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Self-assembly of cyclodextrins: The effect of the guest molecule

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ABSTRACT

The principle action by which cyclodextrins solubilize compounds is via inclusion complex formation. However, data suggest that cyclodextrins and their complexes also aggregate in solution and this aggregation contributes to their ability to solubilize poorly water-soluble materials. The current effort aims at better understanding the role of guest molecule nature (i.e. its structural and functional peculiarities) in cyclodextrin complex aggregation as well as in the aggregate stability assessed using a cellophane membrane permeability assay. A test set of 11 acidic, basic and neutral drugs and antibacterial agents (i.e. guests) were examined with regard to their interaction with hydroxypropyl-β-cyclodextrin (HPβCD) and the resulting ability of the formed aggregates to move through a semi-permeable membrane of various molecular weight cut-off values. The data suggested that the interaction of HPβCD with certain guests resulted in the formation of structure large enough to poorly penetrate semi-permeable membrane. The aggregates appeared to be highly dynamic in that there were no qualitative differences between systems that were diluted immediately prior to permeation experiments and those allowed to equilibrate. Pharmaceutical polymers which have been shown to enhance solubilizing efficiency of cyclodextrins had little or no effect on the stability of the aggregates using the permeability paradigm as an endpoint with the exception of carboxymethylcellulose.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides that are commonly used solubilizing complexation agents during screening and formulation of new drug candidates (Frömming and Szejtli, 1994; Brewster and Loftsson, 2007; Loftsson and Duchêne, 2007). CDs are cone-shaped molecules with a hydrophilic outer surface and a hydrophobic central cavity. CDs (i.e. the hosts) can form inclusion complexes with increased water solubility by interacting with lipophilic molecules (guests), or more frequently lipophilic moieties of poorly water-soluble compounds, into the central cavity. In most cases an apparent 1:1 guest/host complex is formed (Duchêne, 1991; Duchêne and Wouessidjewe, 1996; Loftsson and Brewster, 1996; D'Souza and Lipkowitz, 1998; Saenger et al., 1998; Brewster and Loftsson, 2007). During the last decade CDs and their complexes have been studied intensively and wealth of information has been garnered on their structure, abilities to form complexes and the forces involved (Bodor and Buchwald, 2002; Liu and Guo, 2002; Katritzky et al., 2004; Dodziuk, 2006; Douhal, 2006). Self-association of the natural CDs in aqueous solutions has been recognized and investigated whereas information on their derivatives is somewhat more limited (Szente et al., 1998; Szente and Szejtli, 1999; González-Gaitano et al., 2002; Bonini et al., 2006; Rossi et al., 2007; He et al., 2008; Messner et al., 2010). In case of the positively charged CD, sugammadex, it has been shown that this hydrophilic CD derivative does not self-associate to form aggregates at concentrations up to 100 mg/ml (unpublished results). However, inclusion complexes of other hydrophilic CD derivatives have been shown to form aggregates (Loftsson et al., 2002a,b; Magnusdottir et al., 2002; Sigurdsson et al., 2002; Messner et al., 2010, 2011). In this present study we investigated how the guest influences the formation and properties of the complex aggregates using the hydrophilic βCD derivative, 2-hydroxypropyl-β-cyclodextrin (HPβCD) as a sample CD. Eleven guest molecules covering wide spectrum of pharmaceutical compounds including acidic, basic and neutral species were selected. Moreover, the effect of the guest ionization was studied as well as the relationship between the shapes of the phase-solubility behavior and the permeation profiles with regard to the aggregation profile. Attempts were made to stabilize the aggregates through addition of water-soluble polymers pursuing an idea of aggregation phenomenon application for the improvement of existing drug profiles. Self-association of the complexes, and the size distribution of the aggregates formed, was assessed by observing permeation of the

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guest molecule through semi-permeable membranes with a molecular weight cut-off (MWCO) value between 8 and 100 kDa.

2. Materials and methods

2.1. Materials

2-Hydroxypropyl-β-cyclodextrin (HPβCD) with molar substitution of 0.64 (M_W 1400) was purchased from Roquette (Lestrem, France). Ketoprofen (KP) and hydrocortisone (HC) were purchased from Fagron (Nieuwerkerk aan den Ussel, The Netherlands); acetazolamide (AZ), diazepam (DI), ibuprofen (IB), indomethacin (IM) and lidocaine (LI) were obtained from Sigma (St. Louis, MO, USA); methylparaben (MPB) was purchased from Norsk Medisinaldepot (Oslo, Norway); triclocarban (TCC) from Bayer (Ludwigshafen, Germany); triclosan (TCS) and dextromethorphan (DMP) were obtained from Procter & Gamble (Egham, United Kingdom). Carboxymethylcellulose sodium salt 400–800 cP, 2% in H_2O (25 °C) (CMC), (hydroxypropyl)methyl cellulose 2600–5600 cP, 2% in H_2O (20 °C) (HPMC) and hexadimethrine bromide (HDMB) were purchased from Sigma (St. Louis, MO, USA). Milli-Q water (Millipore, Billerica, MA) was used for the preparation of all solutions.

2.2. Solubility determinations

Solubility of guests (e.g., drug or an antibacterial agent) in pure water and aqueous 0.1 M HCl and 0.1 M NaOH solutions containing HPβCD was determined by the heating method as previously described (Loftsson and Hreinsdóttir, 2006). Briefly, an excess amount of the guest compound to be tested was added to 0–105 mM (0–15%, w/v) HPβCD solution and the suspension formed was treated in a sonicator (Cole-Parmer Instrument Company 8892, Niles, IL, USA) at 60 °C for 60 min in a sealed glass vial and then allowed to cool to room temperature. A small amount of the solid guest was then added to the suspension and the mixture allowed to equilibrate in the resealed vials at room temperature (23 ± 1 °C) for 7 days protected from light under constant agitation (KS 15 A shaker, EB Edmund Bühler GmbH, Germany). After equilibrium was attained, the suspension was filtered through a 0.45 μm RC media membrane filter (Spartan 13/Whatman, Germany), the filtrate was diluted 100 times with the mobile phase and analyzed by HPLC. All values were obtained from the mean of twelve replications.

Phase-solubility profiles were determined according to the method of Higuchi and Connors (1965). The complexation efficiency (CE) was determined by using the slope of the linear phase-solubility diagram (i.e., a plot of the concentration of guest in solution ($[G]_{total}$) versus the total CD concentration ($[CD]_{total}$) in moles per liter) (Loftsson et al., 2005a):

$$CE = \frac{\text{slope}}{1 - \text{slope}} = \frac{[\text{G/CD complex}]}{[\text{CD}]} \quad (1)$$

