NMR

We used nuclear magnetic resonance (NMR) spectroscopy to gain structural information about the CD-ASA complex. ^{1}H-NMR measurements were performed with Bruker AV500

and AV400WB spectrometers. The samples were prepared as decribed in the *sample preparation* section, but instead of water deuterium oxide (D_2O, Wako Chemical, 99.0%) was used as solvent in order to diminish the water OH peak. An internal standard such as TSP was not used to avoid the formation of an inclusion complex with CD. Instead of TSP, the DHO peak was standardized as 4.7 ppm.[58]

The NMR diffusion measurements were performed by Bruker Diff-50 probe with PFG-STE sequence.[59] The attenuation curve was analyzed by the Stejskal-Tanner equation[60] and the diffusion coefficient D was obtained for all peaks with,

$$\frac{I(g)}{I_0} = \exp\left(-(\gamma g \delta)^2 \left(\Delta - \frac{\delta}{3}\right) D\right), \tag{3}$$

where γ is the proton gyromagnetic ratio, g is the field gradient pulse intensity, δ is the gradient pulse duration, Δ is the diffusion time, $I(g)$ is the peak intensity at g, and I_0 is the intensity at minimum field gradient g of the experiment, respectively. In order to analyze the steric structure of the β-CD/ASA inclusion complex, the phase sensitive ROESY technique was used.[61] A spinlock mixing was set to 200 ms. Measurement was performed by changing the g value up to 600 gauss/cm. Δ and δ were fixed to 5 ms and 1 ms, respectively. All NMR measurements were performed at 25.0±1.0 °C.

Thermal diffusion forced Rayleigh scattering

The thermal diffusion coefficients were measured in an optical quartz cell (Hellma) with optical path length of 0.2 mm by Infra-Red Thermal Diffusion Forced Rayleigh Scattering (IR-TDFRS), a laser-induced transient grating technique, which has been described in detail before.[62,63] IR-TDFRS was measured in a temperature range from 10 to 50 °C, with steps of 5 °C. At least two measurements for each sample concentration were done. The error bars represents the standard deviation of the mean. All measurements have been performed in pure water. Since ASA changes the pH of the solution, we performed additional experiments

in buffered solution, which are discussed in detail in the supporting information (section 4). We found that the thermal diffusion coefficient is not influenced by pH and only the translation diffusion coefficient shows a small effect due to the higher ionic strength at lower pH. Therefore, we concluded that effects due to pH-change can be neglected and changes of the thermophoretic behaviour are solely due to complex formation.

The refractive index increments with mass concentration $(\partial n/\partial c)_{p,T}$ was measured by an Anton Paar RXA 156 refractometer. The refractive index was measured for 4 concentrations (1, 0.75, 0.5, and 0.25 wt%) at 5 different temperatures (10-50°C). Since the slope $(\partial n/\partial c)_{p,T}$ of refractive index against concentration was equal within their errors for all temperatures, we used their mean value for the evaluation of our IR-TDFRS data. The resulting value has an error of up to 10% due to the small refractive index changes at these low concentrations. The precision of the refractometer is 0.00002 nD for the refractive index and $\Delta T = \pm 0.03K$ for the temperature control. Systematic errors in the refractive index increment due to the shorter wavelength used by the refractometer are in the order of 0.5-1%[64,65] and therefore relatively small.

The refractive index increment with temperature $(\partial n/\partial T)_{p,c}$ was measured interferometrically.[66] It was measured in the temperature ranges 7-15 °C, 27-35 °C, and 47-55 °C and interpolated for the values inbetween. The solution was heated and cooled automatically with typical rate of 1 mK/sec. The $(\partial n/\partial c)_{p,T}$ and $(\partial n/\partial T)_{p,c}$ for the studied systems are summarized in the supplementary information.

