International Journal of Pharmaceutics 407 (2011) 174–183

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Self-assembly of cyclodextrin complexes: Aggregation of hydrocortisone/cyclodextrin complexes

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ARTICLE INFO

Article history:
Received 6 October 2010
Accepted 10 January 2011
Available online 13 January 2011

Keywords:
Cyclodextrins
Permeation
Complex aggregates
Nanoparticles
Hydrocortisone

ABSTRACT

Cyclodextrins (CDs) are well known functional excipients for solubilization and stabilization of drugs in aqueous formulations as well as enabling adjuncts for increasing the oral bioavailability of solid dosage forms. More recently a number of the valuable properties of these CDs have been ascribed to nanoparticulate aggregation in addition to its ability to form molecular inclusion complexes. The purpose of this study is to identify and characterize the aggregation of CD inclusion complexes with a model drug, hydrocortisone, in saturated solutions which are more relevant to drug formulation than highly dilute systems. Penetration studies of complexes through membranes and phase solubility relationships were assessed for saturated hydrocortisone solutions with the parent CDs, namely αCD, βCD, γCD or with various water-soluble derivatives, i.e., 2-hydroxypropyl-βCD (HPβCD), 2-hydroxypropyl-γCD (HPγCD) or sulfobutyl ether-β-CD (SBEβCD). The data indicate that βCD and γCD form micro-aggregates with hydrocortisone resulting in non-linear phase-solubility relationships. By contract, the other studies of CDs or CD derivatives were found to form nanoaggregates with hydrocortisone resulting in linear solubilization relationships. Permeability profiles were evaluated for the systems formed and are described in three sections specifically a section (section I) where flux is linear (Fickian) as a function of CD concentration, a section (section II) where flux deviates in a negative fashion from linearity but still increases as the CD concentration increases and a section (section III) where flux is independent of the cyclodextrin concentration. Diminished values of flux can be interpreted based on the formation of nanoaggregates of hydrocortisone/CD complexes. Extrapolation of section I data made it possible to obtain theoretical flux values which could be used to estimate the fraction of complexes and drug which participate in aggregation. The CDs which appeared to demonstrate the lowest tendency to form complex aggregates were αCD and SBEβCD, due to their low complexation efficacy and repulsive forces, respectively. Complex aggregates with these CDs are also smaller with maximum size between 50 and 100 kDa. HPβCD and HPγCD complex aggregates manifested a maximum size above 100 kDa and the fraction of drug which participates in complex aggregation with these species is higher than for the other materials assessed. In the case of 90 mM HPγCD solution, data suggest that 87% of all hydrocortisone is tied up in the form of aggregates. These high concentrations were confirmed by TEM which found most particles in the 3–5 nm range but rarely particles as large as 10 and 20 nm. Speculation on the mechanism of the aggregation processes and equilibrium constants are provided but these tend to punctuate our limited understanding of these potentially important processes.

1. Introduction

Cyclodextrins (CDs) are excipients that are commonly used during the screening and formulation for new drug candidates as well as the reformulation of mature products to improve their functionality (Frömming and Szejtli, 1994; Loftsson and Duchêne, 2007; Brewster and Loftsson, 2007). CDs are cyclic oligosaccharides, with a hydrophilic outer surface and a somewhat lipophilic central cavity. In aqueous solutions CDs (i.e., the hosts) are able to form water-soluble inclusion complexes with many lipophilic, poorly water-soluble compounds (i.e., the guests) by taking up the lipophilic molecule or more frequently some lipophilic moiety of the molecule, into the central cavity of the CD molecule. In most cases, an apparent 1:1 guest/host complex is formed although higher order complexes are not uncommon (Duchêne, 1991; Duchêne and Wouessidjewe, 1996; Loftsson and Brewster, 1996; Saenger et al., 1998; D'Souza and Lipkowitz, 1998; Brewster and Loftsson, 2007). CDs and CD complexes have been studied

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doi:10.1016/j.ijpharm.2011.01.011

intensively and these studies have generated a wealth of information on the structural requirements for complex formation and the driving forces associated with complex formation (Bodor and Buchwald, 2002; Liu and Guo, 2002; Katritzky et al., 2004; Dodziuk, 2006; Douhal, 2006). However, most of these studies have been performed in dilute aqueous solutions under close to ideal conditions. These situations may poorly mimic those found in the relevant formulation processes and may add little value in understanding formulation preparation or manufacture. In addition to inclusion complexes, lipophilic drug molecules, CDs and CD complexes are known to form aggregates in aqueous solutions (Loftsson et al., 2005; Bonini et al., 2006; He et al., 2008; Messner et al., 2010; Jansook et al., 2010a). The diameter of the aggregates is frequently about 100 nm and as such not detected by the naked eye. Formation of such aggregates can have important effect on both physicochemical and biological properties of the complexes and thus has to be considered during investigation of CD containing solutions (Messner et al., 2010). The purpose of the present study was to investigate formation of drug/CD (D/CD) complex aggregates and the influence of the CD structure and concentration on the aggregate formation. Hydrocortisone was selected as a model drug and the CDs tested were the natural αCD, βCD and γCD as well as the CD derivatives, 2-hydroxypropyl-βCD (HPβCD), sulfobutyl ether βCD (SBEβCD) and 2-hydroxypropyl γCD (HPγCD). Formation of aggregates was detected by studying hydrocortisone permeation from aqueous CD solutions through semi-permeable cellophane membranes.

Semi-permeable cellophane membranes have previously been used to study formation of D/CD complexes and complex aggregates as well as competitive CD binding and the results obtained have been verified by different techniques (Ono et al., 1999, 2002; Loftsson et al., 2004; Jansook et al., 2010a; Kurkov et al., 2010). This method is based on determination of permeation of the free drug molecules (MW usually <1000 Da), free CD molecules (MW between about 1000 and 2200 Da), individual D/CD complexes (MW between about 1400 and 3000 Da) and D/CD complex aggregates (MW > 3000 Da) through semi-permeable membranes of different molecular weight cut-offs (MWCOs).

2. Materials and methods

2.1. Materials

Hydrocortisone was purchased from Fagron (Nieuwerkerk aan den IJssel, Netherlands). α-Cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and 2-hydroxypropyl-γ-cyclodextrin (HPγCD) MS 0.6 (MW 1576 Da) were obtained from Wacker Chemie (Burghausen, Germany) while 2-hydroxypropyl-β-cyclodextrin (HPβCD) with molar substitution of 0.64 (MW 1400) was purchased from Roquette (Lestrem, France) and sulfobutyl ether β-cyclodextrin sodium salt (SBEβCD) with molar substitution of 0.9 (MW 2163) was kindly donated by CyDex Pharmaceuticals (Lenexa, KS). Milli-Q water (Millipore, Billerica, MA) was used for preparation of all solutions.

