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ORIGINAL ARTICLE

Characterization and control of the aggregation behavior of cyclodextrins

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Abstract Photon correlation spectroscopy has been employed for the purpose of characterizing the aggregation behavior of cyclodextrin molecules in aqueous solutions. This optical method is generally intended to study particle size distribution of colloidal particles, associates and macromolecules. Herein we report on some general methodological issues of photon correlation spectroscopy aiming to illustrate aggregated and non-aggregated state of parent cyclodextrins and cyclodextrin derivatives, such as (2-hydroxy)propyl-β-cyclodextrin and tetraamino rhodaminyl (2-hydroxypropyl)-β-cyclodextrin in different aqueous media. Based on particle size analysis data we have demonstrated that the tendency of cyclodextrin molecules to form aggregates may be controlled by temperature and by various additives, e.g. urea, citric acid and polyvinylpyrrolidone. In the case of (2-hydroxypropyl)-β-cyclodextrin the effect of degree of substitution was also studied.

Keywords Photon correlation spectroscopy · Non-complex forming additives · Self-association · Optical properties

Introduction

Revealing the aspects of the aggregation behavior of cyclodextrin (CD) molecules has attracted increasing attention in the last two decades parallelly with their expanding use in the field of pharmacology and biotechnology. In these applications intermolecular interactions are often manifested in phenomena of real practical relevance e.g. evolvement of visible particulates, seeding process in crystallization. The physical state of dissolved CD molecules is anticipated to be of high importance especially when studying the properties of these in biological systems [1]. It is thought that the aggregation behavior may greatly affect the transport route consequently modifying the biological effects triggered [2]. Induction or prevention of aggregation contributes to better understanding the mechanism of cyclodextrin self-assembly that may find its use in practical terms also upon the development of CD-based injectables and ophthalmic solutions [3]. Coleman et al. [4] found that β-CD forms aggregates with an average diameter of approx. 200 nm in a concentration dependent manner. The first in-detail study of cyclodextrin aggregation performed with photon correlation spectroscopy (PCS, also known alternatively as Dynamic Light Scattering method) may be attributed to González-Gaitano et al. [5], wherein the effect of filtration, chemical substitution and the ionic strength or pH of the solvent were evaluated. Loftsson et al. [6] reported that micelle-like aggregates may be formed from CD molecules. This process was shown to inhibit the transmembrane permeation of cyclodextrins and their complexes across cellophane membranes [6–8]. Major relevant physico-chemical properties of self-assembled CDs and CD-complexes (associate dimensions and micelle-like structuring) are also reviewed elsewhere [9]. It is suggested that these nanostructures are able to solubilize hydrophobic drugs via non-inclusion complexation. Similar phenomenon is thought to play significant role upon cyclodextrin-aided solubilization of carotenoids [10]. The research group of Baglioni used various techniques including dynamic and static light scattering, cryo-TEM and electron spin resonance

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to investigate the characteristics of the aggregation behavior of β-CD and to elucidate the main structural and geometrical features of the resulting associates [11, 12]. A recent study was devoted to the exploration of the self-association character of sugammadex which is a cyclodextrin derivative of peculiar medical significance [13]. The authors found that three independent methods (permeation test, dynamic light scattering and sedimentation equilibrium analytical ultracentrifugation) brought results each corroborating one another indicating that these molecules do not have a tendency to self-associate in a wide concentration range. Discussion on various types of cyclodextrin aggregates (native and modified cyclodextrins, inclusion complexes and their aggregates, CD-rotaxanes and polyrotaxanes, CD-nanotubes) and other high-order aggregates of cyclodextrins have been complied in a review article by He et al. [14]. Some more publications also dealt with the self-assembly of cyclodextrin complexes recently [15–20].

In the present paper we aimed to elucidate some general methodological issues of PCS technique aiming to characterize aggregated and non-aggregated state of parent cyclodextrins as well as cyclodextrin derivatives in aqueous media of different compositions. Based on aggregate size analysis we have shown that the tendency of CD molecules to form self-associative structures may be effectively controlled by temperature and by various water-soluble additives.