Results

NMR

Fig. 3 shows the ^{1}H-NMR spectrum of the β-CD/ASA/D_2O mixture at 25°C. The assignments of the proton peaks of β-CD and ASA have been obtained from previous reports.[58,67] The protons of ASA are assigned with letters (H_a to H_e), the protons of β-CD with numbers

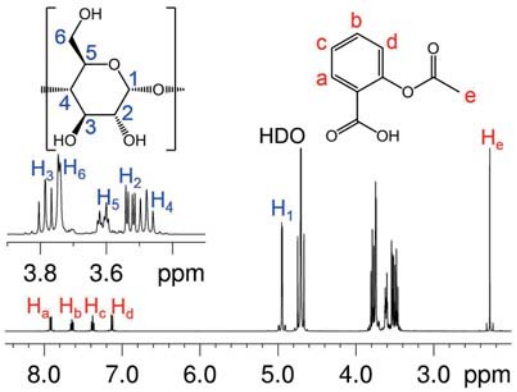

Figure 3: ^{1}H-NMR spectrum of equimolar β-CD/ASA mixture in D_2O. H_a to H_e belong to ASA, H_1 to H_6 to the CD.

(H_1 to H_6). At room temperature only H_3 and H_5 of β-CD in the ternary mixture compared to the binary mixture are shifted by 0.0760 ppm and 0.1335 ppm, respectively.

Complex formation is confirmed by a ROESY spectrum shown in Figure 4(a). Figure 4(b) shows an enlargement of the spectrum with the cross peaks. Using the assigned names displayed in figure 3 we observe coupling for the protons H_3(β-CD) and H_5(β-CD) pointing to the inner of the ring. The strongest coupling is found for H_5(β-CD)-H_a(ASA) and H_5(β-CD)-H_d(ASA). Both ASA-protons are located in the phenyl ring. An approximately 40% weaker coupling exists for the same protons of ASA with H_3(β-CD). The stronger coupling of H_5(β-CD) compared to H_3(β-CD) might be related the the slightly smaller diameter of β-CD at that part of the CD. A stronger coupling, which is only 25% weaker than the coupling with H_5(β-CD) occurs for H_3(β-CD) with H_e(ASA) located in the methyl group. This group has some rotational degree of freedom and can probably come closer to the ring.[68]

The NMR diffusion measurements show that the diffusion constants of ASA and β-CD become smaller, but not equal. If the complex was infinitely stable, the diffusion of ASA and β-CD would be slowed down to the same value: the diffusion constant of the heavier complex. In reality, the complex has a limited life time, which is shorter than the duration of the NMR measurement of 5 ms. The observed diffusion constant is therefore an average over the diffusion of the individual ASA or β-CD and the slower diffusion of the ASA-β-CD-

complex. Similar observation have been made for CDs with surfactants.[69] The self diffusion coefficients can be used to determine the association constant K_a, which can be expressed for an equimolar mixture as

$$K_a = \frac{p_{\mathrm{com}}}{x_0 \cdot (1 - p_{\mathrm{com}})^2}, \tag{4}$$

with the initial molal concentration x_0 and the fraction of the formed complexes p_{com} (for further details see SI section 1). The fraction p_{com} can be expressed with the self diffusion coefficients D_{ASA} and $D_{\beta CD}$ measured in binary solutions of ASA/D_2O and $\beta-$CD/D_2O and the corresponding self diffusion coefficients $D_{\mathrm{ASA,obs}}$ and $D_{\beta\mathrm{CD,obs}}$ observed in the ternary mixture $\beta-$CD/ASA/D_2O,

$$p_{\mathrm{com}} = 1 - \frac{D_{\beta\mathrm{CD,mix}} - D_{\mathrm{ASA,mix}}}{D_{\beta\mathrm{CD}} - D_{\mathrm{ASA}}}. \tag{5}$$

Table 1 lists the self diffusion coefficients found by NMR and compares them with literature results and collective diffusion coefficients measured by IR-TDFRS. Note that for the investigated dilute solutions the collective diffusion coefficients agree with the self diffusion coefficients. Except for D_{ASA}, all values are in excellent agreement with the literature. The diffusion of ASA determined by a diffusion cell is 20% too large,[70] while a DOSY experiments leads to a 25% too small value,[71] but the average value of the literature results agrees within the error bars. From our NMR results and the solute concentrations (given in Table 4 in the supporting information), we can calculate the association constant using Eq. 4. The determined complex fraction of $p_{\mathrm{com}} = (45 \pm 12)\%$ corresponds to an association constant of $K_a = (174 \pm 135)\ \mathrm{kg \cdot mol^{-1}}$. It is known that the complex formation depends strongly on the pH. Fukahori *et al.* measured a ten times higher association of nonionized (pH=1.7) compared to ionized ASA (pH=6).[17]