2.2. Solubility determinations

Solubility of hydrocortisone in water or aqueous CD solutions was determined by the heating method (Loftsson and Hreinsdóttir, 2006). Excess amount of hydrocortisone was added to an aqueous solution containing 0–15% (w/v) CD and the suspension was heated in an autoclave (Astell MXN 472, UK) at 121 °C for 20 min in sealed glass vials and then allowed to cool to room temperature. Small amounts of solid drug were then added to the suspension and the suspensions were allowed to equilibrate in the resealed vials at room temperature (23 ± 1 °C) for 7 days in the dark under constant agitation (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 diluted with mobile phase and analyzed by HPLC.

Phase-solubility profiles were constructed according to the method by Higuchi and Connors (1965). The apparent stability constant ($K_{1:1}$) and the complexation efficiency (CE) were determined from the slope of the linear phase-solubility diagrams (plots of the total drug solubility ($[D]_t$) versus total CD concentration ($[CD]_{total}$) in moles per liter) (Loftsson et al., 2005):

$$K_{1:1} = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

$$\text{CE} = \frac{\text{slope}}{1 - \text{slope}} = \frac{[\text{D/CD complex}]}{[\text{CD}]} = K_{1:1} \cdot S_0 \quad (2)$$

where S_0 is the intrinsic solubility of the drug.

2.3. Permeation studies

The permeability studies of hydrocortisone from aqueous CD solutions (the donor phase) were carried out in unjacketed Franz diffusion cells with a diffusion area of 2.27 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 hydrocortisone. The hydrocortisone 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 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 1 kDa, 8 kDa, 15 kDa, 50 kDa or 100 kDa (Spectrum Europe, Breda, Netherlands) that had been treated with the receptor phase solution over night. The study was carried out at room temperature under continuous stirring of the receptor phase (by 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 30, 60, 120, 180, 240, and 300 min and replaced immediately with an equal volume of fresh receptor phase. The hydrocortisone concentration in the receptor sample was determined by HPLC. The steady state flux (J) was calculated as the slope (dq/dt) of linear section of the amount of hydrocortisone in the receptor chamber (q) versus time (t) profiles, and the apparent permeability coefficient (P_{app}) was calculated from the flux according to Eq. (3) (i.e., Fick's first law):

$$J = \frac{dq}{A \cdot dt} = P_{app} \cdot C_d \quad (3)$$

where A is the surface area of the mounted membrane and C_d is the initial hydrocortisone concentration in the donor phase.

2.4. Quantitative determination of hydrocortisone

Quantitative determination of hydrocortisone 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.

2.5. Transmission electron microscopy (TEM) analysis

The morphology and size of the aggregates in hydrocortisone saturated aqueous 10% (w/v) HPγCD solutions were analyzed using a transmission electron microscope. The aggregates were visualized by TEM using the uranyl staining method (Bugler et al., 1999; Jansook et al., 2010a). Initially, formvar-coated grids were floated on a droplet of the saturated preparation on parafilm, to permit the adsorption of the nanoparticles onto the grid. After blotting the grid with filter paper, the grid was transferred onto a drop of the negative stain. Following this, the grid was blotted with a filter paper and air dried. Aqueous uranyl acetate solution (2%) was used as a negative stain in these experiments. The process of preparation was done under constant vacuum. Finally, the samples were examined in a Model JEM-2100 transmission electron microscope (JEOL, Japan).

3. Results and discussions

Currently applied methods for drug solubility determinations do not distinguish between true solutions, where individual drug molecules are fully dispersed in the aqueous media, and solutions of aggregated drug molecules, that are water-soluble dimers, trimers and small drug oligomers (Loftsson, 2010). Likewise determination of CD solubilization of poorly soluble drugs in aqueous solutions by the phase-solubility method does not distinguish between individual D/CD complexes and water-soluble complex aggregates. A linear phase-solubility profile (A_L-type) only indicates that there is a linear relationship between drug solubility and the CD concentration and that the D/CD complex formed is soluble in the aqueous complexation medium. B_S-type phase-solubility profile indicates that the D/CD complex has limited solubility in the aqueous complexation medium (Higuchi and Connors, 1965). Thus, the first step in this present investigation was method development for detection of complex aggregates in aqueous solutions and estimation of the fraction and size of D/CD complex aggregates.

3.1. The permeation method

The phase-solubility of hydrocortisone in aqueous HPβCD solutions is of A_L-type indicating that the hydrocortisone/HPβCD complex is soluble in the aqueous complexation media over the concentration range investigated and that the total concentration of dissolved hydrocortisone ($[D]_{total}$) is the sum of the concentration of dissolved hydrocortisone molecules ($[D]_0$) and the concentration of the CD inclusion complex ([D/CD]). If the individual drug molecules are fully dispersed and do not form dimers, trimers and water-soluble drug oligomers then $[D]_0$ will be constant and equal to the intrinsic solubility (S_0) in hydrocortisone saturated CD solutions:

$$[D]_{total} = [D]_0 + [D/CD] \quad (4)$$

assuming that one hydrocortisone molecule forms a complex with one HPβCD molecule. Likewise, the total hydrocortisone flux (J_{total}) from hydrocortisone saturated CD solutions through a semipermeable membrane with MWCO greater than the MW of the D/CD complex is the sum of the flux of free dissolved hydrocortisone (J_D) and the flux of the complex ($J_{D/CD}$):

$$J_{total} = J_D + J_{D/CD} \quad (5)$$

according to Eqs. (3) and (5), a linear profile should be obtained when J_{total} is plotted against the CD concentration where the *Y*-intercept is equal to J_D. The flux $J_{D/CD}$ is depended on the total CD concentration and can be calculated by the slope times $[CD]_{total}$. However, many systems are not linear. Fig. 1 shows the J_{total} versus

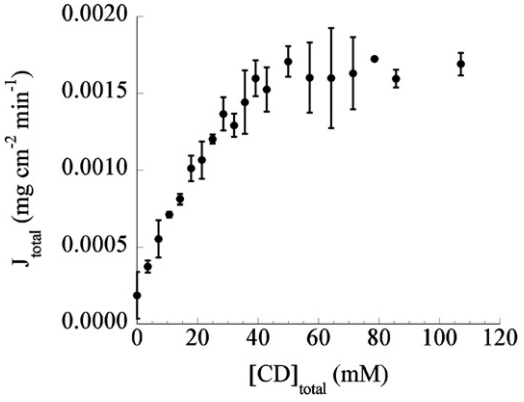

Fig. 1. Permeability profile of hydrocortisone/HPβCD complexes through semi permeable cellophane membrane MWCO 15 kDa.