Method

The aggregate size measurements were carried out using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, United Kingdom) equipped with the manufacturer's standard 633 nm laser source. The samples were analyzed having the solutions filled inside a quartz cuvette of 1.00 × 1.00 cm dimensions. The particle sizes were obtained from the autocorrelation functions recorded and processed by Zetasizer Software version 6.2.

The aggregate sizes were calculated according to the following formulae [21]

$$G(\tau) = \exp\left(\frac{-\tau}{\tau_c}\right) \tag{1}$$

G, autocorrelation function; τ, time; τ_c, relaxation time

$$\tau_c = \frac{1}{DK^2} \tag{2}$$

D,diffusion coefficient; K, scattered wave vector

$$K = \frac{4\pi n}{\lambda}\sin(\theta/2) \tag{3}$$

n, refractive index of the solvent/dispersant; λ, wavelength of the laser; θ, scattering angle

$$D = \frac{kT}{3\pi\eta d} \tag{4}$$

k, Boltzmann constant; T, temperature; η, viscosity of the solvent/dispersant; d, (hydrodynamic equivalent) aggregate diameter.

The distribution profile that may be derived directly from the autocorrelation function is the *intensity size distribution*. Since the scattered intensity is proportional to the diameter of the particles/aggregates on the sixth power, the intensity size distribution is largely weighed in respect to the aggregates, therefore the associates are overrepresented. Upon PCS analysis, the sizes of the species actually undergoing Brownian motion (i.e. the hydrated molecules or hydrated aggregates) all together yield the particle size distribution. Should the particles/aggregates have irregular, non-spherical shape (each dimension corresponds to different 'sizes'), PCS provides a result expressed as hydrodynamic equivalent diameter which exhibits the size of the species to that of a hypothetic spherical particle. In other words, the hydrodynamic equivalent diameter is identical to the diameter encountered in the Stokes' law (see Eq. 4). The morphology of CD-associates also has been discussed in earlier works; for example Bonini et al. [11] confirmed that β-CD monomers aggregate in water at room temperature in differently shaped particles depending on the concentration.

It is possible to transform the intensity-based data to a volume or mass distribution using Mie theory, which describes the scattered intensity as a function of the particle size, the observation angle, and the refractive index properties of the particle [22]. The *volume distribution* and the *volume average size* (M_v) both reflect the physical state of the dissolved molecules or dispersed particles in volume% (which is approximately equivalent with mass%). M_v is a weighed average of N_i (number of particles) having V_i (volume), as referred in Eq. 5.

$$M_v = \frac{\sum N_i V_i^2}{\sum N_i V_i} \tag{5}$$

Although the volume average size obtained by PCS analysis seems to be easier to correlate with results obtained by other methods (e.g. ultracentrifugal sedimentation), due to the necessary mathematical transformation these data should be subject to critical evaluation especially in the light of the validity of the physico-chemical properties of the dispersed material required for the calculation.

Sample preparation

The samples were pre-filtered through a precision woven monofilament polyester filter fabrics of 10 μm nominal mesh size (Medifab, SEFAR AG, Switzerland) which

corresponds to the upper detection limit of the Zetasizer in order to remove large particulate contaminations from the sample, still allowing subject-matter aggregates to pass through. Filtration was chosen as an alternative method to overcome the problem encountered also in the work of Bonini et al. [11] wherein sedimentation was applied.

Materials

α-, β-, γ-cyclodextrins and (2-hydroxypropyl)-β-cyclodextrin samples used throughout the experimental work were the products and research samples of CycloLab Ltd, Budapest. All other reagents and solvents were commercial fine chemicals of analytical grade.

Results

Application of photon correlation spectroscopy for the characterization of aggregation state in cyclodextrin solutions