To our best knowledge there are no literature values, which have been measured at pH=3.5 for a direct comparison of our results. Using isothermal titration calorimetry, Castronuovo and Niccoli[16] find an association constant of $K_a = (210 \pm 30)\ \mathrm{kg \cdot mol^{-1}}$ in a

Table 1: Diffusion coefficients of β-CD and ASA in D_2O, and in the ternary mixture β-CD/ASA/D_2O, determined by IR-TDFRS and NMR at 25 ± 0.1°C.

D / $10^{-10}m^2s^{-1}$	NMR	TDFRS	lit.
$D_{\beta CD}$	2.60 ± 0.03	2.9 ± 0.5	2.91 [a]
D_{ASA}	6.4 ± 0.3	7 ± 1	8.02 [b]
			5.0 [c]
$D_{\beta CD,mix}$	2.53 ± 0.05	3.9 ± 0.5 [d]	–
$D_{ASA,mix}$	4.6 ± 0.3		–
D_{D_2O}	18.3 ± 0.1	–	18.72 [e]
			19.02 [f]

[a] obtained by an optical measurement.[72]

[b] obtained with a diffusion cell.[70]

[c] DOSY measurements.[71]

[d] Due to the sign change at 25°C the concentration signal is very low, so that the value has been obtained by interpolating the D-values at low and higher temperatures.

[e] mass extrapolation of a diaphragma cell measurements.[73]

[f] tracer diffusion measurement.[73]

phosphate buffer solution with a pH=9.3, which seems to be rather high if we compare it with $K_a = 51$ kg $\cdot$ mol^{-1} at pH=6 determined by Fukahori *et al.* using an ultrasonic relaxation method.[17] Interpolating the values determined by Fukahori *et al.*[17] we can estimate $K_a = (360 \pm 70)$ kg $\cdot$ mol^{-1} at pH=3.5, which just agrees within the error bars.

NMR experiments were performed to assess the structural arrangement of ASA within the CD cage. The position of ASA within the CD and the chemical properties of the outward pointing groups will have an influence on the structure and extent of the hydration layer, leading to a change in the thermophoretic response of the complex as compared to the free CD. In literature, different configurations for ASA in CD have been suggested. While circular dichroism measurements at pH=2 in H_2O suggest that the phenyl ring of the ASA points outwards of the CD[8] (compare Fig.1(a)), NMR measurements in D_2O favor a configuration with the phenyl inside the CD[9] (compare Fig.1(b)). In both studies, the stoichiometric ratio was 1:1, but the pH in the NMR study was most likely around 3-4. Probably also

the configuration of the complex changes as function of pH, since Fukahori *et al.*[17] found a 10-times higher equilibrium constant for ionized ASA (pH≈ 6) compared to nonionized ASA (pH≈ 2) in solutions with $\beta-$CD. The authors speculate that the charged group in the ionized ASA can be easily drawn to solvent bulk water, which is supported by the 10-times larger backward rate constant. Based on these results, one would expect that the ASA is located deep inside the CD cavity at the low pH in contrast to the finding of Dahab *et al.*[8] Our NMR study clearly supports the outcome of Loftsson *et al.*,[9] so that we expect that the hydroxygroup and an oxygen point outwards from the cavity.

IR-TDFRS

Apart from ASA, which shows a linear decrease of S_T with rising temperature (Figure 5b, margenta stars), all investigated systems can be described with eq. 2.

Figure 5a shows the Soret coefficient S_T of the unmethylated CDs - α, β, and γ. These three CDs differ only in the number of glycopyranose units and show a very similar behaviour. At low temperatures, they are thermophilic ($S_T < 0$); S_T increases with decreasing size of the cyclodextrin ring. Complex formation with ASA raises the Soret coefficient in all systems by the same amount (0.003 K^{-1} at 10°C). The inversion temperature T*, where the system goes from a thermophilic to a thermophobic behaviour ($S_T = 0$), lies around 35 °C for the CDs alone and is lowered by complex formation with ASA to about 25°C. At high temperatures neither the ring size, nor the complex formation have a strong impact on the Soret coefficient.

