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Evaluation of sugammadex self-association

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ABSTRACT

Sugammadex, a thiolated γCD derivative used as an antagonist of steroidal blockers, was studied with regard to its tendency to self-associate in aqueous solution. Three independent methods – permeation through semi-permeable cellophane membranes, dynamic light scattering, and sedimentation equilibrium analytical ultracentrifugation – were used for this purpose. The results were in agreement with each other and showed no evidence of self-association in a wide sugammadex concentration range from 0.25 to 100 mg/ml.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides whose molecules have a configuration resembling a truncated cone or a doughnut, with a somewhat lipophilic central cavity. These compounds have gained general acceptance as excipients in food, cosmetic and pharmaceutical industries. Due to their favorable physicochemical and pharmacokinetic properties, favorable toxicological profiles, and high aqueous solubility cyclodextrins are generally regarded as safe excipients. For example, the natural α- (αCD), β- (βCD) and γ-cyclodextrin (γCD) have been included in the 'generally recognized as safe' list of the FDA as flavor stabilizers and the cyclodextrin derivatives 2-hydroxypropyl-βCD and sulfobutyl ether-βCD both are cited in the FDA's list of inactive pharmaceutical ingredients. The key property of cyclodextrins is their ability to encapsulate a variety of lipophilic guest molecules. This is enabled by the structural flexibility of cyclodextrins which can possess a cavity of varying width and depth.

Sugammadex is a γCD derivative, which was selected out of many synthesized thiolated cyclodextrin derivatives as the best host for aminosteroids (namely, rocuronium and vecuronium (Naguib, 2007)) used as neuromuscular blocking agents (NMBA) (Adam et al., 2002). Prior to the introduction of sugammadex (Bridion®) reversal of neuromuscular blockade was achieved by cholinesterase inhibitors like neostigmine, edrophonium or pyridostigmine, but major drawbacks of these drugs are their cholinergic (side) effects and their inability to reverse profound blockade. One of the promising strategies chosen to solve this problem was neutralization of the NMBA effect by encapsulating the aminosteroidal blocker into the central cavity of a very hydrophilic cyclodextrin. The pharmacologically inactive and hydrophilic NMBA/cyclodextrin complex would then be rapidly eliminated from the body through glomerular filtration. In this context sugammadex has obvious advantages: its cavity has optimal dimensions for strong nonspecific interaction with the rocuronium skeleton, while negatively charged edges of the cyclodextrin molecule provide further strengthening of the complexation via Coulombic interaction with the positive charge of the guest quaternary nitrogen (Fig. 1). The strength of the rocuronium/sugammadex complex is impressive as the value of its association constant is estimated to be approximately $1.8 \times 10^7\ M^{-1}$ (Adam et al., 2002; Yang and Keam, 2009), whereas association constants for complexes involving parent cyclodextrins mostly do not exceed the $100–500\ M^{-1}$ range (Connors, 1997). Moreover, polar moieties of sugammadex make it more water soluble than its precursor γCD. Thus, the mechanism of sugammadex action administered intravenously is based on efficient drug entrapment within the vascular system and extracellular water compartment, which gives rise to the NMBA concentration gradient and consequent outflow of the NMBA from body tissues, in particular striated muscles, accompanied by liberation of acetylcholine receptors (Booij, 2009). It should be emphasized that sugammadex is unique in the sense that is reg-

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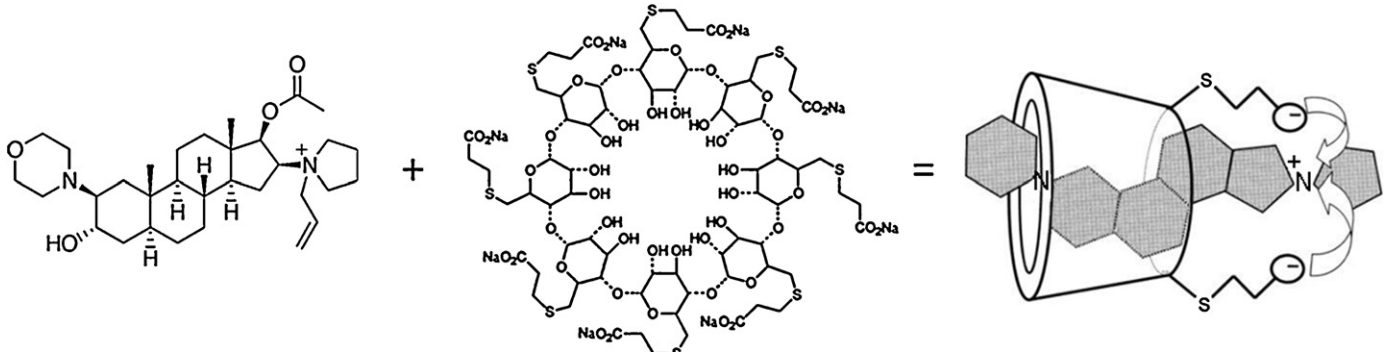