Although the pioneering results reported on the aggregative behavior of CDs by PCS method has been available for two decades [4], due to the improvement of the instrumental setup and the data processing, it was plausible first to revisit the determination the aggregate size distribution functions of the parent CDs in their aqueous solutions. The obtained size distribution curves are the averages of six measurements. The relatively low applied concentration (1.0 wt%) still yielded sufficient scattered light intensities. Figures 1, 2 and 3 show the aggregate size distributions obtained for α-, β-, and γ-cyclodextrins, respectively. Some similar distribution profiles were reported in an earlier works of González-Gaitano [5] and Wu et al. [23] obtained for the aqueous solutions of parent cyclodextrins as determined in concentrations approximately equivalent with those used herein, but they obtained results immediately after filtration through membrane filters of very fine pore sizes (0.1 and/or 0.2). In contrast, during our measurements only visible particulates were removed, consequently notable aggregates of higher order were also observable. Aggregates sized over 1 μm were already reported by Bonini et al. [11] but only for a β-CD solution of 9 mM concentration from which sedimenting particulates were previously removed. The validity of the intensity distribution functions (Figs. 1a, 2a, 3a) is almost entirely dependent on the system suitability of the instrument (subject to be verified by latex standard dispersions and temperature check) since there is no need for sample preparation providing attention has been paid for avoiding sample contamination. The intensity size distribution

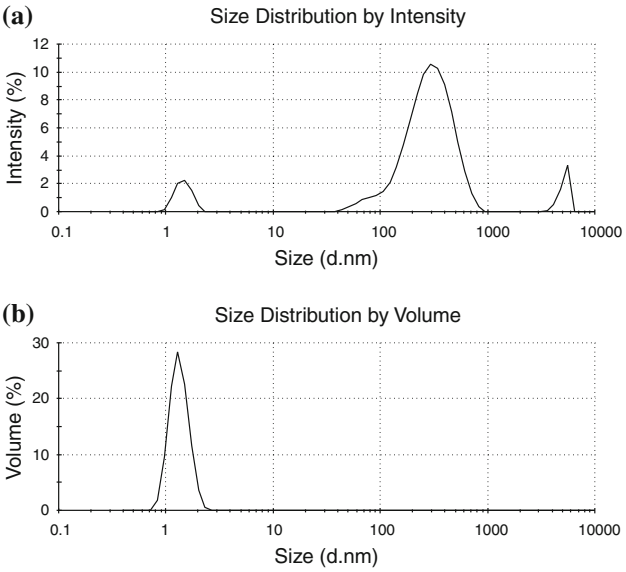

Fig. 1 Aggregate size distributions of 1.0 wt% aqueous α-cyclodextrin solution at 25.0 °C: **a** by intensity, **b** by volume

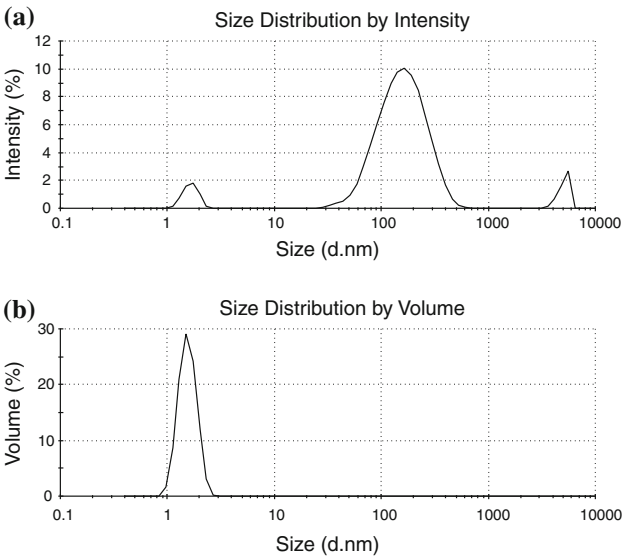

Fig. 2 Aggregate size distributions of 1.0 wt% aqueous β-cyclodextrin solution at 25.0 °C: **a** by intensity, **b** by volume

functions all show that aggregates are formed in the samples within 50-1,000 nm range and even in the μm-scale.

However, the transformation of the results into volume-related data requires some considerations. For the calculation both the refractive index and the absorbance of the scattering objects are needed. Only the magnitude of the absorbance component is required for the calculation. The scattering species undergoing Brownian motion in CD solutions may be both hydrated individual molecules as well as hydrated aggregates presumably formed by means of intermolecular hydrogen bonds. Nevertheless these