The temperature dependent Soret coefficient of the methylated systems is shown in Figure 5b. For easier comparison the results of β-CD are reproduced from Figure 5a. In the investigated systems 23, 53, 55 and 66% of the hydroxyl groups are methylated and there is a clear rise of the S_T-value with increase in methylation (see Figure 5c). The position of the methyl groups (randomly at any position, defined at two out of three positions or through a mixture of β-CD and m-β-def) does not influence the result. Complex formation with ASA has qualitatively the same result as for the unmethylated CDs, but the increase of S_T is not

as pronounced.

Diffusion coefficients for β-CD and ASA were calculated and are listed in table 1, errors are determined by multiple measurements. In ternary mixture β-CD/ASA/D_2O we observed only one diffusion coefficient of the formed complex β-CD/ASA. It was not possible to differentiate individual diffusion coefficients of β-CD and ASA by IR-TDFRS.

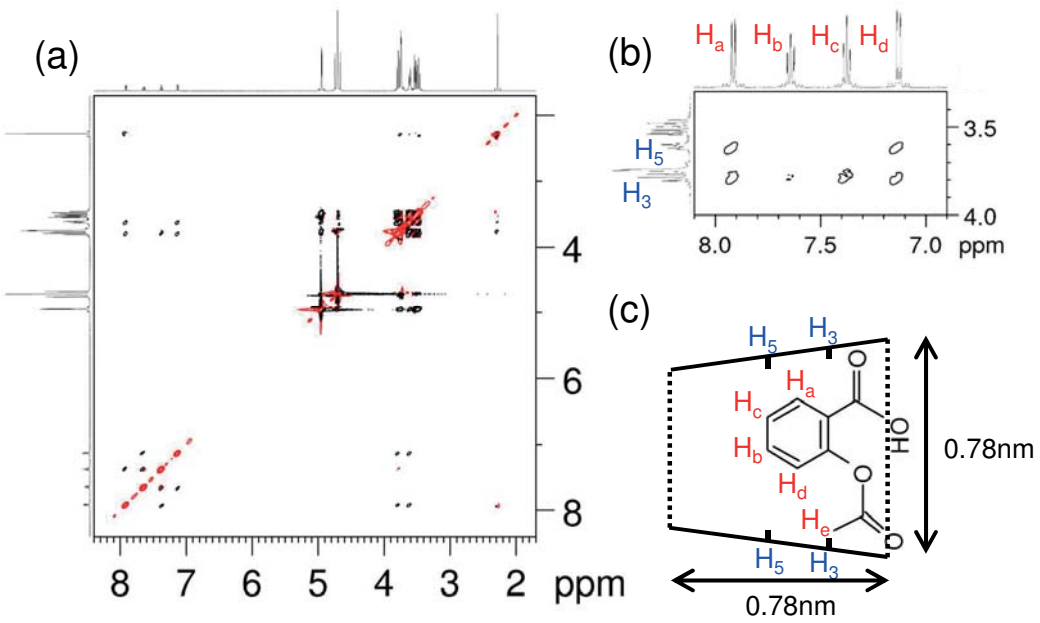

Figure 4: ROESY contour map of equimolar βCD/ASA mixture in D_2O (a) and it enlarged view (b), mixing time of 200 ms at 25°C. Black and red line show positive and negative peak, respectively. (c) A possible structure of the βCD/ASA inclusion complex. Ketone group has a rotational degree of freedom, therefore the double bond face the outer side of the CD.