Fig. 1. Rocuronium/sugammadex 1:1 inclusion complex formation.

istered not as an excipient but as a drug and does not contain any guest molecule in the drug product formulation.

In spite of numerous favorable properties of sugammadex, the peculiarities of its molecular structure and chemical composition may cause certain unfavorable effects. Recently it has been shown that cyclodextrins can self-assemble in aqueous media to form aggregates (Messner et al., 2010). In particular, turbidity of aqueous γCD solutions is related to aggregate formation, which is supported by results obtained with e.g., dynamic light scattering (Coleman and Nicolis, 1992), transmission electron microscopy (Wu et al., 2006) and some analytical methods (Szente et al., 1998). Additionally, substituted cyclodextrins with attached long hydrophobic moieties may possess some surface activity and form micelles or vesicles (Witte and Hoffman, 1996; Roux et al., 2007). The appearance of nano-scale particles would modify the physicochemical properties of aqueous sugammadex solutions which might entail undesirable modification of the cyclodextrin's pharmacological and toxicological properties upon intravenous administration.

The objective of the present work is to investigate whether sugammadex does self-aggregate in an aqueous parenteral solution. For this purpose three conventional methods based on independent physicochemical principles were applied, methods that have been shown to be reliable ways of aggregation detection.

2. Materials and methods

2.1. Materials

Sugammadex (Org 25969, $C_{72}H_{104}Na_8O_{48}S_8$, MW 2178, pharmaceutical batch code 529679001) in the form of 100 mg/ml aqueous stock solution was supplied by N.V. Organon (currently Merck, Sharpe & Dohme (MSD), The Netherlands). Semi-permeable cellophane membranes Spectra/Pore® (Spectrum Europe, The Netherlands) with molecular weight cutoffs (MWCOs) of 3500, 6000–8000, and 12,000–14,000 Da were used for permeation studies. The organic solvents used (Sigma–Aldrich, USA) were of HPLC grade. All other chemicals used were commercially available products of special reagent grade. Milli-Q (Millipore, USA) water was used to prepare all aqueous solutions.

2.2. Permeation studies

Permeation studies were carried out at room temperature ($23 \pm 1\,^{\circ}C$) using unjacketed Franz Diffusion cells (SES Analysesysteme, Germany). The receptor phase contained 12 ml of 1% (w/v) NaCl in water and donor phase contained 2 ml of sugammadex aqueous solution. Sodium chloride served to diminish the volume shift (i.e. escape of water from receptor to donor phase) due to osmotic phenomena. Both phases were separated by a monolayer cellophane membrane of appropriate MWCO. The concentrations of studied sugammadex solutions in the donor phase varied from 10 to 100 mg/ml and were prepared from the stock solution identical to the commercial drug product and water. Prior to the experiment the membranes were soaked in receptor phase for 24 h. At certain time intervals (30, 60, 120, 180, 240 and 360 min) single 150 μl samples were taken from the receptor compartment of each cell and replaced by an equal volume of pure receptor phase. Two to four replicates were done for every donor phase concentration for each membrane. The concentrations of sugammadex in the samples were analyzed by HPLC. The method was supplied by MSD and was adapted from the original sugammadex HPLC assay method to achieve shorter run times.

The permeation profiles (i.e. time dependencies of sugammadex concentration in receptor phase) were constructed for every initial sugammadex concentration and every MWCO used. The slopes of the linear permeation profiles (dq/dt) and membrane surface area ($A = 1.77\ cm^2$) were used to calculate the sugammadex flux (J) using Eq. (1) and plot it versus initial concentration of sugammadex in the donor phase.

$$J = \frac{dq}{Adt} \tag{1}$$

The apparent permeability coefficient (P_{app}) values were calculated from sugammadex initial concentration (C_d) dependencies of flux:

$$J = P_{app} C_d \tag{2}$$

2.3. Dynamic light scattering (DLS)

DLS was performed using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) at a detection angle of 173° at 22 °C, and a He–Ne ion laser ($\lambda = 633$ nm) for the incident beam. Aqueous solutions containing 25–100 mg/ml of sugammadex were measured. Concentrations lower than 25 mg/ml were tested; however, the lower limit for proper use of this technique for this typical molecule is around 25 mg/ml. For assessing self-association the samples were filtered through a 0.1 μm filter prior to the measurement using low volume disposable plastic cuvettes.