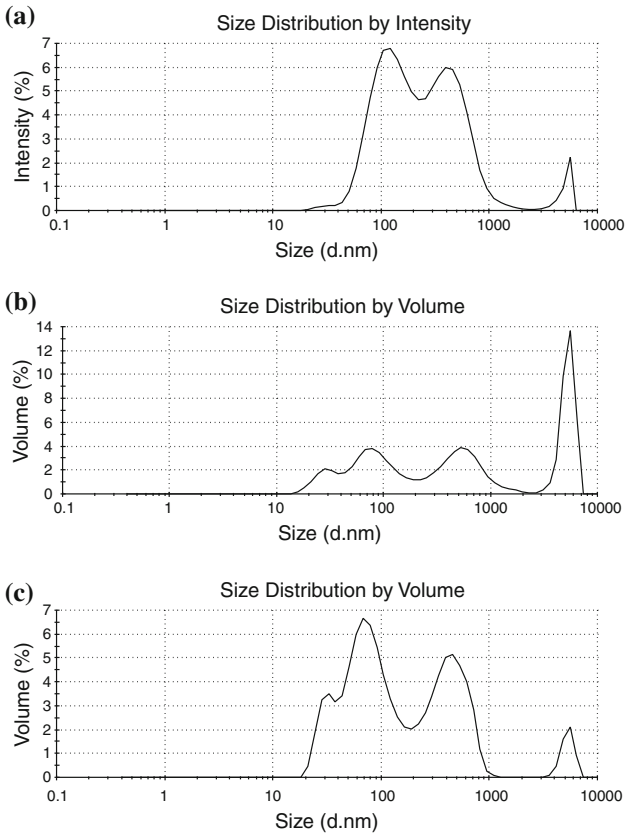

Fig. 3 Aggregate size distributions of 1.0 wt% aqueous γ-cyclodextrin solution at 25.0 °C: **a** by intensity, **b** by volume (applying $n = 1.37$), **c** by volume (applying $n = 1.51$)

assemblies might include considerable portion of solvate sheet within their structure, therefore the determination of the exact refractive index (n) for these complex associates does not promise considerable success. In addition, the instrument's transformation algorithm based on the Mie theory applies to a case wherein all particles are spherical having a homogeneous and equivalent density, so its direct application should be handled with some criticism.

The transformations to volume based size data were performed using an n equal to 1.37 (a typical value for organic compounds) which is an arbitrary, but still credible value. Herein it must be noted that in the case of Rayleigh scatterers ($d \ll \lambda$), n is not critical in terms of transformation. For the calculation absorbance value of 0.01 was used. During the aggregate size analysis of α-CD and β-CD solutions the transformations to volume based data showed the absence of aggregate fraction (a second modality is only plotted by the instrument above the amount of 0.1%). The medians of the monomodal size distribution functions were 1.3 and 1.6 nm for α-CD and β-CD samples, respectively (Figs. 1b, 2b). These values well correspond to the calculated molecular sizes of individual cyclodextrin rings. Szejtli [24] reported that at the wider rim the diameter of α-CD is 1.4 nm while that of β-CD is 1.5 nm. The size obtained also corroborates that the refractive index approximation was appropriate, since the individual CD molecules are true Rayleigh scattering species. The transformations were performed by changing the hypothetic refractive index values by 10% to lower and upper range, still yielding the originally obtained mean sizes within 0.1 nm error.

The same transformation method was applied to the size distribution function obtained for the 1.0% γ-CD solution. The obtained volume distribution function indicated that the aggregates represent considerable amount even in terms of volume (see Fig. 3b). Therefore the scattering particles are not in the Rayleigh-range thus omitting the effect of refractive index is no longer justifiable. By calculating with the hypothetic refractive index value of 1.37 an intense modus appears in the volume distribution around 5,600 nm (Fig. 3b). This calculation yields non-realistic result since actually higher (hypothetic) volume mean size was obtained compared to that of the intensity-based data. Changing the arbitrary refractive index by 10% (to the value of 1.51), the volume size distribution changes to a profile that is at least similar to the intensity-based function (see Fig. 3c). A realistic volume-based size distribution curve represents particle populations (peaks) at definitely smaller size ranges compared to the intensity function.

It may be concluded that derived volume distributions of the cyclodextrin aggregates are best used rather for comparison purposes and should not be considered absolute within the above limitations. Consequently in the foregoing primarily the intensity size (aggregation) distribution functions and the corresponding mean calculated sizes are represented.

