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Self-assembly of cyclodextrin complexes: Effect of temperature, agitation and media composition on aggregation

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ABSTRACT

Recently it has been shown that aggregation of drug/cyclodextrin inclusion complexes is strongly influenced by the drug molecule in addition to self-assembling tendencies of the cyclodextrin itself in aqueous media. Whereas the mechanistic basis of cyclodextrin self-assembly is known, the driving forces for complex aggregation are still unknown. In the present study, the influence of temperature on hydrocortisone/2-hydroxypropyl-β-cyclodextrin complex aggregation is investigated as are influences associated with the addition of ethanol or water soluble polymers to the aqueous systems. Furthermore the effect of stirring on the aggregation is assessed. Size exclusion permeability studies were conducted to estimate complex aggregation tendencies. The results indicate that self-assembled complex aggregates are metastable and notably become smaller with increasing temperature and the addition of ethanol. Water soluble polymers also reduce the size of the complex aggregates. Specifically, hexadimethrine bromide had the greatest impact, since addition of this compound eliminated aggregates from the systems or reduced their size below the molecular weight cut-off of the sizing membrane (8 kDa). Similar observations are made when aqueous solutions of hydrocortisone and 2-hydroxypropyl-β-cyclodextrin are equilibrated by stirred.

1. Introduction

Natural cyclodextrins (CDs) are known to self-assemble in aqueous solutions to form aggregates with intermolecular linkage attributed to the OH groups located at the rims of the donut-shaped CD molecules (Coleman et al., 1992; Szente et al., 1998; González-Gaitano et al., 2002). Substitution of the OH groups reduces their ability to form aggregates (González-Gaitano et al., 2002; He et al., 2008; Messner et al., 2010). However, unexpected phenomena have been observed during investigation of guest molecules (i.e., drugs) in aqueous CD solutions and these were explained by aggregation of the inclusion complexes formed in the solutions (Sigurdsson et al., 2002; Loftsson et al., 2004; Jansook et al., 2010; Messner et al., 2010, 2011a,b). Based on the data generated using permeability and TEM methods, it was suggested that aggregation of inclusion complexes is guest induced (Jansook et al., 2010; Messner et al., 2011b). The mechanisms of aggregation at the molecular level in the solutions studied remain poorly understood. Particularly, the role and strength of the forces involved in the aggregate formation.

Previously we have shown that permeability studies constitute an effective and easy-to-execute experimental technique that uses an observed negative deviation from Fick's first law to detect and provide quantitative analysis of nano-sized aggregates. The present work is a continuation of our ongoing effort to develop an efficient method for characterization of the aggregation phenomenon in aqueous solutions containing drug and CD. Our previous results showed that complex aggregation is indeed heavily influenced by the guest and that aggregate formation depends on the availability of the complex (i.e., the total drug and CD concentrations and the complexation efficacy) and the ability of the guest molecule to interact with neighboring complexes (Messner et al., 2011a,b). In the present work, the influence of temperature, media composition and stirring of the complexation medium are studied, using hydrocortisone/2-hydroxypropyl-β-cyclodextrin complex (HC/HPβCD) as a model inclusion complex system applying the permeation technique using semipermeable membranes of different molecular weight cut off (MWCO) (Messner et al., 2011b).

2. Experimental

2.1. Materials

2-Hydroxypropyl-β-cyclodextrin (HPβCD) with molar substitution of 0.64 (MW 1400) was purchased from Roquette (Lestrem,

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France) and hydrocortisone (HC) from Fagron (Nieuwerkerk aan den Ussel, The Netherlands). 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), hexadimethrine bromide (HDMB) and ethanol were purchased from Sigma (St. Louis, MO, USA). Milli-Q water (Millipore, Billerica, MA) was used for preparation of all solutions.

2.2. Solubility determinations

Solubility of HC in unbuffered water containing HPβCD was determined by the heating method as described previously (Loftsson and Hreinsdóttir, 2006). Briefly, an excess amount of the HC was added to an aqueous 0–105 mM (0–15% (w/v) HPβCD solution; the suspension formed was heated in an autoclave (Astell MXN 472, UK) at 121 °C for 20 min in sealed glass vial and then allowed to cool to room temperature. Next, a small amount of HC was added to the suspension and the mixture was allowed to equilibrate in the sealed vial at room temperature (23 ± 1 °C) for 7 days protected from light and under constant agitation (KS 15A shaker, EB Edmund Bühler GmbH, Germany) at 250 rpm. Preliminary experiments showed that 7 days are more than enough time to reach solubility equilibrium (Jansook et al., 2010). After equilibrium was attained, the suspension was filtered through a 0.45 μm RC media membrane filter (Spartan 13/Whatman, Germany), the filtrate diluted with the mobile phase and analyzed by HPLC.