2.3. Permeation studies

The permeability studies of the guests from aqueous HPβCD solutions (the donor phase) were carried out in an unjacketed Franz diffusion cells with a diffusion area of 1.77 cm^2 (SES GmbH-Analysesysteme, Germany). The receptor phase (12 ml) consisted of an aqueous CD solution that was identical to the donor phase except that it did not contain guest. The guest saturated donor phase solution (2 ml) was prepared as described in Section 2.2 and added to the donor chamber after filtration through the 0.45 μm RC media membrane filter. The donor chamber and the receptor chamber were separated by a single layer semi-permeable cellulose ester membrane (Biotech CE) with a MWCO of 8 kDa, 15 kDa, 50 kDa or 100 kDa (Spectrum Europe, Breda, The Netherlands) that had been pretreated in the receptor phase solution overnight. The study was carried out at room temperature under continuous stirring of the receptor phase using a magnetic stirring bar rotating at 300 rpm (Variomag Poly 15, H + P Labortechnik, Oberschleissheim, Germany). A 150 μl sample of the receptor medium was withdrawn at 120, 180, 240, and 300 min and replaced immediately with an equal volume of fresh receptor phase. Less than 6% of the guest in the donor phase permeated the membrane during the 300 min study period and, thus, steady state was maintained during the experiment. The guest concentration in the receptor sample was determined by HPLC. The steady state flux (J) was calculated as the slope (dq/dt) of linear component of the amount of guest in the receptor chamber (q) versus time (t) profiles, and the apparent permeability coefficient (P_{app}) was calculated from the flux according to Eq. (2) (i.e. Fick's first law):

$$J = \frac{dq}{A \cdot dt} = P_{\text{app}} \cdot C_{\text{d}} \quad (2)$$

where A is the surface area of the mounted membrane and C_d is the initial guest concentration in the donor phase. All values are the mean of three replications.

2.4. Stability studies

Stability of the aggregates was evaluated by measuring the permeation of hydrocortisone/HPβCD complexes through a cellulose membrane with MWCO of 8 kDa as described in Section 2.3. The choice of a guest molecule for stability test rests on the fact that hydrocortisone is a widely used pharmaceutical which as shown by different methods (Loftsson et al., 2002b; Jansook et al., 2010; Messner et al., 2011) has a strong ability to promote aggregation of hydroxypropylated cyclodextrins. The donor solutions were prepared immediately before the permeation study by the dilution of a hydrocortisone saturated stock solution containing 114 mM (16%, w/v) of HPβCD. Three different donor phases were thus obtained by a 2-fold, 4-fold or 8-fold dilution of the stock solution at time zero. The receptor phase consisted of an aqueous 14 mM (2%, w/v) HPβCD solution. A cellulose membrane with a MWCO of 8 kDa was used. The hydrocortisone concentration in the receptor phase was determined by HPLC. For comparison, hydrocortisone saturated 14.29, 28.57 and 57.14 mM (2, 4 and 8%, w/v) HPβCD solutions were also prepared by dissolving an appropriate amount of solid HPβCD in pure water and tested.

The effects of three different polymers, i.e., CMC, HPMC and HDMB, on the stability of the hydrocortisone/HPβCD complex aggregates were evaluated by determining the stability of the aggregates after addition of 0.5% (w/v) of the polymers to the aqueous complexation media. To maintain the concentration of the polymer in the donor phase, 0.5% (w/v) aqueous solution of the corresponding polymer was used to dilute the stock solution.

2.5. Quantitative determination of guests

Quantitative determination of the concentration of the guests was performed using a reversed-phase high performance liquid chromatographic (HPLC) component system from Dionex Softron GmbH (Germany) Ultimate 3000 Series, consisting of a P680 pump with a DG-1210 degasser, an ASI-100 autosampler, a VWD-3400 UV-Vis detector and Phenomenex Luna C18 100 × 4.60 mm, 5 μm column (Phenomenex, UK) with a matching guard column (Phenomenex, UK). The mobile phases, flow rates, wavelengths and retention times of the guests are provided in Table 1.

Table 1
HPLC conditions for quantitative determination of the guest compounds (AcN – acetonitrile, SOS – 1-octanesulfonic acid sodium salt, MeOH – methanol, THF – tetrahydrofuran, Et_3N – triethylamin).

Guest	Mobile phase (volume ratios)	λ (nm)	Flow rate (ml/min)	Retention time (min)
Azetazolamide	AcN:acetic acid:H_2O containing 0.015% (w/v) SOS (12:2:86)	254	1.3	2.7
Dextromethorphan	MeOH:H_2O containing 0.115% (w/v) $(NH_4)_2HPO_4$ (90:10)	227	1.5	3.0
Diazepam	MeOH:H_2O (75:25)	226	1.5	2.6
Hydrocortisone	MeOH:H_2O:THF (70:29:1)	254	1.0	3.1
Ibuprofen	AcN:acetic acid:H_2O (65:1:34)	265	1.5	3.0
Indomethacin	AcN:aqueous 2% (v/v) acetic acid solution (70:30)	283	1.5	2.3
Ketoprofen	AcN:aqueous 2% (v/v) acetic acid solution (60:40)	265	1.5	2.5
Lidocaine	MeOH:H_2O:Et_3N (80:19.5:0.5)	264	1.2	2.8
Methylparaben	AcN: H_2O (50:50)	254	1.5	1.9
Triclocarban	MeOH:H_2O:THF (90:9:1)	283	1.2	2.4
Triclosan	MeOH:H_2O:THF (90:9:1)	283	1.2	2.3

3. Results

3.1. Phase solubility

According to the Higuchi and Connors (1965) classification system of water-soluble complexes, all the phase-solubility diagrams obtained for the guests in aqueous HPβCD solutions belong to either the A_P-type (Fig. 1) or the A_L-type (Figs. 2–4). The determined intrinsic solubilities are shown in Table 2 and vary between 1×10^{-4} mM (triclocarban) and 30.9 mM (methylparaben). The inclusion complexes are characterized by their CEs, which is a measure of solubilizing potential of CDs and calculated for the systems yielding A_L-type diagrams (Loftsson et al., 2007). The CE values obtained in this study are shown in Table 2 and range from 5×10^{-3} (indomethacin) to 2.26 (ibuprofen). For the guests demonstrating A_P-type diagrams, triclosan is solubilized by HPβCD more effectively than triclocarban that is while the intrinsic solubility difference between the two guests is about 30 fold, the observed solubility difference increases to 100 fold in the presence of 105 mM (15%, w/v) HPβCD.

3.2. Permeation profiles

The permeation profiles of the guests from aqueous HPβCD solutions are shown in Figs. 1–4. The collected data appears to fall into two categories, i.e., the profiles that match the guest's phase-solubility diagram, as predicted by Fick's first law, and the ones which show negative deviation from the expected trend.