HPβCD concentration profile for hydrocortisone through a semipermeable membrane with MWCO of 15 kDa. The profile starts to deviate from linearity at about 10 mM HPβCD (i.e., 1.4% w/v) and does not show any increase in J_{total} at HPβCD concentrations beyond 50 mM (i.e., 7% w/v). It has been suggested that such negative deviation from linearity is due to formation of D/CD complex aggregates (Loftsson et al., 2004; Jansook et al., 2010a,b; Messner et al., 2010). The MW of hydrocortisone is 362 Da, that of HPβCD is 1400 Da and that of the 1:1 complex is 1762 Da or 1.76 kDa. Thus, aggregates of up to eight anhydrous D/CD complexes (MW 14 kDa) should be able to permeate the MWCO 15 kDa membrane. In the hydrocortisone saturated HPβCD solutions, the concentration of free drug molecules is constant and equal to the drug intrinsic solubility at all HPβCD concentrations. Eq. (3) states that the flux (here J_D) will be constant if the drug concentration in the donor phase (C_d) is constant and indeed the flux of the free hydrocortisone through the membrane (J_D) was determined to be constant and equal to $0.18 \pm 0.15 \times 10^{-3}$ mg cm^{-2} min^{-1} throughout the J_{total} versus $[CD]_{total}$ permeation profile shown in Fig. 1. The profile can be divided into three sections, i.e., section I–III (Fig. 2 and Table 1). In section I (i.e., $[CD]_{total} < 10$ mM) the drug is present as free drug molecules as well as D/CD complexes and perhaps complex aggregates with total MW of less than 15 kDa. Since all the drug species are able to permeate the membrane, a linear relationship between J_{total} and $[CD]_{total}$ is observed in section I and J_{total} is equal to the theoretical flux (J_{theo}) as predicted by Eq. (3). At 10 mM HPβCD complex aggregates with MW greater than 15 kDa start to form and the flux profile displays negative deviation from linearity (Fig. 2). In this section II (10 mM < $[CD]_{total}$ < 50 mM), the concen-

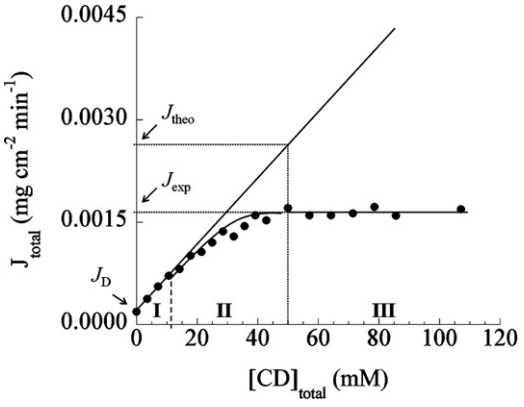

Fig. 2. Description of permeability profile from Fig. 1. J_{exp}: the experimentally determined flux; J_{theo}: the theoretical flux; J_D: the flux of the free drug.

Table 1
Drug permeation from drug saturated aqueous CD solutions through semi-permeable membranes.

Section	MW of the largest aggregates	J_{exp}	Flux	f_A
I	<MWCO	$\uparrow[CD]_{total} \Rightarrow \uparrow J_{exp}$	$J_{exp} = J_{theo}$	0
II	>MWCO	$\uparrow[CD]_{total} \Rightarrow \uparrow J_{exp}$	$J_{exp} < J_{theo}$	>0
III	>MWCO	J_{exp} = constant	$J_{exp} < J_{theo}$	$\gg 0$

MW: molecular weight; MWCO: molecular weight cut off; J_{exp}: experimentally determined drug flux through the membrane; J_{theo}: theoretical drug flux, i.e., J_{total} when the free drug molecules as well as all complex aggregates being formed are able to permeate the membrane; f_A: fraction of drug molecules that is present in aggregates with MW greater than the MWCO of the membrane.
$[D]_0$ is constant and equal to the intrinsic solubility (S_0) of the drug assuming that no aggregates of free drug molecules are being formed. The flux of the free drug (J_D) is constant throughout sections I–III. It is assumed that only 1:1 D/CD complexes are being formed and that these then form complex aggregates.
$[D]_{total} = [D]_0 + [D/CD] + 2\cdot[D/CD]_2 + 3\cdot[D/CD]_3 + \cdots + n\cdot[D/CD]_n$.
$[CD]_{total} = [CD]_{free} + [D/CD] + 2\cdot[D/CD]_2 + 3\cdot[D/CD]_3 + \cdots + n\cdot[D/CD]_n$.
$J_{total} = J_D + J_{D/CD} + 2\cdot J_{(C/CD)_2} + 3\cdot J_{(D/CD)_3} + \ldots + n\cdot J_{(D/CD)_n} = J_{theo}$ or J_{exp}.

trations of D/CD complexes, as well as complex aggregates with total MW of less than 15 kDa, continue to increase with increasing HPβCD concentration but larger aggregates are also being formed resulting in negative deviation from the theoretical flux. When the HPβCD concentration increases beyond 50 mM (section III, $[CD]_{total} > 50$ mM) J_{total} becomes constant (mean ± standard deviation = $1.65 \pm 0.06 \times 10^{-3}$ mg cm^{-2} min^{-1}). If all the drug species are able to permeate the membrane then, according to Fick's first law (Eq. (3)), J_{total} should be equal to J_{theo} and constitute 2.70×10^{-3} mg cm^{-2} min^{-1} at $[CD]_{total} = 50$ mM. However the experimental value (J_{exp}) is only 1.65×10^{-3} mg cm^{-2} min^{-1} (Fig. 2). Thus, the fraction of hydrocortisone that is present in aggregates with MW greater than 15 kDa (f_A) and too large to permeate the membrane, is:

$$f_A \approx 1 - \frac{J_{exp}}{J_{theo}} \quad (6)$$

According to this equation the fraction of hydrocortisone that is present in hydrocortisone/HPβCD aggregates with diameter greater that 15 kDa is about 0.4 at 50 mM HPβCD but about 0.6 at 68 mM HPβCD. The characteristics of sections I–III are shown in Table 1.