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 total concentration of dissolved HC in solution ($[HC]_{total}$) vs the total HPβCD concentration ($[CD]_{total}$) in moles per liter) (Loftsson et al., 2005a):

$$CE = \frac{\text{slope}}{1 - \text{slope}} = \frac{[HC/HP\beta CD]}{[CD]} \quad (1)$$

2.3. Permeability studies

The permeability studies of HC from aqueous HPβCD solutions (the donor phase) were carried out in 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 HPβCD solution that was identical to the donor phase except it did not contain HC. The HC saturated donor phase solution (2 ml) was prepared as previously described 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 MWCO of 8 kDa, 15 kDa, 50 kDa or 100 kDa (Spectrum Europe, Breda, The Netherlands) that had been equilibrated with the receptor phase solution overnight. The study was carried out at room temperature under continuous stirring of the receptor phase using 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 5% of HC in the donor phase permeated the membrane during the 300 min study period and, thus, steady state was maintained during the experiment. The HC 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 guest amount (q) in the receptor chamber vs time (t) profiles, and the apparent permeability coefficient (P_{app}) was calculated from the flux according to the following equation (i.e., Fick's first law):

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

where A is the surface area of the mounted membrane and C_d is the initial HC concentration in the donor phase. Obtained flux vs cyclodextrin concentration dependencies are analyzed according our previously described method (Messner et al., 2011b). Linearity is described in section I_L and negative deviation from linearity in sections II_L and III_L. Extrapolation of the graph present in section I_L will give the theoretical flux (J_{theo}) at any cyclodextrin concentration. Together with the experimentally obtained value (J_{exp}) it is possible to calculate the fraction aggregates (f_A) using Eq. (3).

$$f_A \approx 1 - \frac{J_{exp}}{J_{theo}} \quad (3)$$

In order to analyze the aggregation process, the fraction of drug participating in certain aggregate population (f_D) is calculated using Eq. (4):

$$f_D = f_A^i - f_A^j \quad (4)$$

where i and j are incremental MWCO values and $i < j$.

To study the effects of additives 0.50% (w/v) HDMB, HPMC or CMC was added to the donor phase solutions. In another study, 3, 5 and 30% (v/v) ethanol was added to both the donor and receptor phase.

2.4. Temperature effects

Permeability studies at ambient temperature (23 ± 1 °C), in an incubator (36 ± 0.5 °C) (Gallenkamp, Germany) and in a refrigerator (10 ± 0.5 °C) (Kelvinator scientific, Conway, AR, USA) were carried out as described in the previous section.

2.5. Mechanical effects

Permeability studies in side-by-side diffusion cells (PermeGear, Hellertown, PA, USA) with a diffusion area of 1.13 cm^2 were preformed. Membranes, donor and receptor phase composition (both 3 ml), sampling intervals and other conditions were as previously described in the permeability studies section.

2.6. Quantitative determination of hydrocortisone

Quantitative determination of HC was performed on 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 Kintex C18 100 mm × 4.60 mm, 2.6 micron column (Phenomenex, UK) with a matching HPLC KrudKatcher Ultra Column In-Line Filter (Phenomenex, UK). The mobile phase consisted of methanol, water and tetrahydrofuran 79:20:1 (volume ratios). The flow rate was 1.0 ml/min and the retention time was 1.4 min.

3. Results and discussion

3.1. Solubility determinations

The intrinsic solubility of HC in pure water is 0.84 ± 0.16 mM and its phase solubility in aqueous HPβCD solutions is of the A_L-type with a CE of 1.27. Addition of 5% (v/v) ethanol increases the intrinsic solubility to 1.27 ± 0.06 mM, in other words, the solubility of HC increased by approximately 50% relative to the aqueous solution. Addition of 0.5% (w/v) of the water-soluble polymers to the aqueous solution also enhanced the hydrocortisone solubility (Table 1). HDMB or HPMC were, however, not as effective solubilizers as CMC. The shape of the phase-solubility diagram did not change with the introduction of the polymers. However, the