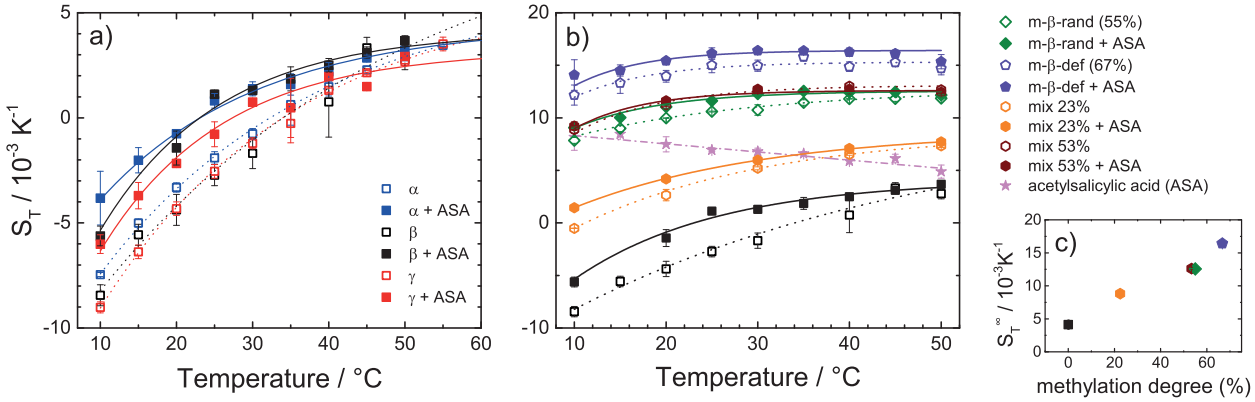

Figure 5: Soret coefficient S_T against temperature for CDs with and without ASA (full and empty symbols, respectively) for a) unmethylated CDs and b) methylated CDs, with β-CD (black sqares) reproduced for easier comparison. Percentages in the legend give degree of methylation. Systems called 'mix' (orange and dark red hexagons) consist of a mixture of β and m-β-def resulting in the given degree of methylation. Addition of ASA results in a more thermophobic behaviour (raised S_T). The dotted and solid lines in (a) and (b) are fits of eq. 2 for the CDs and the CD-ASA-complexes, respectively. c) The Soret coefficient at high temperatures S_T^∞ shows a linear dependence on the degree of methylation.

Discussion and Conclusions

In this work we use cyclodextrins (CDs) and acetylsalicylic acid (ASA) in water as a model system to study the role played by hydrogen bonding upon complex formation. This is a particularly suitable model system for such a study, since the core of the CDs does not structurally change when ASA is embedded within the CD ring, so that the major change of the thermodiffusion coefficient upon complex formation is due to changes in the degree of hydrogen bonding. Since the thermophoretic mobility is sensitive to hydrogen bonding, we performed systematic thermophoresis experiments for various types of CDs, non-complexed and in complexed form, as a function of temperature.

Comparing different CDs, we see a stronger thermophobicity (higher S_{T}) for higher degrees of methylation (see Figure 5c). For homologous groups, S_{T} can be predicted with the donor-acceptor concept.[30] It states that for such similar compounds, the Soret coefficient at a fixed temperature depends linearly on the difference of the number of hydrogen bond donors and acceptors in the molecule. Figure 6 shows S_{T} against $N_{don} - N_{acc}$ for the investigated systems at 20 and 50°C. Counting the donor and acceptor sites for our CDs, we find that methylation turns a hydroxyl group (hydrogen-bonding donor) into an ether group (hydrogen-bonding acceptor), thus reducing $N_{don} - N_{acc}$ (further information on how the number donor and acceptor sites are determined is given in the supporting information section 3). In a previous work,[74] we speculated that donor and acceptor sites block each other through intramolecular hydrogen bonds, leading to $N_{don} - N_{acc}$ as the number of hydrogen-bonding sites open to interaction with the solvent. This first idea does not seem likely, because with this interpretation a positive or negative value for $N_{don} - N_{acc}$ would simply denote whether donor or acceptor sites are left free. Since there is no inherent difference between hydrogen-bonds, whether the solute is a donor or acceptor, the sign of $N_{don} - N_{acc}$ should have no influence on S_{T}. It is clear, however, that hydrogen-bonding acceptors seem to give a positive contribution to S_{T}, while donors give a negative contribution. In the investigated systems, the addition of an acceptor group is always accompanied