3.2. The phase-solubility profiles

The phase-solubility profiles (Fig. 3) were classified according to Higuchi and Connors (1965). The water-soluble CD derivatives and αCD displayed A_L type profiles which conventionally suggest that one drug molecule forms a complex with one CD molecule (Al-Sou'od, 2008). The phase-solubility profiles of both βCD and γCD were of B_S type with initial linear increase in the solubility indicating that the hydrocortisone/βCD and hydrocortisone/γCD complexes and complex aggregates have limited solubility in water. The apparent stability constant ($K_{1:1}$) and the complexation efficiency (CE) were calculated according to Eqs. (1) and (2) (Table 2). For the three natural CDs both $K_{1:1}$ and CE increased with increasing cavity size while for the CD derivatives the substitution leads to either increased or decreased values. We believe the larger the cavity of the CD is, the deeper the hydrocortisone molecule will move into it and the higher is the CE. The derivatives have extended cavity as a result of their side chains, which provides some extra solubilization power (Mosher and Thompson, 2002) but steric hindrance may reduce its effect compared to the native CDs. Among the CD derivatives, SBEβCD has the highest stability constant which indicates either the longer side chain or the polar head group or both improve inclusion complexation of hydrocortisone.

Table 2
Apparent stability constant ($K_{1:1}$) and complexation efficiency (CE) values of hydrocortisone/CD complexes.

Cyclodextrin	Slope	Correlation coefficient	$K_{1:1}$ (M^{-1})	CE
αCD	0.062	0.993	72	0.07
βCD[a]	0.542	0.999	1360	1.18
γCD[a]	0.632	0.999	1970	1.72
HPβCD	0.558	0.998	1450	1.26
SBEβCD	0.580	0.999	1580	1.38
HPγCD	0.516	0.998	1270	1.07

[a] Calculated from the initial linear section of the phase-solubility graph.

3.3. The permeation profiles

The permeation profiles are shown in Fig. 3. The fluxes (J_D) of hydrocortisone from saturated aqueous hydrocortisone solution through the semi-permeable membranes are shown in Table 3. Although there is no statistical difference between the experimental values, a trend can be observed showing that J_D increases with increasing MWCOs of the membrane. Large pores are expected to give less permeation resistance than small pores. None of the hydrocortisone/CD complexes tested are able to permeate the semi-permeable membrane with MWCO of 1 kDa and therefore J_{total} through the 1 kDa membrane is constant and independent of the total drug concentration ($[D]_{total}$) or total CD concentration ($[CD]_{total}$) in the hydrocortisone saturated donor phase, i.e., $J_{D/CD} = 0$ and consequently $J_{total} = J_D$. The hydrocortisone/CD complexes (MW from 1.3 to 2.5 Da) are able to permeate the other membranes tested (MWCOs from 8 to 100 kDa) and thus J_{total} should increase with increasing amount of dissolved hydrocortisone, free or in a CD complex. For CDs displaying A_L type phase-solubility profiles a negative deviation from a linear J_{total} versus $[CD]_{total}$ profile indicates formation of water-soluble complex aggregates that are too large to permeate the membrane.

The phase-solubility profiles of hydrocortisone in aqueous βCD and γCD solutions are of B_S type which is reflected in their permeation profiles (Fig. 3B and C), i.e., the J_{total} increases while the hydrocortisone solubility increases but then levels off or even decreases when the complexation media becomes saturated with the D/CD complex. The phase-solubility and permeation profiles indicate that main difference between βCD and γCD on the one side (B_S type phase-solubility diagrams) and αCD, HPβCD, SBEβCD and HPγCD on the other side (A_L type phase-solubility diagrams) is the size of the aggregates. βCD and γCD form large visible complex aggregates that precipitate from the aqueous complexation media whereas the other CDs tested form nanosized aggregates that are smaller than 0.45 μm and sufficiently soluble to remain in liquid phase.

3.4. Formation of complex aggregates

βCD and γCD inclusion complexes are not discussed in this section due to their ability to form microparticles. Thus, it is difficult to estimate how many of the complex aggregates are eliminated due to filtration.

Table 3
The flux of hydrocortisone (J_D) from saturated aqueous solution through semi-permeable cellophane membranes.

The MWCO of the membrane (kDa)	J_D (mean ± SD) (μg cm^{-2} min^{-1})
1	0.4 ± 0.3
8	0.4 ± 0.3
15	0.4 ± 0.3
50	0.8 ± 0.4
100	1.1 ± 0.5