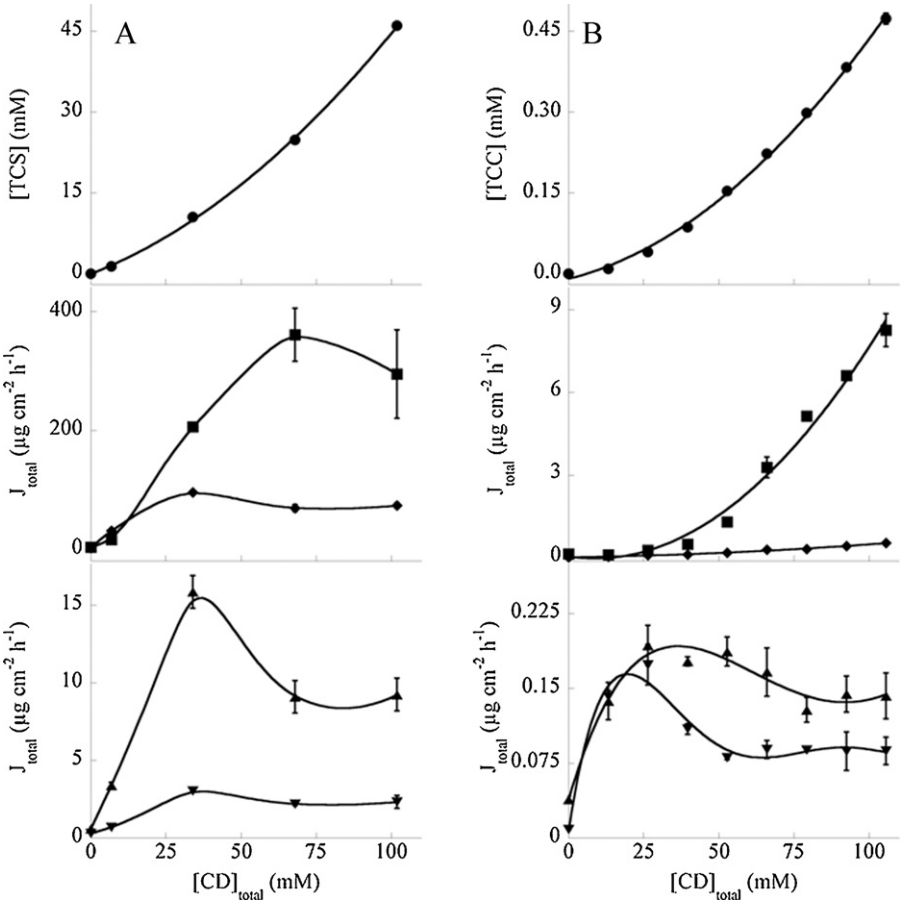


Fig. 1. Phase-solubility (top, ●) and permeation profiles (middle, bottom) of the triclosan (A) and triclocarban (B) through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Both compounds display A_P-type phase-solubility diagrams in pure aqueous HPβCD solutions. Error bars represent standard deviation (solubility: $n = 12$; flux: $n = 3$).

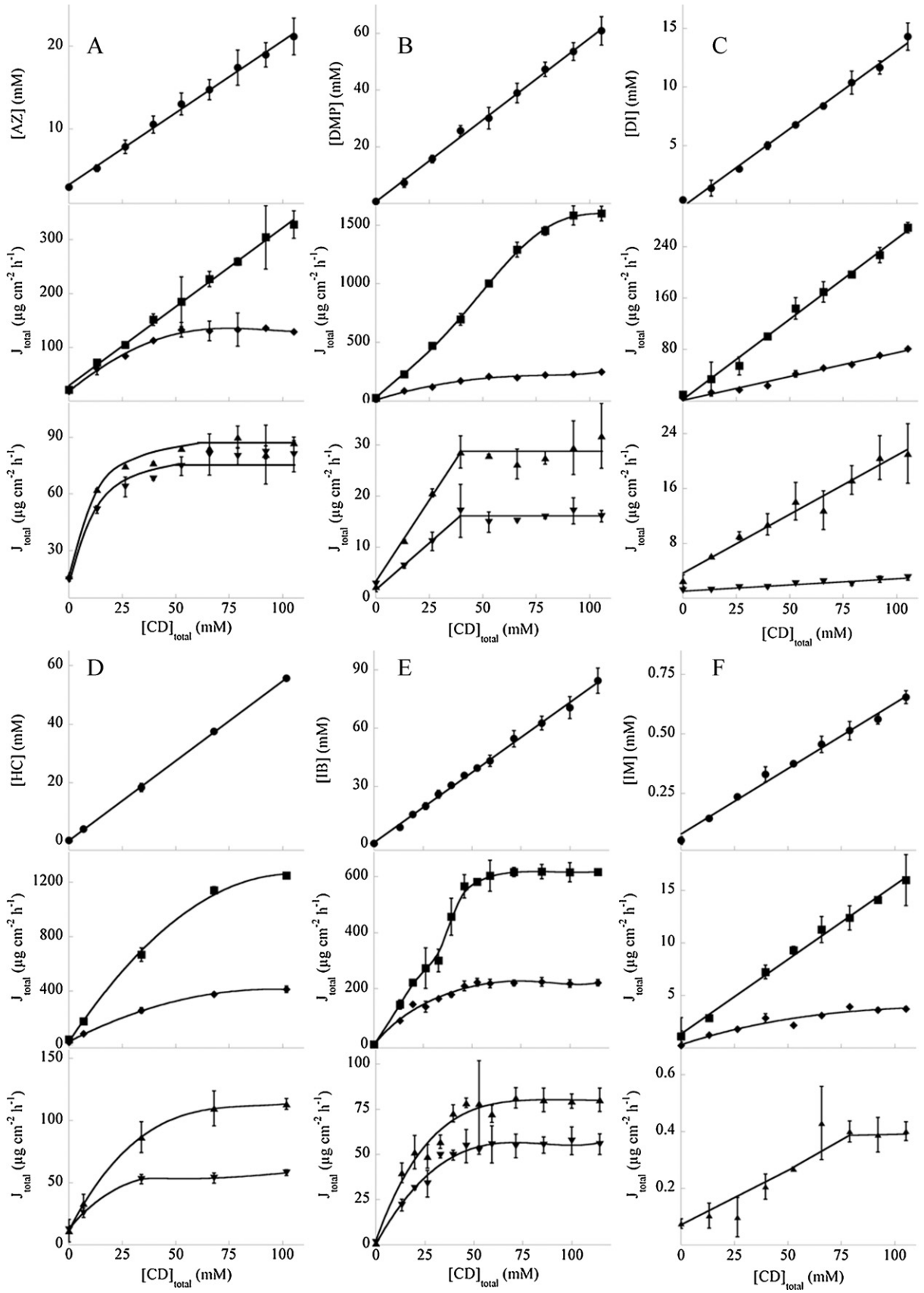


Fig. 2. Phase-solubility (top, ●) and permeation profiles (middle, bottom) of the phase solubility A_L-type guests acetazolamide (A), dextromethorphan (B), diazepam (C), hydrocortisone (D), ibuprofen (E), indomethacin (F), and methylparaben (G) in aqueous HPβCD solution through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Error bars represent standard deviation (solubility: $n = 12$; flux: $n = 3$).