by addition of hydrophobic groups (-CH_3 for the CDs, -CH_2-CH_2- for the ethylene glycols). A more convincing way to interpret the data is therefore that N_{don} is counting hydrophilic groups, while N_{acc} is really counting hydrophobic contributions. Greater hydrophilicity leads to a stronger thermophobic response. This observation holds for 20°C as well as for 50°C, although the effect is less pronounced at the higher temperature, indicating a weakening of hydrogen bonds. What is may be surprising about these findings is that this simple method also predicts the Soret coefficients of the complexes reasonably well, just by adding donor and acceptor numbers of ASA. This further substantiates our NMR interpretation, because donor and acceptor sites of ASA are accessible to the solvent.

Assuming that the temperature dependence of S_T in aqueous systems is mainly due to the temperature dependent formation and breaking of hydrogen bonds, we propose a slightly adapted form of eq.(2),

$$S_T(T) = S_T^{\infty} - C_H \exp(-A_H T) \ . \tag{6}$$

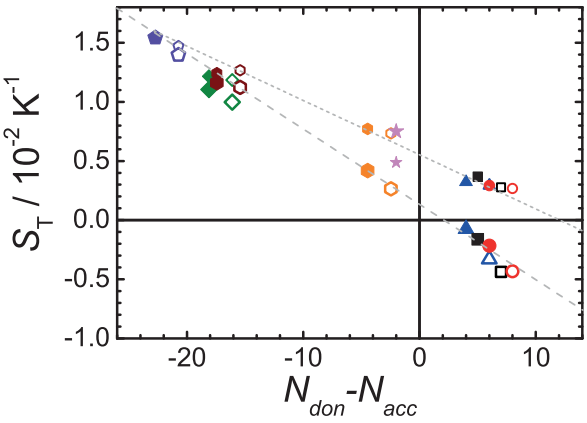

Figure 6: Soret coefficient S_T at 20°C (larger symbols) and 50°C (smaller symbols) as a function of the difference between the number of hydrogen bond donors (N_{don}) and the number of hydrogen bond acceptors (N_{acc}). The open symbols refer to the CDs without ASA and the solid symbols refer to equimolar mixtures of CD and ASA: α-CD (blue triangle), β-CD (black square), γ-CD (red circle), β-CD/m-β-def (23%) (orange hexagon), β-CD/m-β-def (53%) (brown hexagon) m-β-rand (55%) (green diamond), m-β-def (67%) (violet pentagon), and ASA (pink star). The percentages give the degree of methylation.

The interpretation of this empirical formula is as follows. The contribution S_{T}^{∞} to the Soret coefficient stems from thermal properties of the core material, possible charges, etc., without the presence of hydrogen bonds. The second term accounts for the presence of hydrogen bonds, where C_H is a measure for the number of hydrogen bonds. The temperature dependent factor that multiplies C_H describes the diminishing contribution of hydrogen bonds as they weaken with increasing temperature. The parameter $A_H > 0$ measures the temperature dependent strength of a hydrogen bond. For larger values of A_H, hydrogen bonds weaken more strongly with increasing temperature. Note that both C_H and A_H have the dimension T^{-1}. The contribution of hydrogen bonds is expected to be the main cause of the temperature dependence of the Soret coefficient, so that S_{T}^{∞} is essentially temperature independent. Fitting the experimental data with eq. 6 reveals a quite significant correlation between parameters C_H and A_H. Figure 7a shows that $\ln(C_H)$ is linearly dependent on A_H. Without a more detailed microscopic model, it is impossible to distinguish whether a stronger contribution is due to stronger interaction or a greater number of hydrogen bonds.

Inserting the linear dependence of $\ln(C_H)$ on A_H into eq. 6 leads to a function of the form

$$S_{\mathrm{T}}(T) \;=\; S_{\mathrm{T}}^{\infty} - \exp\left(m + \; A_H^{*}\left(n - T\right)\right) \;, \tag{7}$$

with the constants $m = -3.4 \pm 0.2$ and $n = (255 \pm 5)$ K calculated from the linear fit shown in Figure 7a. The only two fitting parameters left are thus S_T^{∞} and A_H^{*}, where the latter is a measure for the temperature sensitivity of the strength of hydrogen bonds. Note that m is negative and $n < T$, so that a larger A_H^{*} indicates a smaller contribution of the temperature dependent term.