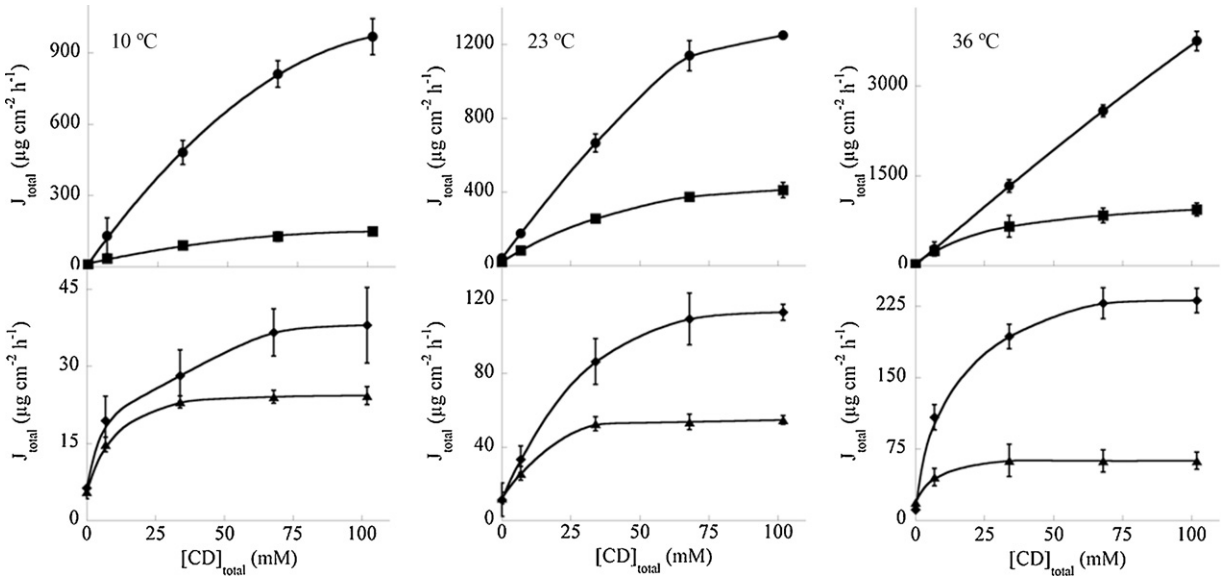

Fig. 1. Permeability profiles of HC in aqueous HPβCD solutions at different temperatures through semi permeable cellophane membranes with MWCO 8 (▲), 15 (♦), 50 (■) and 100 kDa (●). Error bars represent standard deviation.

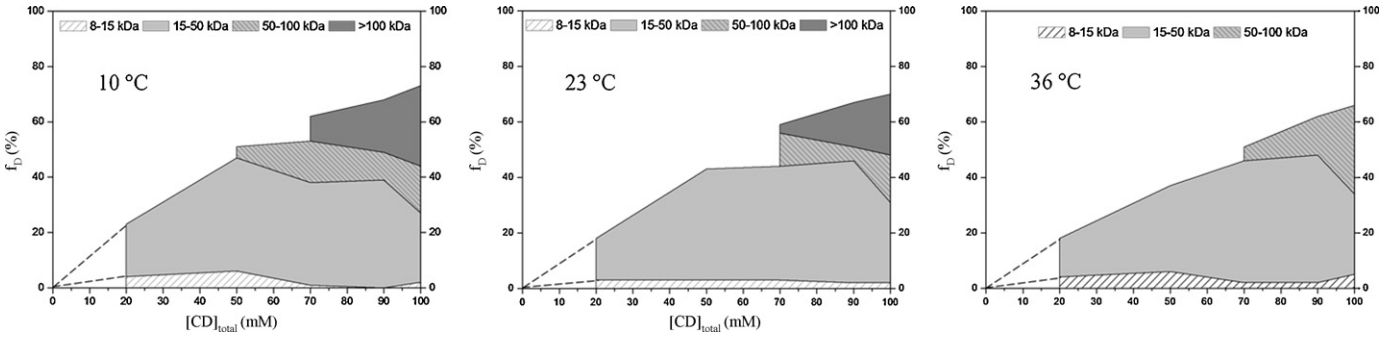

Fig. 2. Size population profiles of HC/HPβCD complex aggregates for different temperatures.

CE values did increase (Table 1). Intrinsic solubility values of HC in pure water and ethanol/water mixture are in agreement with those available from literature (Rytting et al., 2005; Ali et al., 2010) and the solubility values for HC in the aqueous polymer solutions also agree with previously published values (Loftsson and Sigurðardottir, 1994; Loftsson et al., 1994, 1996). A synergistic solubilizing effect of the polymers and the HC/HPβCD complex was observed and the CE values in pure water and aqueous polymer solutions are comparable with previously published values (Loftsson et al., 1994).