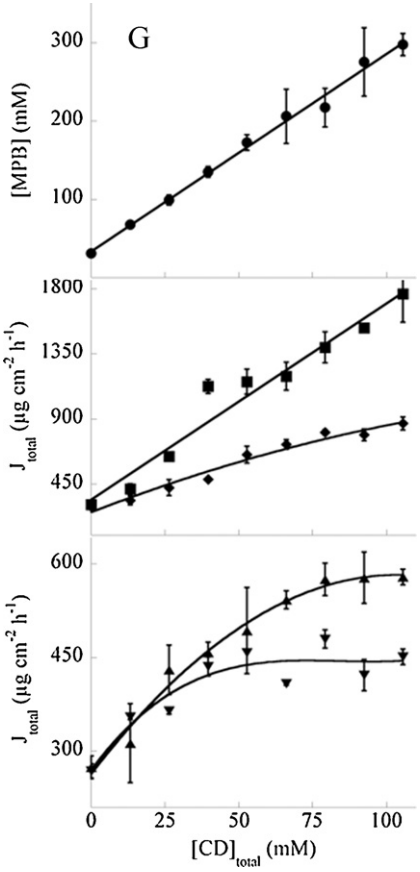


Fig. 2. *Continued*

Specifically, the A_P-type phase-solubility profiles result in somewhat unexpected permeation relationships in that the permeability curves possess a maximum with a consequent flux decrease and leveling off as the HPβCD concentration increases, with the exception of the permeation profiles of triclocarban at MWCO 50 and 100 kDa that match Fick's first law(see Fig. 1A and B). Permeation profiles of the other guest compounds tested are either in agreement with Fick's first law (i.e., match with the corresponding A_L phase-solubility diagram) or are independent of the HPβCD concentration, most after initial increase in accordance to Fick's first law (Figs. 2, 3ii and 4ii). The shape of the permeability profiles is strongly affected by the guest/HPβCD interaction and the consequent complex aggregation. The only guest permeability profile that matches perfectly with Fick's first law, regardless of the membrane MWCO, is that of diazepam.

The apparent permeability coefficients (P_{app}) estimated from the linear permeation profiles according to Eq. (2), are reported in Table 3. The P_{app} values increase up to three orders of magnitude as the membrane MWCO increases from 8 to 100 kDa (see diazepam in Table 3). This is to be expected since the permeation resistance (Loftsson et al., 2002b), provided by the membrane, decreases with increasing pore size (i.e., increasing MWCO).

3.3. Effect of ionized and unionized forms of dissociable guests upon aggregation

The ionization state of the guest molecule on the aggregate formation was also investigated. The solubility and permeability of the basic guest lidocaine and the acidic guest ketoprofen were determined in both aqueous 0.1 M hydrochloric acid and 0.1 M sodium hydroxide solution, in addition to pure water solution. The results obtained show that while lidocaine demonstrates A_L-type phase-solubility diagrams independent of pH (Fig. 4), fully ionized ketoprofen is not solubilized by HPβCD at basic pH as effectively (B_S-type profile) as the partly ionized form in pure water or the unionized form in acidic media (A_L-type profiles, see Fig. 3iii). Thus, ionization strongly affects both the total amount of dissolved guest and the guest/HPβCD complex formation. This again affects the aggregation and consequently the guest permeation through the membranes.

3.4. Stability studies

The stability of the hydrocortisone/HPβCD complex aggregates was evaluated by comparing the flux of hydrocortisone from hydrocortisone saturated solutions containing 57 mM (8%, w/v), 29 mM (4%, w/v) and 14 mM (2%, w/v) HPβCD, that had been allowed to equilibrate for one week, with the flux from comparable solutions obtained by diluting hydrocortisone saturated 144 mM (16%, w/v) HPβCD stock solution right before the hydrocortisone permeability determination. If the aggregates disassembled right after dilution, the hydrocortisone flux from the two corresponding HPβCD solutions should be almost identical. If, on the other hand, the aggregates were stable or disaggregated somewhat slowly the hydrocortisone flux from the diluted solutions should be lower than from the equilibrated solutions.

Fig. 5 shows the flux values for hydrocortisone through a semipermeable membrane with MWCO 8 kDa from the solution diluted immediately prior to testing and the equilibrated solutions. As indicated in Fig. 5, the ladder bars represent the flux of the equilibrated solutions and the filled bars correspond to the solutions obtained after dilution of the stock solution. The results indicate that the

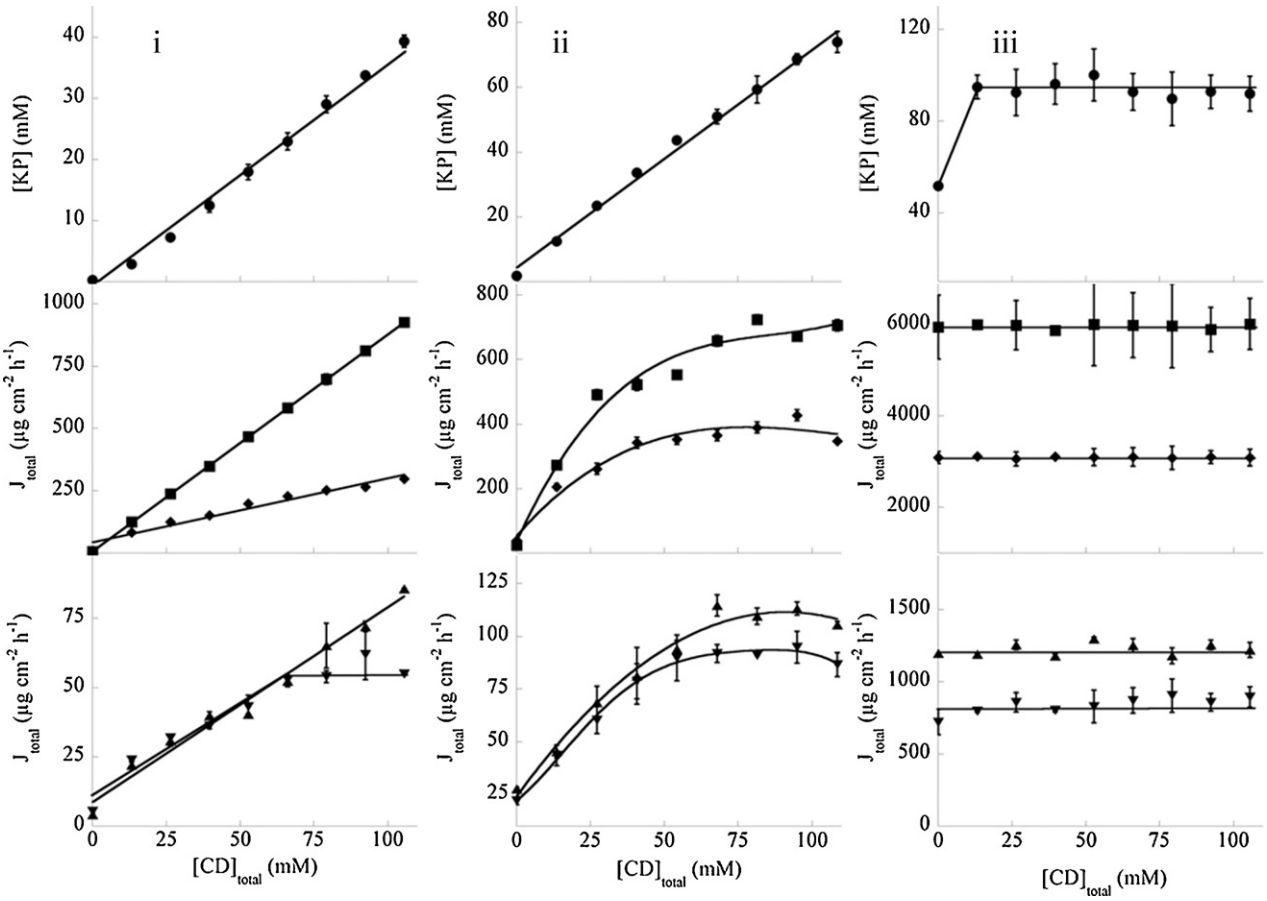


Fig. 3. Phase-solubility (top, ●) and permeation profiles (middle, bottom) of ketoprofen in 0.1 M HCl (i), aqueous (ii), and 0.1 M NaOH solution (iii) of HPβCD through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Error bars represent standard deviation (solubility: $n = 12$; flux: $n = 3$).

complex aggregates readily dissociate upon dilution and of the three polymers tested only the negatively charged CMC affected aggregated stability to any appreciable extent. The uncharged HPMC and the positively charged HDMB did not have a measurable stabilizing effect.