Effect of temperature on the aggregation behavior of cyclodextrins

It has also been evaluated whether changes in temperature trigger the aggregation or de-aggregation of the dissolved native CD molecules. The Zetasizer instrument is suitable for particle sizing also at elevated temperatures. To avoid the effect of the presumably temperature-sensitive variations in the refractive index of the scattering particles (which is already an uncertain factor even at constant temperature), the refractive index independent intensity mean sizes were selected as indicative data for this study. The differences in the viscosities of the samples as well as the temperature-dependent viscosity changes were both taken into account.

The changes of the mean aggregate sizes as functions of temperature are plotted in Fig. 4, wherein it is illustrated that the elevation of temperature decreases the aggregate size. For demonstrating the changes of the aggregation

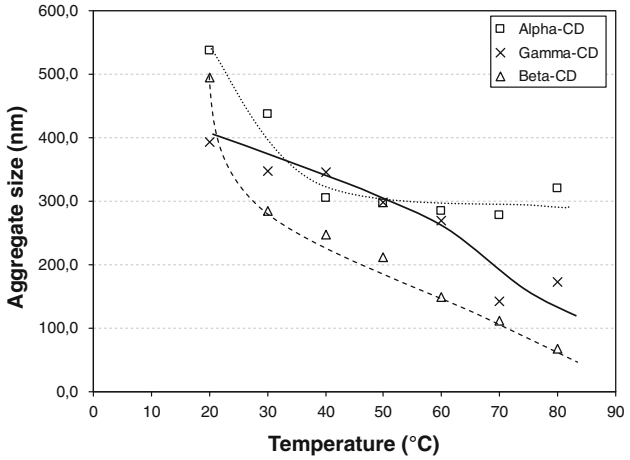

Fig. 4 Intensity average particle (aggregate) sizes of parent cyclodextrins in distilled water (the applied concentrations are 14.5 w/v% for α- and γ-CD and 1.5 w/v% for β-CD)

behavior of CDs with varying temperatures, relatively higher cyclodextrin concentrations were applied (14.5 w/v% for β- and γ-CD and 1.5 w/v% for β-CD). It is notable that the aggregate sizes show sharper drops between 20 and 40 °C in the case of α- and β-CD and at 60–80 °C for γ-CD solution. The aggregate size of α-CD is nearly constant in the range of 40–80 °C. Giving account for the possible mechanism of these characteristic changes is planned in our future work. In the studied temperature range complete deaggregation could not be reached, but it can be seen that increasing the kinetic energy of the individual cyclodextrin molecules may disrupt the aggregate architecture. It must be noted herein that the intensity-based representation in itself does not provide information about the quantitative ratio of cyclodextrin molecules being in aggregated and non-aggregated state.

Even though it is well known that formation of complex associates is not favored upon elevation of temperature in equilibrium, during the preparation of cyclodextrin complexes it is very often experienced that the elevation of temperature facilitates the rate of inclusion process. Amongst other also important concurrent processes, induced cyclodextrin deaggregation might also play a role in facilitating the approach of the guest molecule towards the cyclodextrin cavity at higher temperatures.

Aggregation characteristics of CD-derivatives

The investigation of various cyclodextrin derivatives in respect to aggregation properties was motivated by their extending widespread use. Introducing (2-hydroxypropyl)-β-cyclodextrin in practical use has especially been justified by its high solubility compared to parent β-cyclodextrin which is generally explained by an assumption that these molecules are less susceptible to aggregation due to the presence of the substituents. On the contrary, our PCS analysis showed that HPβCD (having degree of substitution of 4.3) actually forms aggregates in 1.0 wt% aqueous solution (see Fig. 5a). Owing to the statistical composite nature of HPβCD, it is inevitable that a fraction of the sample consists of monosubstituted species. It is known that the solubility of monosubstituted HPβCD is lower [25] than that of β-cyclodextrin which indicates that the presence of these species may account for the occurrence of the aggregates. Analyzing the aggregate properties of a monosubstituted HPβCD it was found that these molecules rather tend to aggregate yielding aggregate populations falling in a very similar size range compared to that found in HPβCD of higher degree of substitution (see Fig. 5b).