3.2. Temperature effects

In addition to room temperature (23 °C) the permeability studies were carried out at 13 K above (36 °C) and below (10 °C) this ambient value. Results show that the flux values increase with increasing temperature, as expected from the temperature dependency of the diffusion coefficient (i.e., increasing with increasing temperature) and the media viscosity (i.e., decreasing with increasing temperature). Furthermore, all permeability profiles demonstrated the expected I_L, II_L and III_L sections for a drug having A_L-type phase-solubility behavior. Negative deviation from Fick's first law was observed at all temperatures and for all membranes tested except in the case of the 100 kDa membrane at 36 °C (Fig. 1). According to the interpretation system previously published for size estimation of complex aggregates (Messner et al., 2011b), HC/HPβCD complex aggregates larger than 100 kDa were observed at 10 and 23 °C, while only smaller aggregates with molecular weight less than 100 kDa were observed at 36 °C. The total fraction of aggregated HC/HPβCD complexes (Fig. 2) decreases with increasing temperature from 72% at 10 °C to 66% at 36 °C. The size population profiles (Fig. 2) show a diminished fraction of drug participating in aggregation with increasing temperature. This leads to the conclusion that the ability of the complexes to form aggregates decreases with increasing temperature. Observations like these are not uncommon in aggregated systems and have also been observed in solutions containing the natural CDs (González-Gaitano et al., 2002). Assuming week non-covalent forces are involved in the aggregate formation, such as hydrogen bonding, van der Waals forces, electrostatic interactions, dipole forces and hydrophobic interactions, the stability is expected to be depressed with increasing temperature and their formation kinetics is enhanced resulting in smaller complex aggregates at higher temperatures.

Table 1
Intrinsic solubility of HC and its CE with HPβCD in pure water and additional of 0.5% (w/v) water soluble polymers.

Solvent	$S_0 \pm$ SD (mM)	CE
Water	0.84 ± 0.16	1.26
HDMB	1.02 ± 0.07	3.26
HPMC	1.04 ± 0.13	1.37
CMC	1.27 ± 0.13	2.90

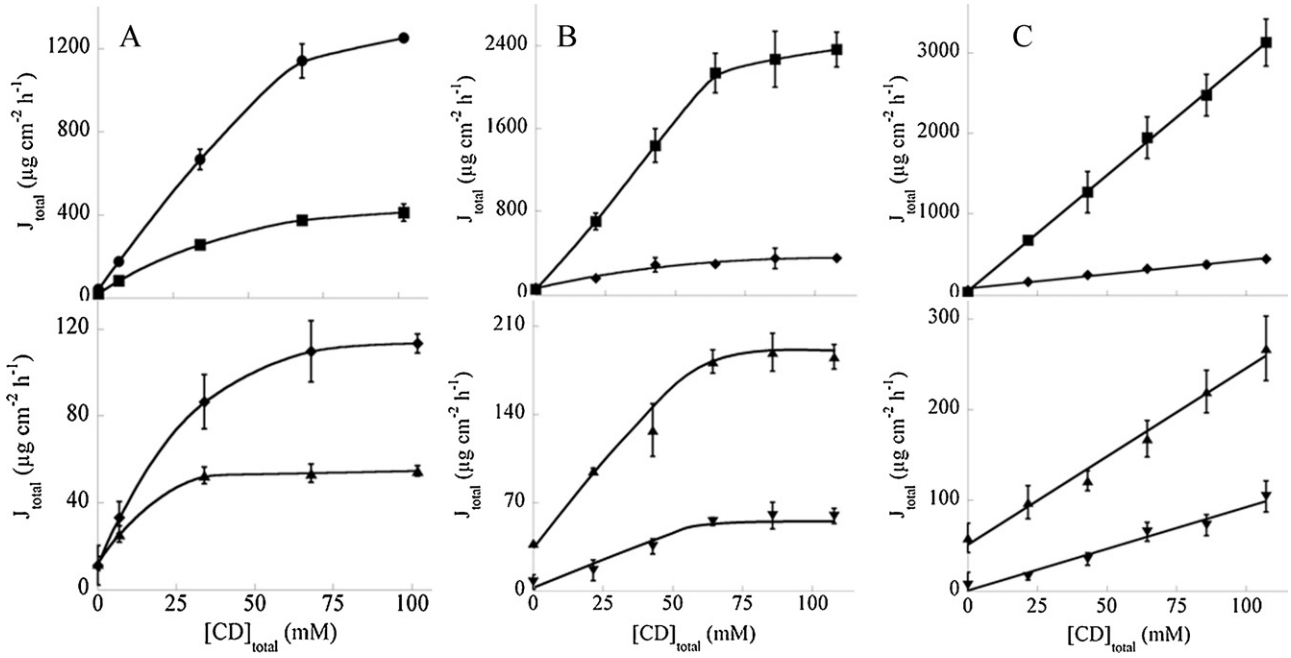

Fig. 3. Permeability profiles of HC in aqueous HPβCD solutions through semi permeable cellophane membranes with MWCO 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■) in Franz diffusion cells (A) and in side-by-side cells without (B) and under constant stirring of the donor phase (C). Error bars represent standard deviation.