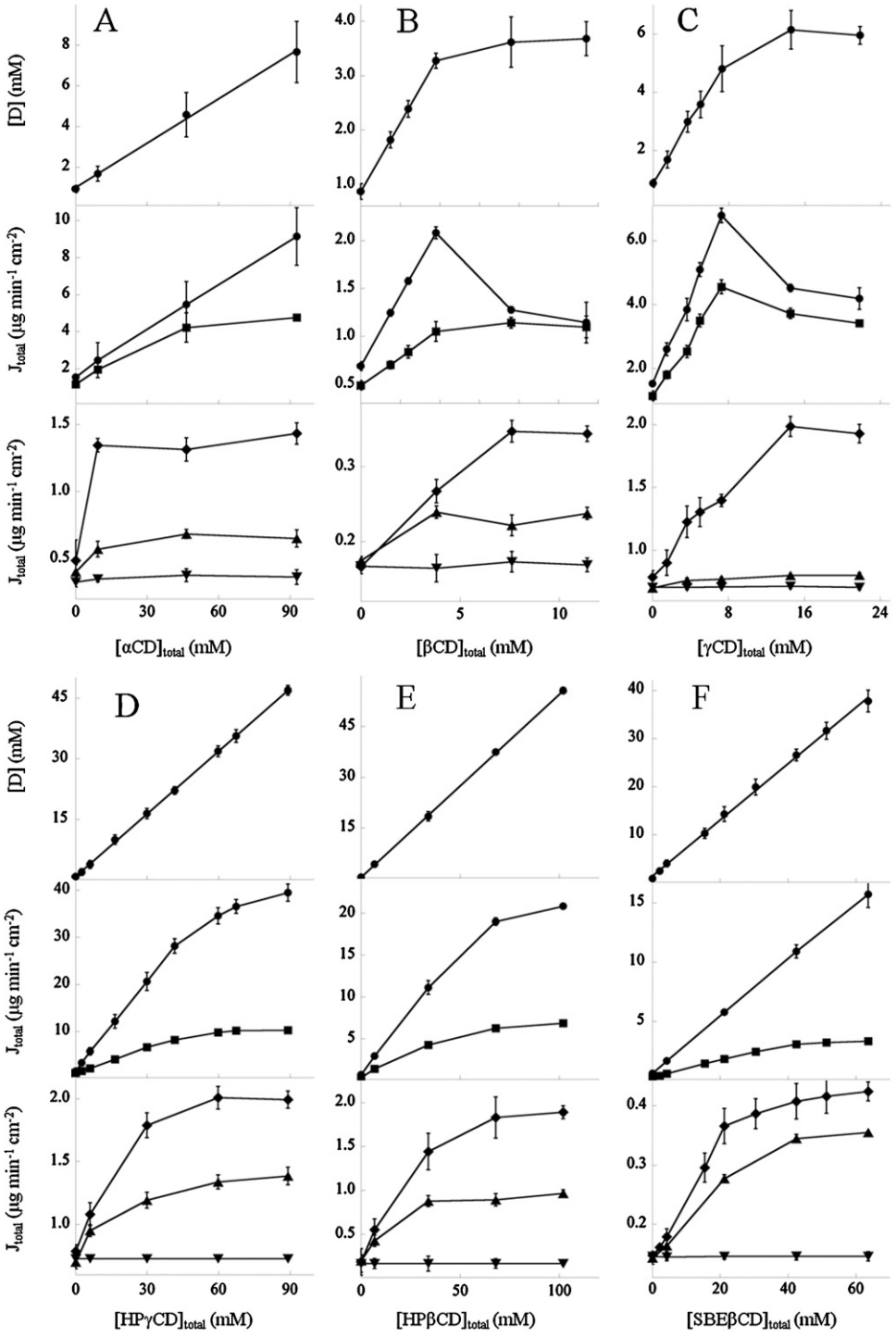

Fig. 3. Phase-solubility (top) and permeability profiles (middle, bottom) of hydrocortisone/CD (A: αCD, B: βCD, C: γCD, D: HPγCD, E: HPβCD, F: SBEβCD) complexes through semi-permeable cellophane membranes with MWCO 100 kDa (●), 50 kDa (■), 15 kDa (♦), 8 kDa (▼) and 1 kDa (▲) membrane.

Within this work it is assumed that the only molecules participating in aggregate formation are the 1:1 hydrocortisone/CD inclusion complexes and not single molecules of hydrocortisone or CD. Since the intrinsic solubility of hydrocortisone is negligibly small in comparison to the concentration of solubilized drug present in studied CD solutions, the fraction of hydrocortisone which takes part in aggregation process can be considered to be approximately equal to the fraction of aggregated 1:1 complexes. The fractions of aggregated complex (f_A) unable to permeate semi-permeable membrane of a given MWCO (Eq. (6)) are displayed in Table 4. It is worth mentioning that the data collected in this table are based on the permeation profiles which in turn are related to the aggregation process. That is, the limiting conditions of $f_A = 0$ and $f_A = 1$ denote that all complexes (not only those participating in aggregates) present in solution are able or unable to permeate the membrane, respectively. In order to analyze the aggregation process the fraction of drug participating in certain aggregate population (f_D) may be introduced, which can be defined as:

$$f_D = {f_A}^i - {f_A}^j \quad (7)$$

Table 4
The fraction of aggregated complexes (f_A) in saturated hydrocortisone/CD solutions that are unable to permeate semi-permeable membrane of given MWCO.

MWCO (kDa)	$[\alpha CD]_{total}$ (mM)					$[HP\gamma CD]_{total}$ (mM)				
	10	20	40	60	80	20	30	50	70	90
100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.12
50	0.00	0.00	0.00	0.08	0.23	0.00	0.00	0.03	0.14	0.43
15	0.00	0.08	0.35	0.45	0.52	0.44	0.52	0.55	0.63	0.71
8	0.02	0.16	0.45	0.50	0.60	0.46	0.62	0.75	0.84	0.87
	$[HP\beta CD]_{total}$ (mM)					$[SBE\beta CD]_{total}$ (mM)				
	20	50	70	90	100	20	30	40	50	60
100	0.00	0.00	0.03	0.16	0.22	0.00	0.00	0.00	0.00	0.00
50	0.00	0.00	0.15	0.21	0.39	0.00	0.00	0.02	0.10	0.22
15	0.15	0.40	0.56	0.65	0.68	0.18	0.29	0.36	0.41	0.52
8	0.18	0.43	0.59	0.67	0.70	0.23	0.35	0.48	0.56	0.59

where i and j are incremental MWCO values and $i < j$. Thus, f_A is the fraction of hydrocortisone participates in formation of aggregates larger than a given MWCO, whereas f_D is its faction for a certain size population, e.g. from 8 to 15 kDa. The results are collected in Table 5 and visualized in Fig. 4 by means of concentration profiles. The total fraction of drug participating in aggregation increases gradually with increasing concentration of each CD in the rank order αCD < SBEβCD < HPβCD < HPγCD and reaches maximum value of 87% (90 mM HPγCD in Table 5). The blank area in concentration diagrams corresponds to a mixture of variable compositions which contains free drug molecules, complexes and complex aggregates with MWCO < 8 kDa. This means that, for example, *at least* 87% of hydrocortisone in 90 mM (~14% w/v) HPγCD aqueous solution participates in aggregate formation at room temperature. In other words, hydrocortisone/HPγCD 1:1 complexes have an extremely high tendency to form aggregates. Even hydrocortisone/αCD com-