The method enabled the aggregate size analysis of a fluorescent cyclodextrin derivative, tetraamino rhodaminyl HPβCD (having average degree of substitution of 3.5) that has recently been evaluated as a potential drug delivery model [26]. Even that this CD derivative has peculiar optical properties due to its fluorophore moiety compared to that of the parent cyclodextrins or HPβCD, still, the use of the same instrumental setup was found adequate, because the wavelength of the applied laser source was far from the wavelength of the molecule's characteristic absorption and emission bands. The aggregate size analysis data of tetraamino rhodaminyl HPβCD determined in distilled water and in physiological phosphate buffered saline solution are shown in Fig. 6. The results show that this fluorescent derivative is present in fully aggregated state both in low ionic strength and in isosmotic media. The

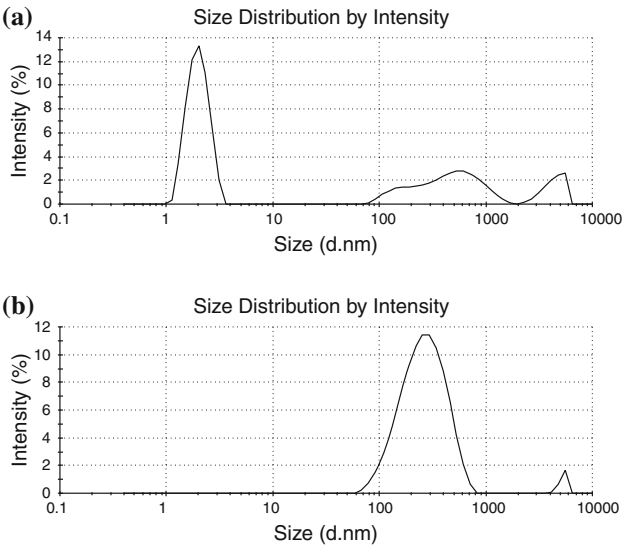

Fig. 5 Aggregate size analysis of 1.0% HPβCD in distilled water: **a** average degree of substitution = 4.3, **b** average degree of substitution ~1

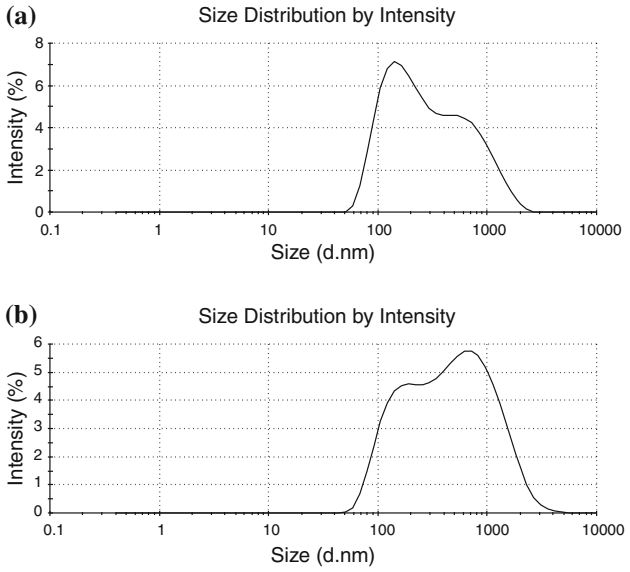

Fig. 6 Aggregate size analysis of tetraamino rhodaminyl HPβCD (in 0.5 wt% concentration): **a** in distilled water, **b** in phosphate buffered saline solution

relevance of the physical state of this potential delivery system may come into view upon the evaluation of the mechanism of its cellular uptake.

Control of the aggregation behavior of cyclodextrins with additives

Probably the most apparent significance of the aggregation state of cyclodextrins is manifested in life sciences. It is therefore important to study the aggregation characteristics of CDs in aqueous media having compositions relevant in pharmaceutical and biological applications. Moreover by studying the behavior of CDs in the presence of different additives it was aimed to survey whether such observations open possibilities in controlling the aggregation state of these molecules. Representative compounds of distinct chemical properties (hydrogen bond breakers/modifiers, macromolecules, acids, cosolvents) were selected as additives. Their effects on the aggregation characteristics of CD molecules in aqueous solutions are summarized in Table 1.