4. Discussion

The guests included five acidic compounds, five basic compounds and one neutral compound, which can be rank ordered by their increasing intrinsic solubility (Table 2) in the following manner:

TCC < TCS < IM < DI = IB < DMP < HC < AZ < KP < LI < MPB.

HPβCD formed water-soluble complexes with all the guest compounds studied. In the aqueous 105 mM (15%, w/v) HPβCD solution the solubility rank was as follows

TCC < IM < DI < AZ < TCS ≈ LI < HC < DMP < KP < IB < MPB.

In general, the experimental values shown in Table 2 agree with published literature values (Backensfeld et al., 1990; Mura et al., 1998; Holvoet et al., 2004; Rytting et al., 2005; Loftsson et al., 2007). The strongest solubilizing effect is observed in the case of ibuprofen and the weakest in the case of lidocaine. If the only solubilizing mechanism is the formation of inclusion complex between the poorly soluble guest and the water-soluble HPβCD, the results would indicate that the ibuprofen molecule has the most favorable physicochemical properties and optimal geometry for fitting into the HPβCD cavity as well as for the formation of non-covalent inclusion formation with HPβCD. The majority of the guests under study gave rise to A_L-type phase-solubility diagrams, with slope less than unity, that could suggest that the complexation has 1:1 guest:HPβCD stoichiometry. Two guest compounds, triclosan and triclocarban, form apparent 1:2 complexes as implicated by a A_P-type phase-solubility profile. However, ^{1}H NMR spectroscopic measurements have indicated that triclosan/HPβCD complexes form aggregates at HPβCD concentrations upwards of about 35 mM (5%, w/v) (Loftsson et al., 2005b). It has also been shown that the Higuchi and Connors phase-solubility classification is not entirely adequate for the evaluation of the CD solubilizing mechanism (Loftsson et al., 2004). Often 1:1 inclusion complexation seems to be accompanied with the formation of complex aggregates, and the aggregates formed are sometimes able to solubilize additional guest molecules through non-inclusion mechanisms. Analysis of guest permeation through semipermeable cellophane membranes offers the possibility to give a better understanding of the solubilizing mechanisms of CDs.

Determination of guest permeation profiles from aqueous CD solutions through semipermeable membranes is a simple but effective experimental technique for aggregate detection and characterization. The basic tenet is that in the absence of aggregation, the largest particles existing in the solution are the guest/CD inclusion complexes. Thus, an appropriate choice of a membrane, one with MWCO exceeding the molecular weight of a 1:1 complex, would give a guest permeation profile that matches the guest phase-solubility profile, or a permeation profile that corresponds to Fick's first law (Eq. (2)). In contrast, if aggregation involving the guest takes place resulting in aggre-

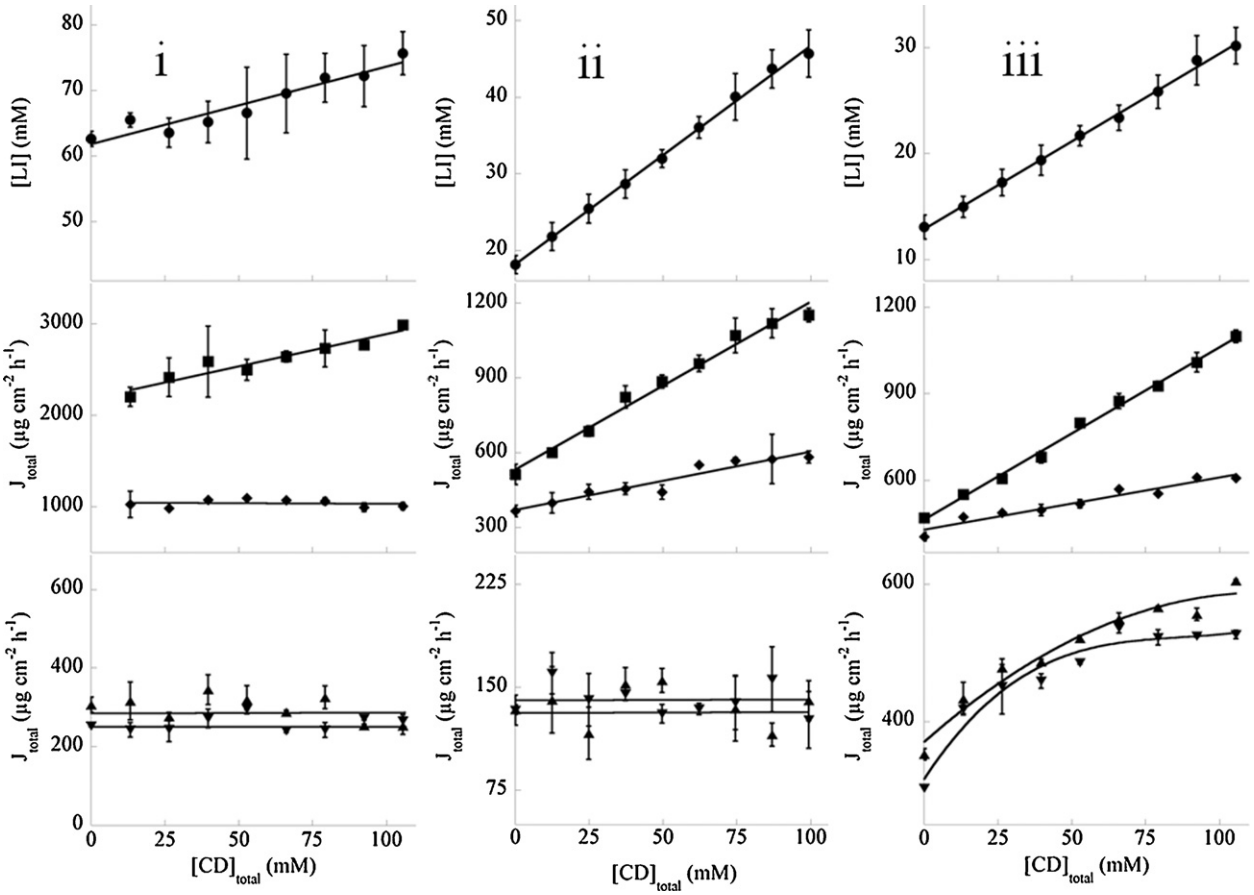


Fig. 4. Phase-solubility (top, ●) and permeation profiles (middle, bottom) of lidocaine in 0.1 M HCl (i), aqueous (ii), and 0.1 M NaOH (iii) solution of HPβCD through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Error bars represent standard deviation (solubility: $n = 12$; flux: $n = 3$).