3.3. Mechanical effects

In all previously reported experiments, the donor phase was unstirred during the permeability studies (Messner et al., 2011a,b). A different diffusion cell system was used to study if and how stirring of the donor phase changes permeability of HC and HC/HPβCD complexes through the membranes. The system consisted of a side-by-side cell apparatus providing donor and receptor chambers, where a magnetic stirring bar can be placed in one or both chambers. The geometry of this system is different from the conventional Franz cell system previously used complicating somewhat comparison of results obtained in the two different experimental set-ups. Therefore, Fig. 3 displays the permeability profiles of HC in aqueous HPβCD solutions in the conventional Franz cells (A) and in the side-by-side cell system with unstirred (B) and stirred (C) donor phase. It can be seen that profiles in Fig. 3A and B display negative deviation from linearity for all the membranes tested (MWCO from 8 to 100 kDa), but the values of the flux in the side-by-side cell with unstirred donor phase are about twice as high as those obtained in the Franz cells. Thus, although the flux values are not identical the shapes of the profiles are not affected. The higher flux values obtained in the side-by-side system can be related to a larger volume of the donor phase (3 ml vs 2 ml), smaller surface area (1.13 cm^2 vs 1.77 cm^2) and smaller size of the receptor phase (3 ml vs 12 ml) of the system compared to the conventional Franz cells. However, if the donor phase is stirred the permeability profiles (Fig. 3C) through all membranes tested become linear. This indicates that the size of complex aggregates dramatically attenuates upon stirring from more than 100 kDa to less than 8 kDa, which again indicates that the forces involved in aggregate formation must be reasonably week. Similar effects have been observed for the self-assembly of unsubstituted β-cyclodextrin (βCD) and γ-cyclodextrin (γCD) molecules in diluted aqueous solutions (González-Gaitano et al., 2002; Szente et al., 1998), where aggregates of βCD and γCD re-assembly after filtration. Other micellar-type systems show similar behavior (Cantù et al., 1997). Agitation of the system by stirring or filtration increases the movement of the particles and the bonds responsible for complex aggregation are probably too weak to resist and fall apart. However, previous studies have shown that drug/CD complex aggregates are stable for at least several months upon storage at room temperature as detected by transmission electron microscope and dynamic light scattering (Jansook et al., 2010; Messner et al., 2010). Thus, although the formation of drug/CD complex aggregates are well documented the forces involved in the aggregate formation need to be investigated further.

3.4. Additive effects

Alcoholic co-solvents such as ethanol reduce the polarity and dielectric constant of the complexation media (Pitha and Hoshino, 1992; Pitha et al., 1992; Li et al., 1999; Mrozek et al., 2002; Loftsson and Brewster, 2008) and, thus, can increase the observed intrinsic solubility of the lipophilic drug HC. In solutions containing binary (i.e., drug/cyclodextrin) complexes, addition of alcoholic co-solvents can either increase or decrease the CE. It is known that alcohols are able to form complexes with CDs and their ability to form CD complexes increases with increasing chain length (Matsui and Mochida, 1979; Munoz De La Pena et al., 1991; Van Stam et al., 1996; Mrozek et al., 2002; García-Río et al., 2006). Destabilization of the binary complexes has also been described, the reason being that the solute (i.e., the lipophilic drug) is more likely to remain outside the CD cavity due to reduced solvent polarity with increasing alcohol concentration. It was even suggested that the concentration of binary steroid/HPβCD complexes become negligible at ethanol concentration above 50% (v/v) (Li et al., 1999). This will result in a decreased in the CE upon addition of ethanol, but counter forces were also found resulting in CE increase. Formation of ternary complexes (i.e., steroid/ethanol/HPβCD) has been suggested (Pitha and Hoshino, 1992; Li et al., 1999) and ethanol is believed to act as a space regulating molecule to provide a better fit of the guest to the host cavity (Munoz De La Pena et al., 1991; Zung et al., 1991). Thus, ethanol either reduces or improves the affinity of HC for the CD cavity complicating predictions of the effect on the CE. CE increase and decrease are described in literature for particular guest/CD complexes with alcoholic co-solvents, which can sometimes be concentration dependent (Pitha and Hoshino, 1992; Mrozek et al., 2002; Loftsson et al., 2005b; Hegge et al., 2009). Fig. 4