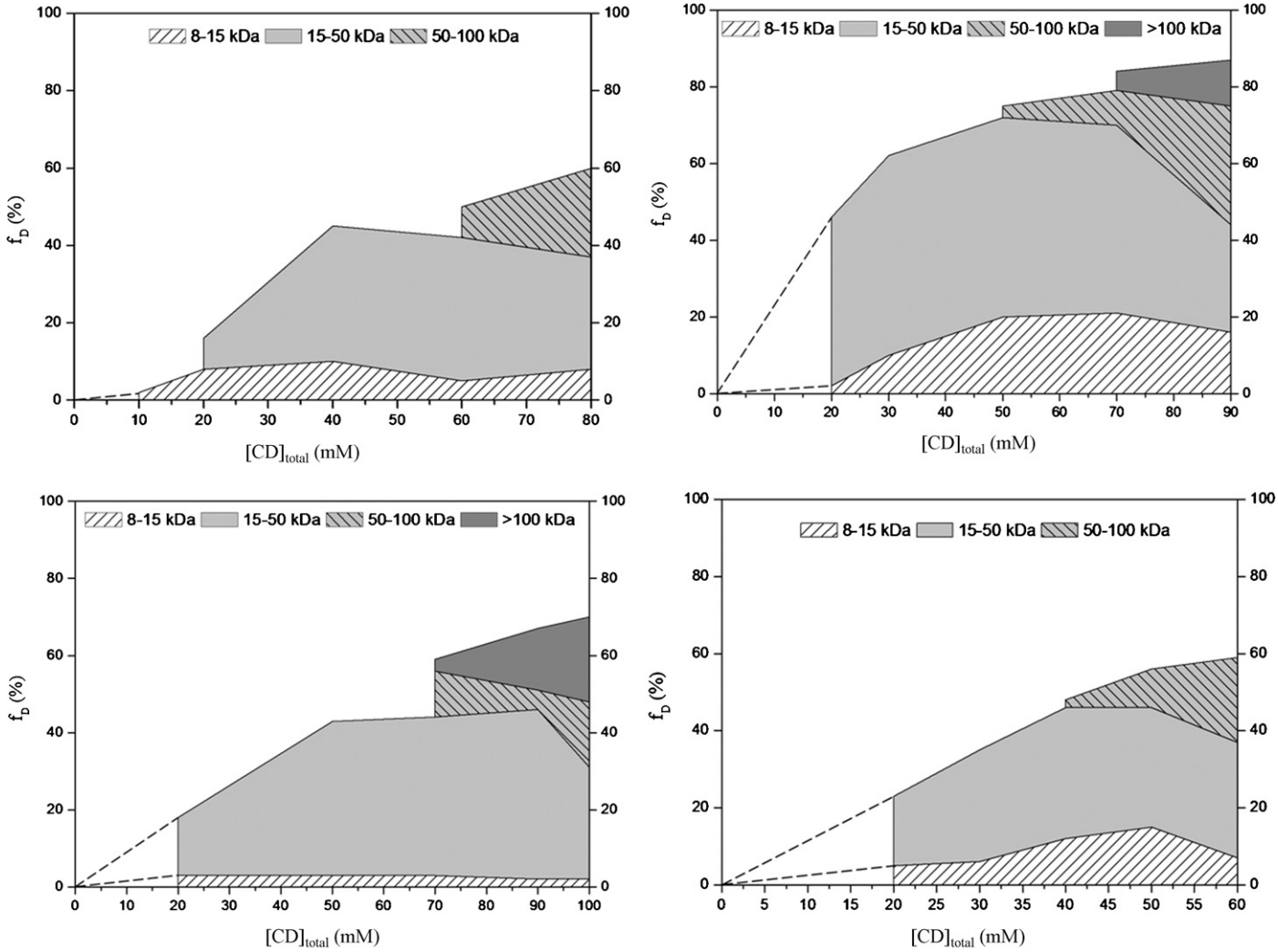

Fig. 4. Effect of CD concentration on the size distribution of D/CD aggregates (A: αCD, B: HPγCD, C: HPβCD, and D: SBEβCD). f_D is drug fraction in %.

Table 5
The distribution of aggregated complexes between size populations at different cyclodextrin concentrations of studied hydrocortisone/CD solutions.

$MW_{aggr.}$ (kDa)	$[\alpha CD]_{total}$ (mM)					$[HP\gamma CD]_{total}$ (mM)				
	10	20	40	60	80	20	30	50	70	90
8–15	2	8	10	5	8	2	10	20	21	16
15–50	–	8	35	37	29	44	52	52	49	28
50–100	–	–	–	8	23	–	–	3	9	31
>100	–	–	–	–	–	–	–	–	5	12
$\sum f_D$ (%)	2	16	45	50	60	46	62	75	84	87
$MW_{aggr.}$ (kDa)	$[HP\beta CD]_{total}$ (mM)					$[SBE\beta CD]_{total}$ (mM)				
	20	50	70	90	100	20	30	40	50	60
8–15	3	3	3	2	2	5	6	12	15	7
15–50	15	40	41	44	29	18	29	34	31	30
50–100	–	–	12	5	17	–	–	2	10	22
>100	–	–	3	16	22	–	–	–	–	–
$\sum f_D$ (%)	18	43	59	67	70	23	35	48	56	59

plexes, which have the lowest propensity to form aggregates, are mainly present as aggregates at similar molar concentrations. Thus, all studied hydrocortisone complexes demonstrate a strong tendency to form aggregates under the given conditions of the assay.

The aggregate formation and their size increase with increasing CD concentration. However, at CD concentrations above 20 mM the largest aggregate fraction is in all case within the 15–50 kDa range. If it is assumed that aggregates are tightly packed, then aggregates within the ranges observed correspond to 12–38 (αCD), 8–25 (HPγCD), 9–28 (HPβCD), and 6–19 (SBEβCD) complexes per aggregate. The CDs tested can be divided into two groups, αCD and SBEβCD that form aggregates of MW less than 100 kDa, and HPβCD and HPγCD that form larger aggregates with MW greater than 100 kDa. Furthermore, hydrocortisone/HPγCD complexes form a relatively large 8–15 kDa population in comparison to hydrocortisone/HPβCD, which is another fact making HPγCD the best CD with regard to its aggregation ability. A possible explanation of the weaker aggregation ability of αCD and SBEβCD can be the relative low affinity of hydrocortisone and αCD (see Table 2) and repulsion forces acting between the negatively charged hydrocortisone/SBEβCD complexes.