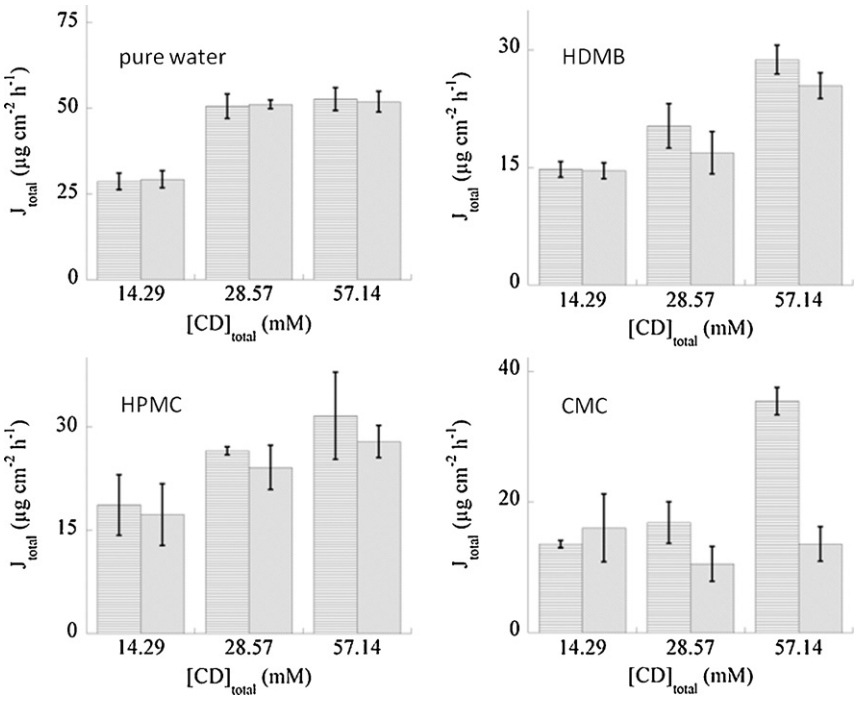


Fig. 5. Fluxes (mean ± SD) of hydrocortisone through MWCO 8 kDa semi-permeable membrane from equilibrated HPβCD solutions saturated with hydrocortisone (ladder column) and the diluted solutions obtained by the dilution of hydrocortisone saturated HPβCD stock solution (filled column) in the presence of the water soluble polymers HDMB, HPMC and CMC.

Table 2
Structure and the physicochemical properties of the guest compounds: molecular weight (M_W), intrinsic solubility (S_0) and complexation efficiency (CE).

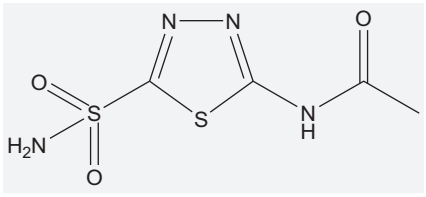
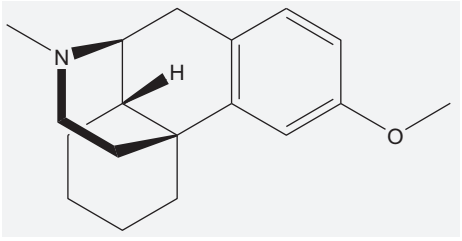
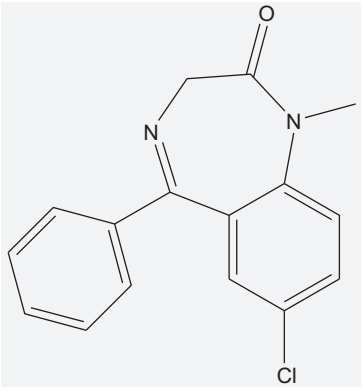
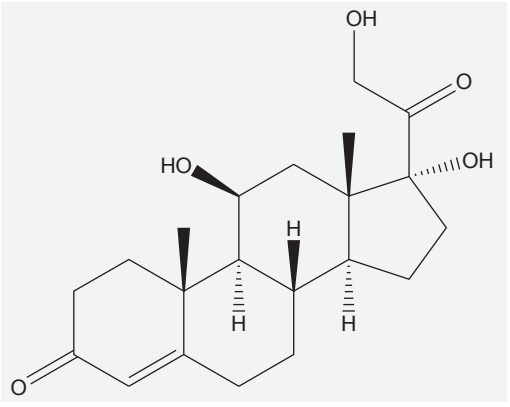
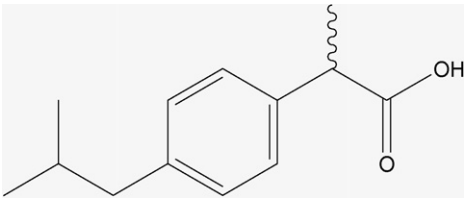
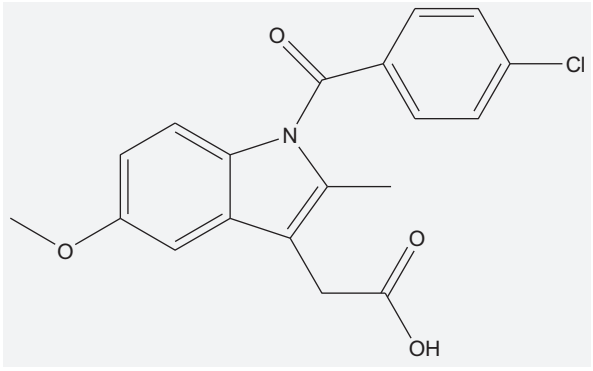
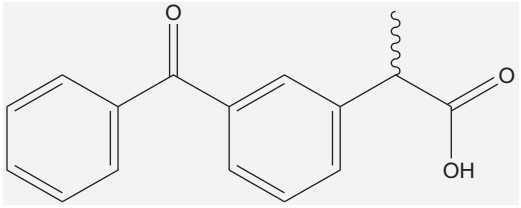
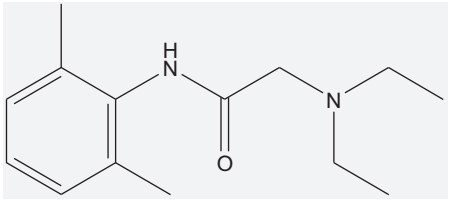
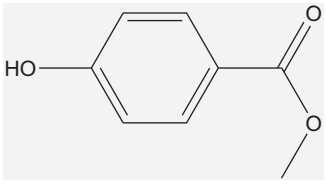
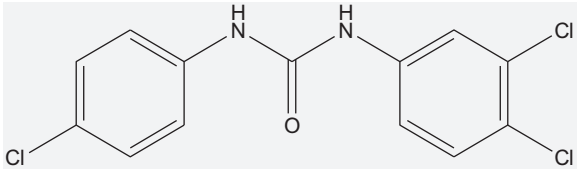
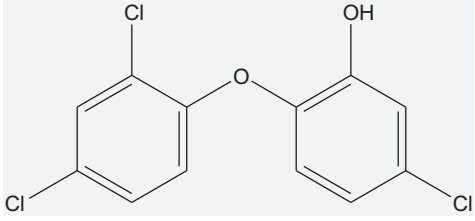
Guest	Structure	M_W (g/mol)	pK_a	Solvent	pH	S_0 (mM)	CE
Acetazolamide (AZ)		222.25	7.2	Water	5.3	2.9	0.21
Dextromethorphan (DMP)		271.40	8.3	Water	7.9	0.6	1.36
Diazepam (DI)		284.74	3.4	Water	6.4	0.4	0.15
Hydrocortisone (HC)		362.47	–	Water	6.8	0.8	1.26
Ibuprofen (IB)		206.28	4.4	Water	4.8	0.4	2.26