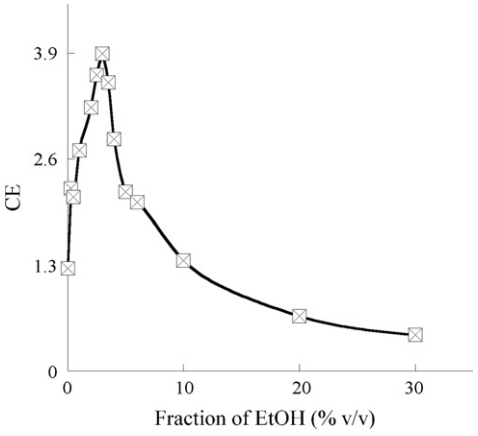

Fig. 4. CE of HC/HPβCD as a function of ethanol concentration in the complexation media.

shows that CE increases significantly upon addition of ethanol up to 3% (v/v) to the aqueous complexation medium. Further addition of ethanol yields in a CE decrease that can, at high ethanol concentrations, be lower than when no ethanol is present. The permeability of HC was determined from complexation media containing 3%, 5% and 30% (v/v) ethanol, that is, from media with maximum, medium and minimum CE. All media gave linear permeation profiles for the MWCO 100 kDa membrane (Fig. 5). The permeability profiles for the 50 kDa membrane show a slight negative deviation from linearity at 3 and 5% (v/v) ethanol and a linear profile that follows Fick's first law at an ethanol concentration of 30% (v/v). All other permeability profiles for the lower MWCO membranes show strong negative deviation from linearity and a leveling off of flux at higher CD concentrations. Furthermore flux values decrease with increasing ethanol concentration (Figs. 3A and 5). By contrast, the opposite is observed for the intrinsic flux (i.e., the flux from media containing no HPβCD) that increases with increasing ethanol concentration. This is due to an increase in the intrinsic solubility of HC in higher ethanol fractions. The results indicate the addition of ethanol to an aqueous solution containing HC/HPβCD complexes decreases the ability of the complexes to form aggregates. The maximum aggregate size decreased from >100 kDa in pure water to between 50 and 100 kDa upon addition of 3 and 5% (v/v) ethanol and to 15–50 kDa upon addition of 30% (v/v) ethanol. Apparently, ethanol both weakens the inter-complex forces involved in the aggregate formation and reduces the overall concentration of drug/CD complexes, both of which will result in decreased aggregation. The observed solubility enhancement at 3% (v/v) ethanol concentration could be due to formation of ternary HC/ethanol/HPβCD complexes and the observed decrease at higher ethanol concentrations could be due to enhanced solubilization of the drug and increased competition of the ethanol molecules for a space in the CD cavity (Pitha and Hoshino, 1992; Li et al., 1999). Based on this property, ethanol can reduce the ability of the HC/HPβCD complexes to form aggregates and further addition of the co-solvent results in further decrease