For heat trend analyses the samples were transferred to glass cuvettes with a path length of 1 cm. The heat trend was performed from 15 to 45 °C with increments of 2 °C. At every temperature the sample was equilibrated for 2 min.

Data analysis was performed using the DTS software (version 6.01, Malvern Instruments Ltd., UK) provided by the manufacturer. The hydrodynamic diameter ($D(h)$) of the molecule at different concentrations was estimated based on the Stokes–Einstein equation:

$$D(h) = \frac{kT}{3\pi\eta D^*} \tag{3}$$

where k is Boltzmann's constant, T is the absolute temperature, η is viscosity, and D^* is the diffusion coefficient.

Table 1
HPLC conditions for sugammadex quantitative determination.

Column temperature, °C	40		
Autosampler temperature, °C	20		
Mobile phase	A: buffer[a] + AN[b], 83 + 20 (v + v)		
	B: AN		
Gradient:	Time (min)	*A* (%)	*B* (%)
	0	100	0
	9	100	0
	10	30	70
	19	30	70
	20	100	100
	30	0	100
	31	100	0
	40	100	0
Run time, min	30		
Flow rate, ml/min	0.27		
Detection wavelength, nm	200		
Injection volume, μl	2.5		
Injection loop, μl	100 or equivalent		
Detector cell, mm	6 or equivalent		
Maximum pump pressure, bar	400		

[a] Phosphate buffer (pH 3).
[b] Acetonitrile.

2.4. Sedimentation equilibrium analytical ultracentrifugation (SE-AUC)

SE-AUC experiments were performed within a concentration range of 0.25–25 mg/ml sugammadex solution using a Beckman XL-I analytical ultracentrifuge at a rotor speed of 60,000 rpm at 20 °C in an AN-60 Ti rotor with aluminum double sector cells of optical path 12 mm and sapphire or quartz windows with absorbance optics at different wavelengths and a radial step size of 0.003 cm. Optimization of the settings was performed at 3000 rpm, subsequently the rotor was stopped before the method was started. The rotor accelerated from 0 rpm to 60,000 rpm. Every 8 h scans were taken until equilibrium was reached. Approach to equilibrium was tested using the SEDFIT software (Schuck, 2000) (version 11.8). Equilibrium was reached when the root mean standard deviation (RMSD) of the last two scans was <0.005. The last equilibrium scan of each sample was used for analysis with SEDFIT. Equilibrium data were analyzed with SEDFIT using the non-interacting Discrete Species model with the following parameters: ν-bar of 0.9 and a density of 0.998 g/ml (density of water at 20 °C). With this model the molecular weight was determined at each concentration.

2.5. The quantitative determinations

Quantitative determinations were performed using a reverse phase HPLC component system from Dionex Softron GmbH (Germering, Germany) Ultimate 3000 Series, consisting of a P680 pump with a DG-1210 degasser, an ASI-100 autosampler, a VWD-3400 UV–Vis detector operated at 200 nm, and Phenomenex Aqua 3 μm C18 reverse-phase column (150 mm × 2.0 mm). The parameters of HPLC method used for sugammadex analysis are summarized in Table 1. The typical chromatogram is illustrated in Fig. 2.

3. Results and discussion

3.1. Permeation

Drug permeation through monolayer semi-permeable cellophane membranes with different MWCO has been shown to be a useful tool for the detection and characterization of drug/cyclodextrin complexes self-association (Loftsson et al., 2002; Jansook et al., 2010). This technique yields a view of association onset and gives a rough estimation of the aggregate size distribution at different concentrations.