Table 1 Effect of additives on the aggregation characteristics of CD molecules in aqueous solutions

Cyclodextrin	Additive (concentration)	Effect
β-cyclodextrin	Citric acid (0.1–1.0%)	Anti-aggregative effect
	Citric acid (0.1–1.0%) + heating to 80 °C	Highly effective anti-aggregative effect
	Dimethyl formamide (0.1–1.0%)	Anti-aggregative effect
	Urea (1.0%)	Ø
	Dimethyl sulfoxide (1.0%)	Ø
	Polyvinylpyrrolidone K-30 (0.1%)	Anti-aggregative effect
γ-cyclodextrin	Citric acid (0.1–1.0%)	Aggregation enhancing effect
	Urea (1.0%)	Ø
	Dimethyl formamide (1.0%)	Ø
	Polyvinylpyrrolidone K-30 (1.0%)	Anti-aggregative effect
HPβCD	1.0% Citric acid/citrate buffer (pH 6)	Ø
	NaCl (0.9%)	
	KH_2PO_4 (1.0%)	
	EDTA (1.0%)	
	Phosphate buffered saline (PBS)	
	Boric acid (1.0%)	
	Arginine (1.0%)	
	Fructose (5.0%)	
	Glycerol (1.0%)	
	Ethanol (1.0–15 V%)	
	Sodium tetraborate (1.0%)	Aggregation enhancing effect
	Polyvinylpyrrolidone K-17 (1.0%)	Anti-aggregative effect
	Polyvinylpyrrolidone K-17 (1.0%) + ethanol (5.0−20 V%)	Highly effective anti-aggregative effect

Ø no detectable effect

Generally, when anti-aggregative effect was observed, the intensity (and not the mean size) of the aggregates was found to decrease. In some cases additives behaved differently if preparation of the solution was followed by heat treatment. Citric acid caused de-aggregation of β-CD especially when the solution was heated to elevated temperature. Surprisingly citric acid affected the aggregation behavior of γ-CD in a controversial way at room temperature. Urea has earlier been reported to deaggregate β-cyclodextrin due to its ability to affect the intermolecular hydrogen-bond network of the CD, however this effect was only significant in somewhat concentrated solutions (i.e. above 1 M) [27]. We have observed that urea applied in dilute solution (1.0 wt%) is practically inefficient.

A polymeric additive—polyvinylpyrrolidone (PVP)—widely used in the pharmaceutical formulations showed well-defined anti-aggregation effect. Solvated PVP macromolecules being in a coiled up conformation scatter light themselves, but their size (10–20 nm) is smaller at least by one order of magnitude than that of the typical CD aggregates. The deaggregation of CD molecules was recorded as the particle population falling within the range characteristic to CD-associates was less or no longer detectable in the presence of PVP. Due to practical applicability purposes the effect of a higher molecular mass PVP suitable for *per os* use (K-30) was evaluated when anti-aggregation of β-CD or γ-CD was studied whereas HPβCD was studied in the presence of PVP K-17 which represents the grade of highest molecular mass still allowed for parenteral administration. Both PVP samples proved to be an effective additive to prevent the self-aggregation of β-CD, γ-CD and HPβCD. The de-aggregative effect of lower molecular mass PVP could be enhanced by using ethanol as a cosolvent. These findings are well in line with previous observations of Messner et al. [15] obtained by membrane permeability studies wherein polymeric additives such as carboxymethylcellulose, (hydroxypropyl)methyl cellulose as well as ethanol were found to reduce the size of hydrocortisone/HPβCD complex aggregates.

Conclusions

Intermolecular bonding interactions of cyclodextrins may be influenced by applying formulation methods at different temperatures and in the presence of various non-complex forming water soluble additives. Such control may have high practical relevance in pharmaceutical development as well as in bioscientific applications.

The determination of the intensity size distribution is required to detect cyclodextrin aggregates even in those cases when these assemblies represent negligible weight fraction of the solute. Should the aggregates be present in small quantity, the sensitive detection of these structures may be critical because they are potential precursors of unwanted visible particulates. On the other hand the nanoparticles formed by aggregation might be readily absorbed via endocytosis. Further experiments are going on with complexes of the studied cyclodextrins.

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