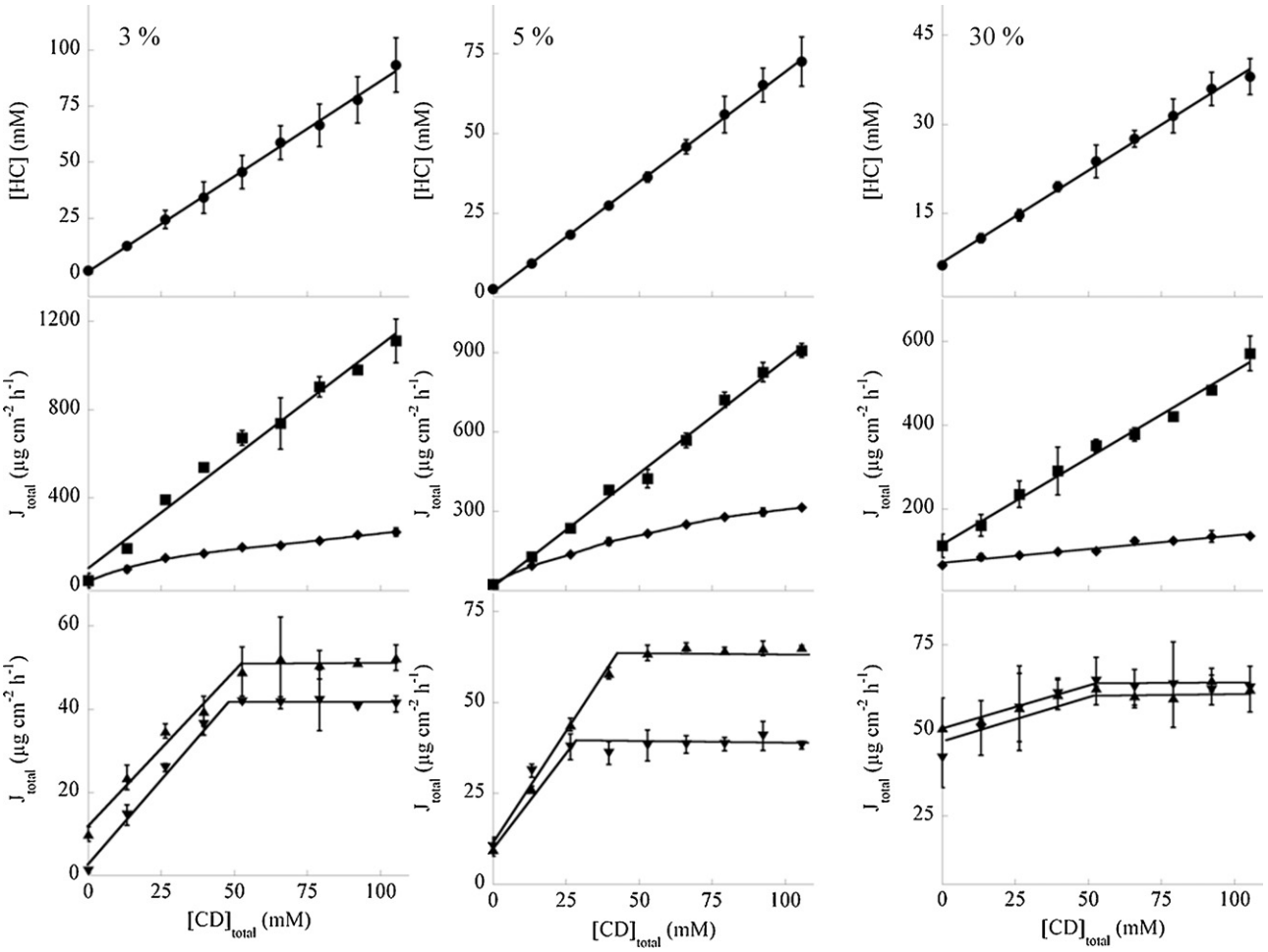

Fig. 5. The effect of ethanol on the phase-solubility profiles of HC in aqueous HPβCD solutions (top, ●) and permeation profiles (middle, bottom) of HC from aqueous HPβCD solutions through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Error bars represent standard deviation.