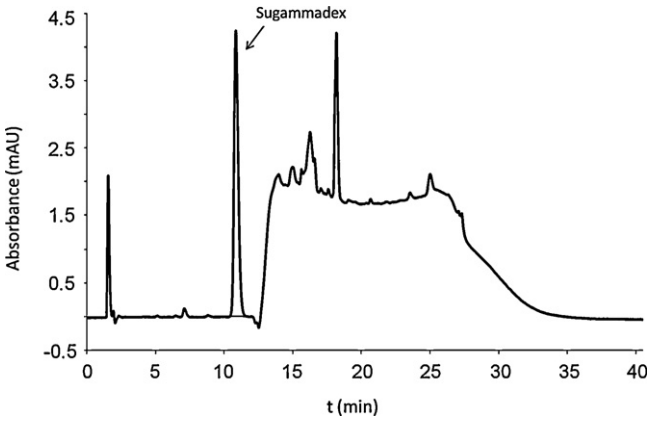

Fig. 2. An example of chromatogram used for sugammadex HPLC quantitative analysis.

Sugammadex permeation profiles were obtained for every concentration and each membrane MWCO. All profiles were of a similar type, as exemplified in Fig. 3, and can be described by a linear equation:

$$q = bt - a \quad (4)$$

where q is the amount of sugammadex permeated, and t is time. It should be noted that in theory the permeation line starts from the origin of coordinates, whereas in our case it starts a little later. This phenomenon is often observed and is known as lag time, which is the time needed to reach steady-state flux through the membrane (Banker and Rhodes, 2002).

The flux values derived from permeation profiles are summarized in Table 2.

The flux concentration dependencies are shown in Fig. 4. It can be seen that the sugammadex flux (J) is directly proportional to its initial concentration in the donor phase (C_d), which is in agreement with Fick's first law (see Eq. (2)).

The linear profiles indicate that (1) P_{app} is constant and independent of sugammadex concentration and (2) that sugammadex is in all cases able to permeate the membranes, even those with a MWCO of 3500 Da. These observations indicate that sugammadex (MW of the octa sodium salt: 2178) does not form aggregates in the studied solutions. An interesting observation is that the flux lines do not pass through the origin of coordinates. This can be explained

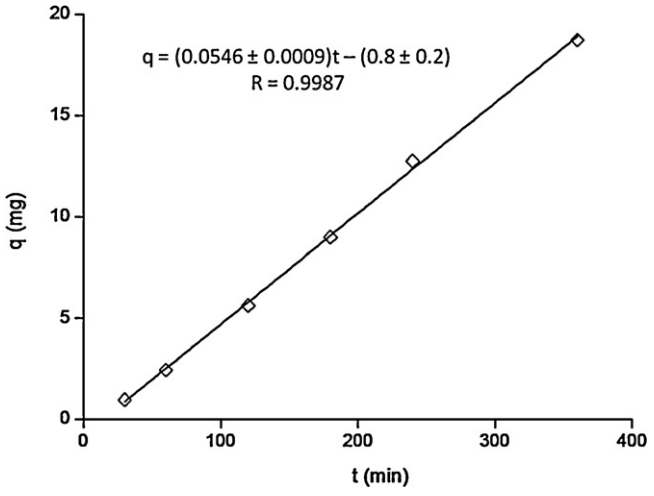

Fig. 3. An example of sugammadex permeation profile for C_d = 90 mg/ml through monolayer cellophane membrane with MWCO 3500 (q is amount of sugammadex permeated, t is time). One of two replicated incubations.

Table 2
The correlation parameters (mean ± *SD*) for a linear equation $J = BC_d + A$ describing sugammadex permeation through monolayer cellophane membranes with different MWCOs in aqueous solutions at room temperature. *R* is the pair correlation coefficient, *SD* is the standard deviation and P_{app} is the apparent permeation coefficient.

MWCO 3500		MWCO 6000–8000		MWCO 12,000–14,000	
A	-0.75 ± 0.09	*A*	-0.40 ± 0.05	*A*	-0.23 ± 0.07
B	0.018 ± 0.001	*B*	0.0207 ± 0.0007	*B*	0.036 ± 0.001
R	0.9960	*R*	0.9986	*R*	0.9984
SD	1.51×10^{-2}	*SD*	4.00×10^{-2}	*SD*	8.28×10^{-2}
$P_{app} = (5.0 \pm 0.3) \times 10^{-6}$ cm/s		$P_{app} = (5.8 \pm 0.2) \times 10^{-6}$ cm/s		$P_{app} = (10.0 \pm 0.3) \times 10^{-6}$ cm/s	