Recently, an empirical correlation between the temperature sensitivity of the Soret coefficient in relation to the partition coefficient $\log P$ has been found for different types of molecules.[74] A_H^{*} is also a measure to characterize the degree of hydrophilicity of a substance.

Its values are plotted against log$P$ in Figure 7b. Note that log$P$ values are only available for the pure CDs (black squares), while the log$P$ for complexes (red circles) are not strictly defined and are estimated from the correlation between the difference of donor and acceptor sites, $N_{don} - N_{acc}$, with log$P$ (for further explanation see SI section 3). The correlation with hydrophilicity shows that our initial assumption that $S_{\mathrm{T}}(T)$ depends on interaction strength between solute and solvent is correct. For more hydrophilic compounds, indicated by small partition coefficients, the temperature dependence of S_{T} is more pronounced (smaller A_H^*) than for the more hydrophobic compounds.

Piazza's equation (2) as well as our adapted expression (6) describe the temperature

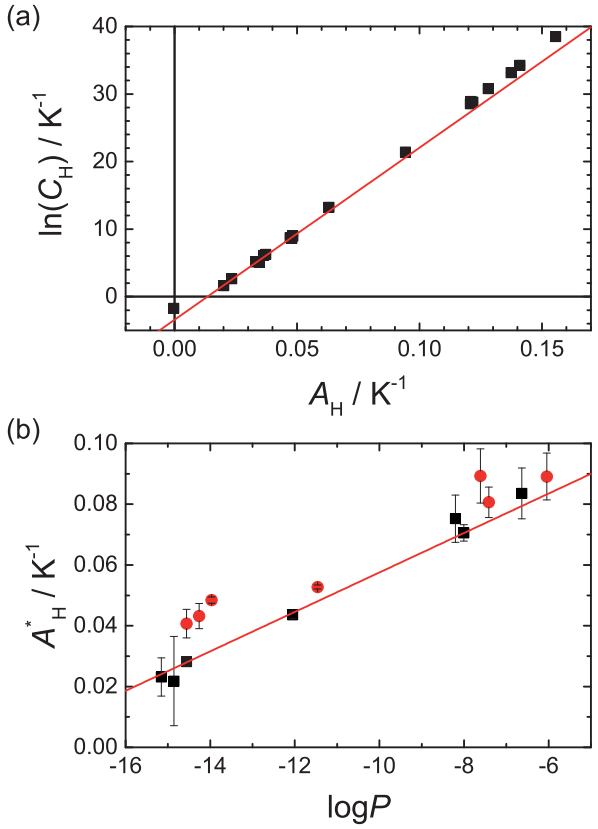

Figure 7: (a) Fit parameters C_H and A_H from eq. 6 show linear correlation. (b) Inserting the linear dependency of C_H into eq. 6, A_H^* becomes the only fit parameter describing temperature dependence in eq. 7. It shows a linear correlation with log$P$. Note that log$P$ values were only available for the pure CDs (black squares), the log$P$ values for the ASA complexes (red circles) are estimated (see SI section 3).

dependence of the Soret coefficient for the investigated CDs and complexes well. Additionally, eq. 6 is able to describe the temperature dependence of the S_T of more hydrophobic compounds like ASA. The interpretation of the results, however, is difficult. With an increase in hydrophobicity, A_H and $\ln(C_H)$ become negative, which might be interpreted as a repulsive interaction between solute and solvent, such as the formation of clathrate-like structures around hydrophobic parts of the molecule. In that case, the homogeneity of the solution on a microscopic scale is questionable, often leading to a decay of S_T as function of temperature.[31,75,76] The temperature dependence is not due to the strength of hydogen bonds between solute and solvent, then, but due to the increasing flexibility of the hydrogen bond network of water at high temperatures.