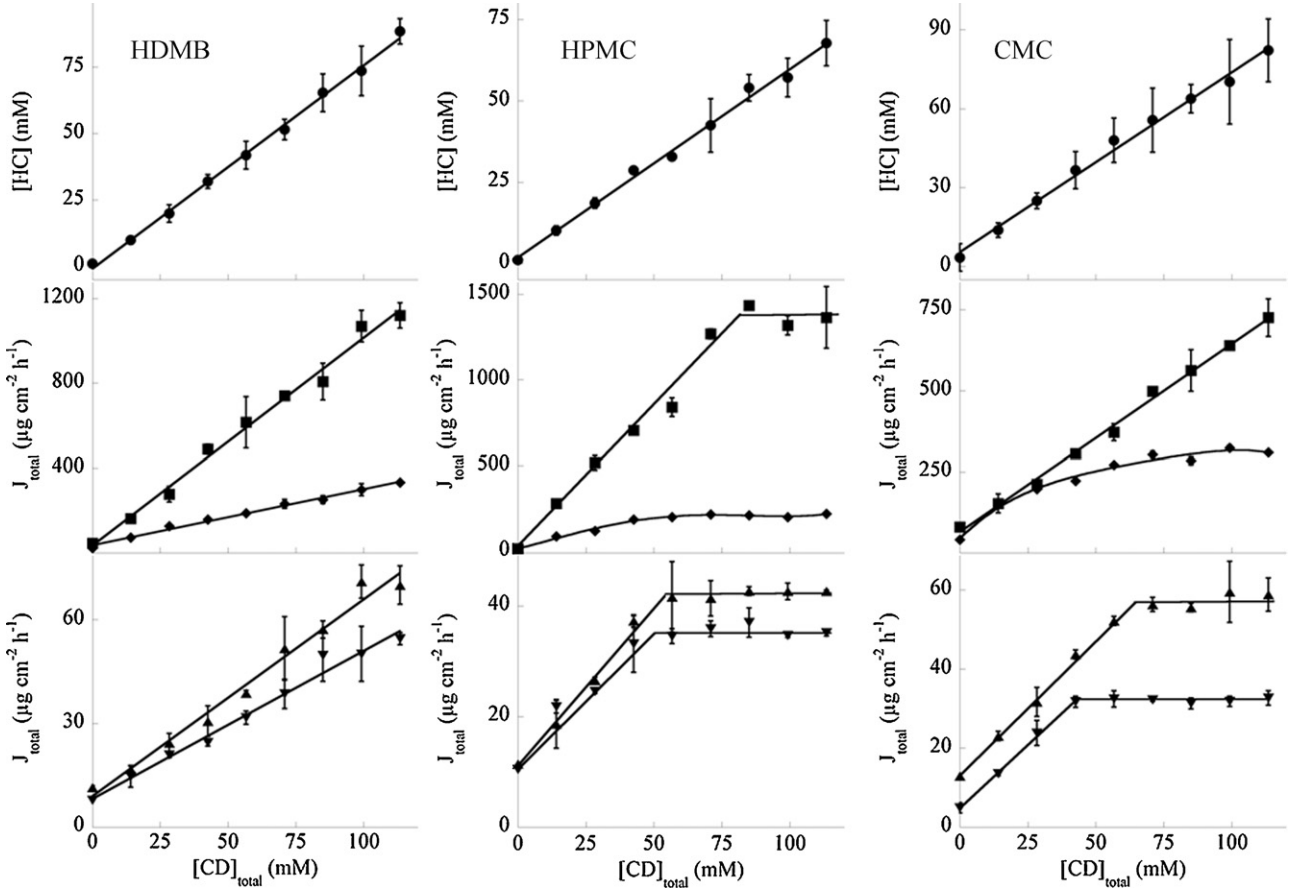

Fig. 6. Phase-solubility profiles (top, ●) and permeation profiles (middle, bottom) of HC in aqueous HPβCD solutions, containing 0.5% (w/v) of a water soluble polymer, through semi-permeable membranes with MWCO of 8 (▼), 15 (▲), 50 (♦) and 100 kDa (■). Error bars represent standard deviation.

in availability of HC/HPβCD complexes. It should be noted that the viscosity increases with the addition of up to 30% (v/v) ethanol to the solution (Songa and Peng, 2008) which affects the permeability coefficient. Therefore the permeability coefficients of the different ethanol/water mixtures of the same membrane are not comparable. However, this will not affect the shape of the permeation profile form complexation media with identical ethanol/water ratio and, thus, is not responsible the observed negative deviation in permeability profiles.

Three different water soluble polymers – one neutral (HPMC), one positively charged (HDMB) and one negatively charged (CMC) – were added to the aqueous HPβCD solutions saturated with HC. Fig. 6 shows the HC permeability profiles from these solutions. The positively charged polymer, HDMB, showed I_L type profiles for all membranes tested. The negatively charged polymer, CMC, showed an I_L type profile for the 100 kDa membrane and negative deviation from linearity for the lower MWCO membranes where as the neutral polymer shows negative deviation from linearity for all membranes tested. Results indicate that HDMB decreased the size of the complex aggregates from >100 kDa to <8 kDa, CMC also decrease their aggregate size to between 50 and 100 kDa, whereas HPMC seem to have little or no effect. Having said that, the appearance of the section III_L profile in the permeability profiles of HPMC indicates that the polymer decreases the extent of aggregation. Thus, all polymers decrease the ability of the HC/HPβCD complex to self-assemble to form aggregates although to a different extent. It might be possible that aggregates present in the solution are bound to the polymer chain in a micellar fashion as has been suggested in the case of water-soluble polymers. This being the case, the accumulation of weak forces may play a role in polymer-aggregate interaction and may severely change morphology of the system (Brackman and Engberts, 1994), which can lead into a inhibition of complex aggregates towards further assembly or to collapse existing aggregates. However, addition of water soluble polymers to the solution leads to much more complex interactions and cannot be unambiguously explained for these three different polymers without further experimental assessments.

Although the amount of dissolved drug is much higher with addition of polymers, the flux is decreased compared to solutions with no additives (Table 1). Reduced flux can be explained by possible interaction of polymer chains with the membrane surface or blockage of pores and consequent higher permeation resistance.

4. Conclusion

The presence of additives such as ethanol, HDMB, CMC or HPMC notably reduces the size of HC/HPβCD complexes aggregates. Whereas ethanol reduces the ability of complexes to self-assemble; the water soluble polymers probably diminish the size of the aggregates. Similar tendencies as those related to ethanol addition can be observed by increasing the system temperature or by stirring the donor phase. All of these suggest that forces involved in complex aggregation are relatively weak and can be easily disturbed. However, the mechanistic basis of the complex aggregation phenomenon remains not completely understood. Based on the findings of this study, hydrogen bonding could be an important interaction type associated with aggregate kinetics and thermodynamics. However, this does not exclude other types of week

non-covalent forces such as van der Waals forces, electrostatic interactions, dipole forces, charge transfer, release of conformational strain and hydrophobic interactions.

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