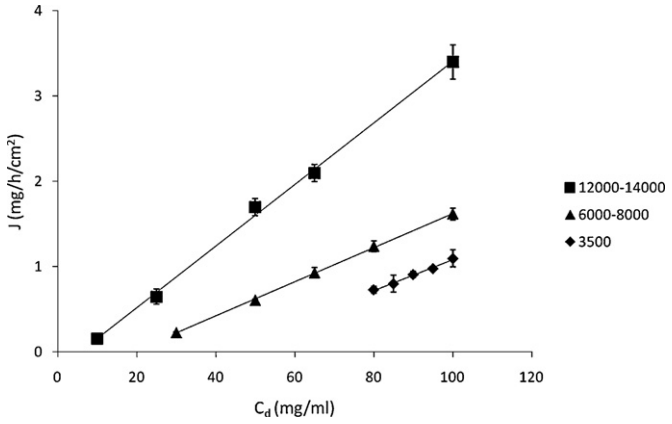

Fig. 4. The effect of initial concentration C_d on the flux *J* of sugammadex through monolayer cellophane membranes with different MWCO (shown is the mean of 2–4 replicates; error bars represent standard deviation).

by the so called membrane resistance. In order to penetrate through the semi-permeable membranes the sugammadex molecules must possess driving forces sufficient to overcome counter forces. It is well-known that the main driving force for permeation processes (i.e. passive transport) is the concentration gradient of the permeating species, whereas the most probable counter force is physicochemical interaction of sugammadex with membranes. Furthermore, the unstirred water layer covering the membrane can be an additional barrier for sugammadex permeation. As follows from Fig. 4, the resistance is related to membrane pore size: the larger the MWCO the less concentration gradient is needed to overcome counter forces. This can be explained as follows: sugammadex molecules can interact non-covalently with pore walls while penetrating. As pores get wider the fraction of sugammadex molecules interacting with pore walls diminishes due to increased distance and the overall membrane resistance gets weaker. Moreover, increase of membrane MWCO leads to an increase in the apparent permeability coefficient (Table 2 and Eq. (2)).

3.2. Dynamic light scattering

DLS is a conventional method used for self-association assessment of cyclodextrins and other oligomers (Gonzalez-Gaitano et al., 2002; Ukhatskaya et al., 2010). If self-association occurs one or several populations of particles with hydrodynamic radius larger than that of a single hydrated molecule emerge and often progressively grow with increasing concentration.

Fig. 5a shows the raw correlation data obtained with DLS from filtrated sugammadex solutions. The difference in concentration between the samples is clearly seen in the ordinate intercept which decreases with decreasing concentration. All correlation curves represent a single species of sugammadex with a $D(h)$ of approximately 0.8 nm as shown in Fig. 5b, which is comparable to the sugammadex molecule dimensions (cavity depth ~1.1 nm and cavity width 0.6–1.1 nm) (Adam et al., 2002) and therefore is related to a monomeric form of sugammadex. In Fig. 5c the $D(h)$ obtained from the volume distributions is plotted against C_d and shows no concentration dependence. Also no increase in *z*-average is observed (data not shown). However, there is a possibility that small associates are present which cannot be separated from the main species using this technique. This is the case when self-associates differ less than roughly a factor 3 in size from the main species. As an indication of the presence of such species the polydispersity index (PDI) of a sample can be used. If self-association of sugammadex would occur then also the PDI would increase with increasing concentration. Fig. 5d shows the PDI at each concentration. No increase in PDI can be observed.

Filtration is a compulsory step for DLS measurements aiming to separate dust appearing in the tested solution during its preparation due to the contact with laboratory environment. Nevertheless, there is a risk of associates rejection whose diameter exceeds filter pore size thus discounting them in solution analysis. To avoid artifactual results unfiltered samples were also measured. It was observed that in unfiltered samples a small amount of large particulates was present. In contrast to Fig. 5a, Fig. 6a shows the raw correlation data which clearly indicates the presence of two species. This is confirmed in Fig. 6c which shows the intensity distribution of this sample, showing two species with a $D(h)$ of the main species of approximately 1 nm (0.8 nm in volume) and vast population of species ranging from 100 to 10,000 nm. Since large species can only be observed in the intensity distribution and not in the volume distribution (Fig. 6b), it is concluded that these species are of very low abundance. Moreover, it is known that the mass fraction of particles detected by DLS is directly proportional to the intensity but reversely proportional to cubic $D(h)$ that makes fractions of big particles with low intensity tending to zero (Gonzalez-Gaitano et al., 2002). DLS is extremely sensitive for small amounts of large species which therefore can only be observed in the intensity distributions.