Figure 8 shows the temperature sensitivity of the Soret coefficient $\Delta S_T = S_T(50°C) - S_T(20°C)$ of several linear sugars, CDs and ASA as a function of log$P$. Taking ΔS_T as a measure of the temperature sensitivity is not principally different from fitting T_0 or A_H^* to the temperature curve, but it necessitates fewer measurements and is therefore easier to obtain. For the complexes, we used the same log$P$ values as for Figure 7. The con-

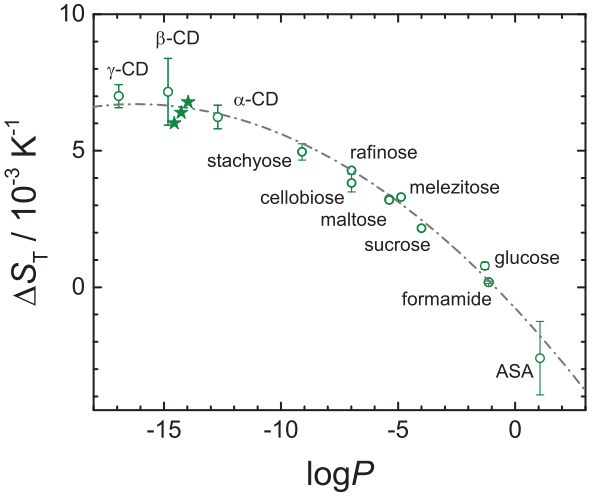

Figure 8: $\Delta S_T = S_T(50°C) - S_T(20°C)$ against log$P$ for oligosaccarides,[77,78] formamide,[31] CDs, and ASA (open symbols) at low concentrations. Additionally we plotted the values of CD/ASA complexes (green stars) by assuming that the S_T observed for CD+ASA is the weighted average of the S_T-values of CD, ASA and CD/ASA complex (see SI section 2 for details). The dashed line is a second order polynomial fit of all open symbols.

centration of solute molecules was quite low with 1wt% (CDs), 5wt% (formamide), 10wt% (oligossacharides), and 20wt%(glucose). We find an increase of temperature sensitivity of ΔS_{T} with an increasing hydrophilicity (corresponding to a more negative log$P$) of the solute molecules. The temperature sensitivity observed for the solutions with CD and ASA $\Delta S_{\mathrm{T}}^{mix}$ is much smaller than expected for the complex, due to the presence of ASA with its negative $\Delta S_{\mathrm{T}}^{ASA}$. With p_{com} determined for β-CD, the temperature sensitivity of the complex $\Delta S_{\mathrm{T}}^{com}$ (green stars in fig 8) was calculated for the unmethylated CDs (see SI section 2). The results agree reasonably well with the trend set by the CDs and the literature compounds (dash dotted line). The methylated CDs without ASA also show a ΔS_{T} that is lower than expected, which might be caused by an overestimation of log P by the algorithm used in this work. To test if this interpretation of the data is correct, further experiments with other drug compounds forming complexes with CDs are required.

Our systematic study of CDs and their complexes with ASA shows that the thermophoretic behaviour is strongly connected with hydrophobicity/hydrophilicity. The unambiguous correlation between the temperature sensitivity of the Soret coefficient and log$P$ and the apparent additivity found for the complex compared to the empty CD might be an interesting alternative to define the hydrophilicity of a complex, for which log$P$-value cannot be measured independently. Although there are still some questions to be addressed, our investigations show that the change of the temperature sensitivity of S_{T} upon complex formation could provide structural information about protein-ligand binding. Combining these findings with the rapid MicroScale Thermophoresis (MST) it will be possible to quantify not only protein-ligand affinities,[1] but also the hydrophilicity of the formed complexes. The temperature sensitivity of thermophoresis also plays a key role to direct drugs into inflamed areas and therefore it would be interesting to perform in the future temperature dependent studies in crowded media.

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Supporting Information Available

A brief derivation how the fraction of complexes (see Eq.5) can be determined from the measured self diffusion coefficients. We describe how the number of donors and acceptors has been determined. Additional experiments in a buffered solution are presented to assess, whether the pH-changes due to the addition of ASA has an effect on the results and we summarize the measured contrast factors. Our data are compared with the master curve of ref.[44] The composition of the investigated solutions is given in detail.

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Graphical TOC Entry