3.5. TEM analysis

As a system with the most profound aggregation ability, hydrocortisone/HPγCD aqueous solution was further studied by TEM analysis. The images showed that spherical nanoparticles of diverse sizes coexist in solution (see representative Fig. 5). Aggregates with diameter of 3–5 nm appear to be dominant in 10% w/v aqueous (64 mM) HPγCD solutions saturated with hydrocortisone with larger aggregates of diameters of 18–20 nm were also detectable. This observation is in agreement with the permeation results (Fig. 4B) showing that at this HPγCD concentration, hydrocortisone is mainly present as three aggregate populations within the size range of 8–100 kDa. The diameter of an anhydrous unsubstituted γCD molecule is 1.75 nm (Dodziuk, 2006). Assuming that the hydrocortison/HPγCD complex can be represented by a 2 nm rigid and tightly packed sphere, an aggregate of 4 nm diameter consisting of 6 complexes can be suggested while a 6 nm aggregate would consist of 12 complexes. As the size of the aggregates increases, the uncertainty in the estimated number of complexes grows considerably. Thus, a nanoparticle of 3–5 nm diameter may contain, on the average, 6 anhydrous hydrocortisone/HPγCD complexes (MW 11.6 kDa), whereas a spherical nanoparticle of 18–20 nm diameter may contain roughly 150–200 complexes with MW exceeding 100 kDa which is inconsistent with the observed size distribution in Fig. 4B. However, the permeation studies only give a rough estimate of the aggregate size distribution. Furthermore, the assumption of tightly packed aggregates can be incorrect since it is known that both CD and CD complexes are hydrated in aqueous solutions (Castronuovo et al., 2007; Jana and Bandyopadhyay, 2009). In addition, the size of particles from TEM image can change as a function of sample preparation. Nevertheless, TEM results support the contention that spherical aggregates of various sizes are present in aqueous hydrocortisone/HPγCD solutions. Moreover, it is likely that the size distribution of aggregates at the concentration chosen is mainly limited to two particle size populations. This finding could be useful in understanding of the aggregation mechanism.

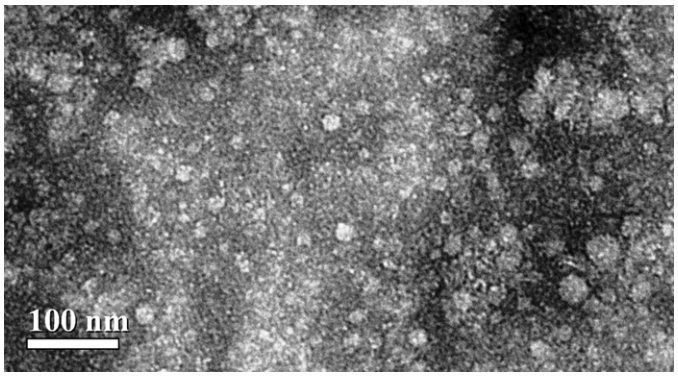

Fig. 5. Transmission electron microscopic (TEM) image of aqueous 64 mM HPγCD solution saturated with hydrocortisone.

3.6. Speculative driving forces for aggregation

Self-assembly of pure unmodified CDs is known and even observable in the visible range in case of γCD. Chemical derivatives, on the other hand side seem to have a reduced ability to undergo self aggregation (Messner et al., 2010). In our studies, we observed aggregates of inclusion complexes for all CDs tested, however αCD and SBEβCD form smaller constructs than HPβCD and HPγCD. The fraction of aggregates is largest for HPγCD. Thus, we believe the CD itself is not primarily responsible for aggregation and that the complexed drug, hydrocortisone, has an important role in this regard. Phase solubility data show a low stability constant of the inclusion complex formation between hydrocortisone and αCD and this result in a dramatically reduced interaction of drug in the solution. If the drug is responsible for the formation of aggregates, we would assume a limited ability of this poorly interacting system to give rise to aggregation, which is what we

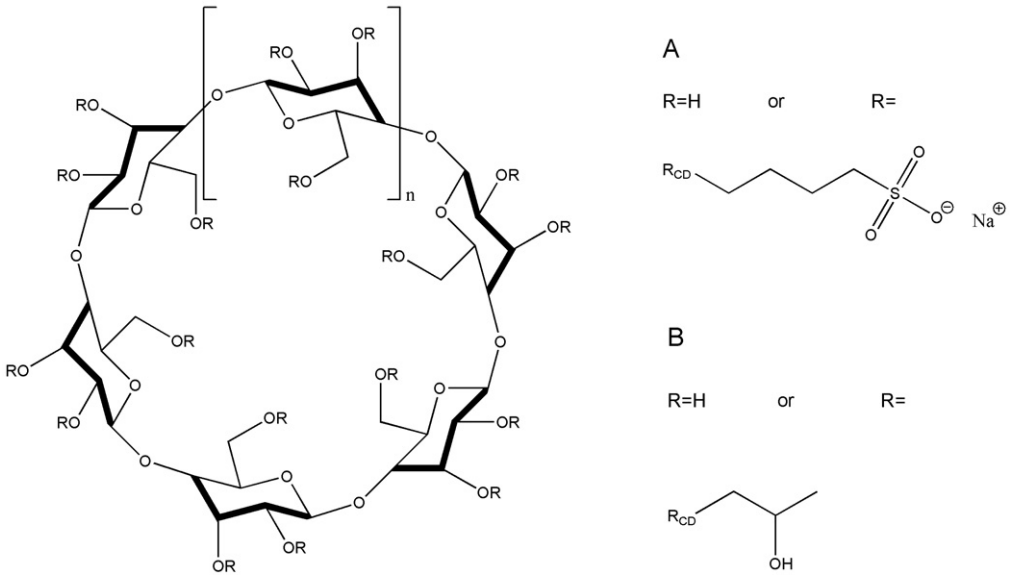

Fig. 6. αCD, βCD and γCD cone for R=H and $n = 1, 2$ and 3, respectively and derivative side chains SEBβCD (A) and HPβCD (B).

observe. An apparent disconnect with this working hypothesis is the fact that SBEβCD also demonstrated a reduced ability to form aggregates but shows a high stability constant for inclusion complexes with hydrocortisone. Assuming that the cavity of the βCD is extended by the SBE side chain (Fig. 6), this should result in a better encapsulation of the drug and therefore the hydrocortisone is sterically shielded, which could diminish the ability to form aggregates. Furthermore, the polar head groups of the side chain are likely to have repulsive coulombic interactions with nearest neighbor complexes. For the HP side chains, the cavity enlargement is smaller and repulsive coulombic interactions are not possible but the CE is high and consequently many drug/CD complexes are available to promote aggregation. In fact we observed the highest tendency to form aggregates in the case of HPβCD and HPγCD. βCD and γCD form the largest aggregates from a dimensional perspective among the CD tested and we believe that this is a combined effect of self-aggregation of the CDs and the very high CE. These interpretations are not advanced as mechanistic proof of a particular aggregation mechanism (Loftsson et al., 2004; Messner et al., 2010) but the results we obtained in this study could be used to further advance our understanding of cyclodextrin-based aggregation. Furthermore, these findings can be understood as additional indication in the growing body of literature that aggregation is drug induced.