Table 2 (*Continued*)

Guest	Structure	M_W (g/mol)	pK_a	Solvent	pH	S_0 (mM)	CE
Indomethacin (IM)		357.79	4.5	Water	4.7	5×10^{-2}	5×10^{-3}
Ketoprofen (KP)		254.28	4.5	Water 0.1 M HCl 0.1 M NaOH	4.2 1.3 6.0	1.6 0.2 51.5	2.04 0.62 –
Lidocaine (LI)		234.34	7.9	Water 0.1 M HCl 0.1 M NaOH	7.9 6.8 12.5	18.1 62.6 13.1	0.40 0.14 0.20
Methylparaben (MPB)		152.15	8.4	Water	7.9	30.9	–[a]
Triclocarban (TCC)		315.60	12.7	Water	8.1	1×10^{-4}	–[b]
Triclosan (TCS)		289.53	7.9	Water	7.8	3×10^{-3}	–[b]

[a] The slope is higher than unity.
[b] A_P-type phase solubility profile.

gates that are larger than the MWCO of the membrane, then negative deviation from expected permeation profile would be observed. The aggregate size distribution can be assessed by the determination of the permeation profile through series of semipermeable membranes with different MWCO (Messner et al., 2011).

The results shown in Figs. 1–4 indicate aggregation formation for ten of the eleven guest compounds. Table 4 categorizes

Table 3
Apparent permeability coefficients (P_{app}) for guests displaying A_L-type phase-solubility profiles and linear permeation profiles that follow Fick's first law. The donor phase (i.e. solvent) was identical to the solubility media displayed in Table 2.

Guest	Solvent	P_{app} (g cm h^{-1} mol^{-1}) for the different MWCO membranes			
		8 kDa	15 kDa	50 kDa	100 kDa
Acetazolamide	Water	–[a]	–[a]	–[a]	0.29
Diazepam	Water	1.8×10^{-3}	15.6×10^{-3}	73.8×10^{-3}	0.25
Indomethacin	Water	–[b]	–[a]	–[a]	12.5×10^{-3}
Ketoprofen	0.1 M HCl	–[a]	68.6×10^{-3}	0.26	0.87
Lidocaine	0.1 M NaOH	–[a]	–[a]	0.19	0.57
	Water	–[a]	–[a]	0.23	0.67
	0.1 M HCl	–[a]	–[a]	–[a]	0.71
Methylparaben	Water	–[a]	–[a]	0.80	1.42

[a] Nonlinear permeability profiles.
[b] Not available.

the permeation profiles obtained in the present work. The guests showing A_L-type diagrams give permeation profiles that contain up to three separate components designated as I_L, II_L and III_L (Messner et al., 2011), whereas the guests displaying A_P-type phase-solubility diagrams give more complex permeation profiles that consist of up to four distinct sections, sections I_P, II_P, III_P and IV_P (Table 4). Based on this classification, triclosan and triclocarban have different aggregation patterns. In the 35–70 mM (5–10%, w/v) HPβCD concentration range, triclosan complexes aggregate to form superaggregates with a total apparent molecular weight (M_W) of 50–100 kDa (i.e., contain approximately 30–60 complexes per aggregate) (Fig. 1A), whereas at HPβCD concentrations higher than 70 mM (10%, w/v) the aggregates merge with newly formed complexes and existing aggregates with complex aggregates to form aggregates containing many more than 60 complexes per aggregate. Based on NMR studies the critical aggregation concentration for HPβCD was found to be 5.4% (w/v) (Duan et al., 2005), thus it could be possible that a chemical shift is due to the formation of some super-aggregates. In contrast, triclocarban complexes form smaller aggregates, which appear at relatively low HPβCD concentration, e.g., at 20–35 mM (3–5%, w/v) HPβCD aggregates with total M_W of 8–15 kDa are formed (i.e., contain 4–8 complexes per aggregate), whereas at HPβCD concentrations above 35 mM (5%, w/v) HPβCD aggregates not exceeding 30 complexes per aggregate exist. The phase-solubility profiles of the two guests show that triclosan is solubilized to a significantly greater extent than triclocarban. The presence of aggregates and the A_P-type profiles indicate that the guests are solubilized both through inclusion complex formation and through some non-inclusion mechanism (e.g., micellar type solubilization). Also, the fact that triclosan is solubilized to a significantly greater extent than triclocarban indicates that aggregate formation depends on the availability of guest/HPβCD complexes and that the larger triclosan/HPβCD complex aggregates are formed due to the greater availability of triclosan/HPβCD complexes than that of triclocarban/HPβCD complexes. This would be in agreement with our previous observation that the size of the aggregates increases with increasing CD concentration (Messner et al., 2011).

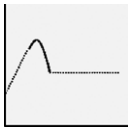
The size distribution of the guest/HPβCD complex aggregates of guest compounds displaying A_L-type phase-solubility diagrams are shown in Scheme 1. It is noteworthy that diazepam/HPβCD complexes do not demonstrate any evidence of aggregation, while lidocaine has a strong tendency to form relatively large aggregates even at low HPβCD concentrations (i.e., below 12 mM (2%, w/v)). Four guests (dextromethorphan, hydrocortisone, ibuprofen and ketoprofen) form large aggregates that are unable to permeate the membrane with MWCO 100 kDa. It is interesting that these guests are characterized with CE values exceeding unity, i.e., the major part of HPβCD present in solution participates in complex formation with only small fraction of unbound HPβCD present in the complexation media. This is another observation showing direct relationship between complexation, i.e., availability of guest/HPβCD complexes, and aggregation.

Two of the guest compounds including an acid (ketoprofen) and a base (lidocaine), were selected for the investigation of the effect of ionization on complex aggregate formation. In pure aqueous solutions, ketoprofen and lidocaine exist as a mixture of the ionized and unionized molecules (pK_a is 4.5 and 7.9, respectively). Performing the solubility and permeation studies in either aqueous 0.1 M HCl or 0.1 M NaOH solution allows dealing with ionized or neutral guest which can provide an understanding of the ionization on aggregation. As expected, the solubility of fully ionized guest compounds is notably higher than that of neutral ones (Figs. 3 and 4). Ionization makes guest molecule more hydrophilic that in turn decreasing the driving force for inclusion complex formation. This decreases affinity for the hydrophobic cavity can lead to poor solubilization as observed in the case of the fully ionized ketoprofen that gives B_S-type diagram (Fig. 3iii). However, analysis of the ketoprofen permeation profiles indicates that the complex aggregation intensifies upon ionization of the guest molecule. Unionized ketoprofen forms small aggregates (i.e., 4–9 complexes per aggregate) and then only at relatively high HPβCD concentrations. In contrast, the fully ionized ketoprofen has a strong tendency to form much larger aggregates that exceed 60 complexes per aggregate, even in relatively diluted HPβCD solutions. Large aggregates precipitate resulting in a B_S-type phase-solubility diagram. HPβCD rarely gives B_S-type phase-solubility profiles and, thus, this is an interesting observation that shows that the aggregation formation can be quite unpredictable.