3.7. Possible aggregation mechanisms

It appears that the aggregate formation can proceed via two different mechanisms. One is stepwise aggregation and another is spontaneous formation of nanoparticles which contain a discrete number of complexation units (Scheme 1). However, the proposed mechanisms of aggregate formation can coexist at any given CD concentration or be concentration dependent. For instance, at low CD concentrations aggregate formation can be dominated by a stepwise mechanism while at higher concentrations, a spontaneous aggregate formation may become dominant and vice versa. This can be explained by a change in the binding properties of aggre-

Formation of a hydrocortisone/cyclodextrin 1:1 complex:

$$\underset{(D)}{\text{Hydrocortisone}} + \underset{(CD)}{\text{Cyclodextrin}} \overset{K_{1:1}}{\rightleftharpoons} \underset{(D/CD)}{\text{Hydrocortisone/Cyclodextrin}}$$

Stepwise formation of complex aggregates:

$$D/CD + D/CD \overset{K_1}{\rightleftharpoons} \underset{\text{Complex dimer}}{(D/CD)_2} + D/CD \overset{K_2}{\rightleftharpoons} \underset{\text{Complex trimer}}{(D/CD)_3} + D/CD \overset{K_3}{\rightleftharpoons} \cdots\cdots \overset{K_{n-1}}{\rightleftharpoons} (D/CD)_n + D/CD$$

or

$$a \times D/CD \overset{K_a}{\rightleftharpoons} \underset{\text{4 nm aggregate}}{(D/CD)_a} + b \times D/CD \overset{K_b}{\rightleftharpoons} \underset{\text{10 nm aggregate}}{(D/CD)_{a+b}} + c \times D/CD \overset{K_c}{\rightleftharpoons} \underset{\text{20 nm aggregate}}{(D/CD)_{a+b+c}} + d \times D/CD \overset{K_d}{\rightleftharpoons} \cdots\cdots$$

Spontaneous aggregate formation:

$$n \times D/CD \overset{K_n}{\rightleftharpoons} (D/CD)_n$$

Scheme 1. Stepwise and spontaneous formation of drug/cyclodextrin (D/CD) complex aggregates. The molecular weight of an anhydrous hydrocortisone/HPβCD complex monomer is 1762 Da and that of an anhydrous hydrocortisone/SBEβCD complex monomer is 2525 Da. $K_{1:1}$ is the equilibrium constant for formation of 1:1 D/CD complex monomer. $K_1, K_2, K_3, \ldots, K_{n-1}$ are equilibrium constants for stepwise formation of the complex aggregates and K_n is the equilibrium constant for spontaneous formation of the complex aggregates.

gating species (e.g. steric hindrance of binding sites or saturation of hydrogen-bonds).

Topchieva et al. (2006) showed that hydroxypropylated CDs self-aggregate via spontaneous (cooperative) mechanism associated with hydrogen-bonding contributions. Thus, it is assumed that the only aggregates present in 8–15 kDa aggregate population are hexamers (see Section 3.5) and that the stepwise aggregation mechanism was excluded from the model presented. If this was the case, spontaneous formation of a nanoparticle containing six 1:1 complexes is described by the equation in Scheme 1 where $n = 6$ and aggregation constant (K_{A_6}) is determined by

$$K_{A_6} = \frac{[(D/CD)_6]}{[D/CD]^6} \quad (8)$$

From Eqs. (1) and (2) it follows that

$$[D/CD] = K_{1:1} S_0 [CD] \quad (9)$$

where

$$[(D/CD)_5] = f_D [D/CD] \quad (10)$$

The combination of Eqs. (8)–(10) yields the following final expression for calculation K_{A_6}:

$$K_{A_6} = \frac{f'_D}{K_{1:1}^5 S_0^5 [CD]^5} \quad (11)$$

where $f'_D \approx 0.2$ if expressed as a fraction (see Table 5). Using analogous reasoning for an aggregate composed of 200 units

$$K_{A_{200}} = \frac{f''_D}{K_{1:1}^{200} S_0^{200} [CD]^{200}} \quad (12)$$

where extrapolated $f''_D \approx 0.25$. After substitution of values from Tables 2 and 5 for appropriate magnitudes $K_{A_6} \approx 1.7 \times 10^5\ M^{-5}$, while $K_{A_{200}} \to \infty$. The high values obtained agree qualitatively with those of Topchieva et al. (2006), who reported aggregation constants for αCD and HPβCD to be on the order of 10^{44} and 10^{19} orders of magnitude, respectively. However, the physical meaning of the large values of the aggregation constants is unclear. The aggregates are known to be unstable, i.e., the aggregation enthalpy tends to zero, and, thus, the only explanation of this enormous increase in the value of the stability constant is an increase of aggregation entropy, which is counterintuitive. The reason for such confusions can be the non-applicability of accepted assumptions as well as lack of an adequate theory for description of aggregation process in CD solutions.

4. Conclusions

Nanoparticulate complex aggregates of hydrocortisone/αCD and hydrocortisone/SBEβCD have a maximum size of between 50 and 100 kDa. Hydrocortisone/HPγCD and hydrocortisone/HPβCD complexes form nanoparticles with maximum size greater than 100 kDa, whereas inclusion complex aggregates with βCD and γCD are larger than 0.45 μm and visible to the naked eye. Estimations on fraction and distribution of complex aggregate particles from permeability profiling showed an increasing fraction participating in aggregation with increasing CD concentration for all CDs. In general CDs which form smaller complex aggregates also have a lower fractional participation. Complex aggregate size population profiles show a continuous growth of aggregates with increasing CD concentration for all tested CDs, although the maximum size is different. However, it remains unclear if formation is stepwise or spontaneously or a combination of both and a useful model for describing complex aggregation is still missing. Nevertheless the results obtained indicate a process which is principally drug-induced. The use of an aggregation theory in concert with complexation of CD with guest molecules is better aligned with experimental findings especially in concentrated solutions that complexation theory alone.

Acknowledgements

This work was supported by the University of Iceland Research Fund. We gratefully acknowledge the support of Scientific and Technology Research Equipment Centre, Chulalongkorn University, Bangkok, Thailand for TEM analysis.

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