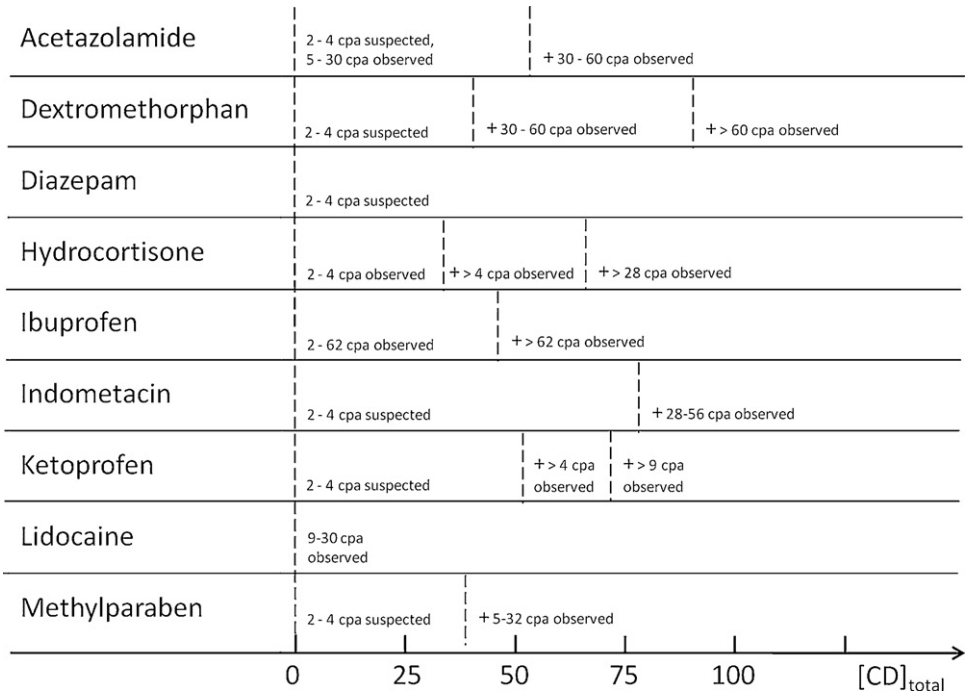
Although both the ionized and the unionized forms display A_L-type phase-solubility diagrams, the size distribution of the lidocaine/HPβCD complex aggregates changes with ionization (Fig. 4). Thus, unionized lidocaine form aggregates with a total M_W of less than 50 kDa (i.e., less than 30 complexes per aggregate) whereas positively charged lidocaine molecules form aggregates with total M_W of 50–100 kDa (i.e., 30–60 complexes per aggregate). These results show that ionization of a guest compound can have significant effect on the complex aggregate formation. Due to charge repulsion one might expect that ionized guest molecules would result in smaller and fewer aggregates but the experimental results with lidocaine show that this is not always the case.

Previous studies of hydrocortisone saturated aqueous HPβCD solutions have shown that the complex aggregation increases with increasing HPβCD concentration with a consequent decrease in the fraction of hydrocortisone participating in simple (i.e., monomeric) hydrocortisone/HPβCD inclusion complexes (Messner et al., 2011). If the aggregates dissociate relatively rapidly upon dilution then, in a permeation study, dilute hydrocortisone/HPβCD complex solution prepared from a

Table 4
Classification of the permeation profiles (Mw_A – aggregate molecular weight; Mw_C – complex molecular weight; MWCO – molecular weight cut-off).

Type of phase-solubility diagram	Type of permeability profile	Illustration of permeability profile	Permeation of solubilized guest	Membrane characteristics	Aggregation data
A_L	I_L		Free	$Mw_C < MWCO \leq 2Mw_C$ $2Mw_C < MWCO \leq nMw_C$	No aggregates No aggregates with $Mw_A \geq MWCO$ aggregates with $2Mw_C \leq Mw_A < MWCO$ suspected
	II_L		Hampered	$Mw_C < MWCO \leq nMw_C$	Some newly formed complexes participate in aggregates with $Mw_A \geq MWCO$
	III_L		Absent	$Mw_C < MWCO \leq nMw_C$	All newly formed complexes participate in aggregates with $Mw_A \geq MWCO$
A_P	I_P		Free	$Mw_C < MWCO \leq 2Mw_C$ $2Mw_C < MWCO \leq nMw_C$	Same as I_L (Fick's first law must be met)
	II_P		Hampered	$Mw_C < MWCO \leq nMw_C$	Same as II_L
	III_P		Absent	$Mw_C < MWCO \leq nMw_C$	Same as III_L
	IV_P		Extremely hampered	$Mw_C < MWCO \leq nMw_C$	Newly formed complexes and existing aggregates with $Mw_A < MWCO$ assemble to form aggregates with $Mw_A \geq MWCO$

concentrated solution of hydrocortisone/HPβCD complexes and a comparable hydrocortisone/HPβCD complex solution prepared by dissolving appropriate amount of HPβCD in water, should give close to identical flux values. On the other hand, if the flux of the diluted sample is significantly lower, it could be concluded that the aggregates have not fully disassociated. Water-soluble polymers affect the solubility and stability of simple guest/CD complexes and, thus, polymers might stabilize complex aggregates as well (Loftsson et al., 1994; Loftsson and Fridriksdottir, 1998). Hydrocortisone/HPβCD complex aggregates are readily dissociated upon dilution. The neutral and the positively charged polymers, HPMC and HDMB respectively, seem to have no effect on the stability of the complex aggregates, since the fluxes of the diluted and the originally prepared solutions are equal within experimental error. The negatively charged polymer, CMC, causes a decrease of the flux in the diluted samples. Since the flux



Scheme 1. Distribution of aggregate size populations vs cyclodextrin concentration in studied solutions (cpa is complexes per aggregate).

value is constant for all measured HPβCD concentrations, we assume that CMC has some stabilizing effect preventing, at least to some degree, the aggregate dissociation during the 5 h of the experiment.

5. Conclusion

All the guest/HPβCD complexes tested, except perhaps the diazepam/HPβCD complex, self-assembled to form aggregates. Medium size aggregates (M_W between 8 and 100 kDa) were formed in the case of triclocarban, methylparaben, acetazolamide, lidocaine and indomethacin whereas dextromethorphan, hydrocortisone, ibuprofen, ketoprofen and triclosan form complex aggregates larger than 100 kDa. The results indicate that the availability of guest/HPβCD complexes is the driving force for aggregate formation. CE values lower than unity lead to complex aggregates smaller than 100 kDa, whereas guests with higher CE form larger aggregates. Deviations from the CE vs. the aggregation relationship can be due to structural differences of the guests and their inclusion complexes. Furthermore, ionization of the guest molecule increases the aggregate formation.

In comparison to simple drug/CD complexes aggregation of CD complexes will not affect therapeutic efficacy of the drugs, kinetics of drug release from CD complexes or drug pharmacokinetics. The forces participating in the aggregate formation are weak "solute–solute" physicochemical interactions such as hydrogen bonding and van der Waals interactions. Thus, the CD aggregates are most likely metastable and will fall apart upon dilution in the blood stream or in other bodily fluids. Furthermore, elevation from room to body temperature might also depress the aggregate formation. However, complex aggregation can be important during drug formulation and manufacturing.

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