To check if large species detected by DLS related to sugammadex associates the measurements of the unfiltered solution were repeated at different temperatures. It is based upon knowledge that cyclodextrin self-associates are normally metastable (since van der Waals forces and hydrogen bonds that play a fundamental role in association are relatively weak) and are notably depressed or completely disappear at moderate heating (Gonzalez-Gaitano et al., 2002). Fig. 7 shows that these species do not disappear upon heating up to 45 °C, indicating that it is unlikely that they are large self-assemblies of sugammadex.

3.3. Sedimentation equilibrium analytical ultracentrifugation

Despite the small size of sugammadex, SE-AUC is very well suited to determine the molecular weight (MW) and association state of sugammadex in solution. To determine association ability of sugammadex a dilution series of sugammadex was analyzed. A concentration range from 0.25 to 25 mg/ml (filtrated) was chosen based on the lower and upper sugammadex concentration limits of this method. Similar as with DLS in the case of self-association the molecular weight (which, in fact, is the species weight) should increase with concentration. For this purpose, the molecular weight at each concentration was determined using the non-interacting discrete species model in SEDFIT.

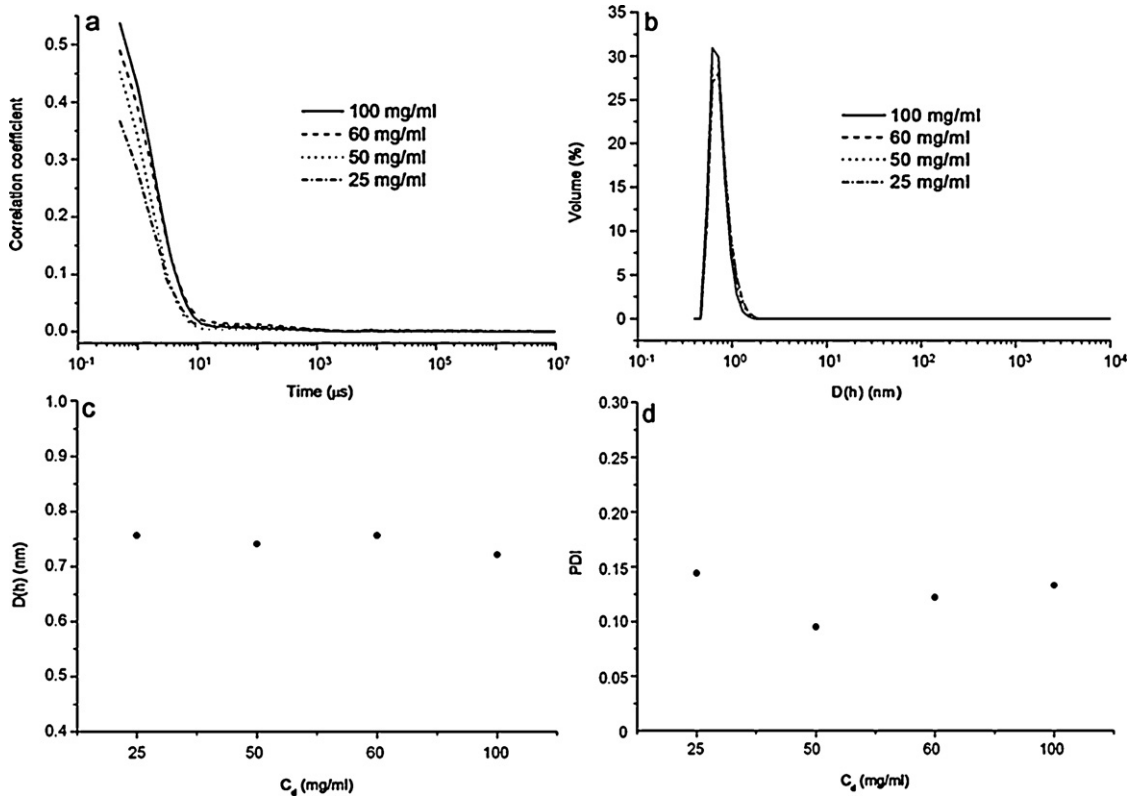

Fig. 5. DLS results of a dilution series of sugammadex filtered solutions: (a) the correlation data obtained at each concentration; (b) the volume distribution at each concentration; (c) the hydrodynamic diameter determined for each concentration plotted against the concentration; (d) the polydispersity index for each concentration plotted against the concentration.

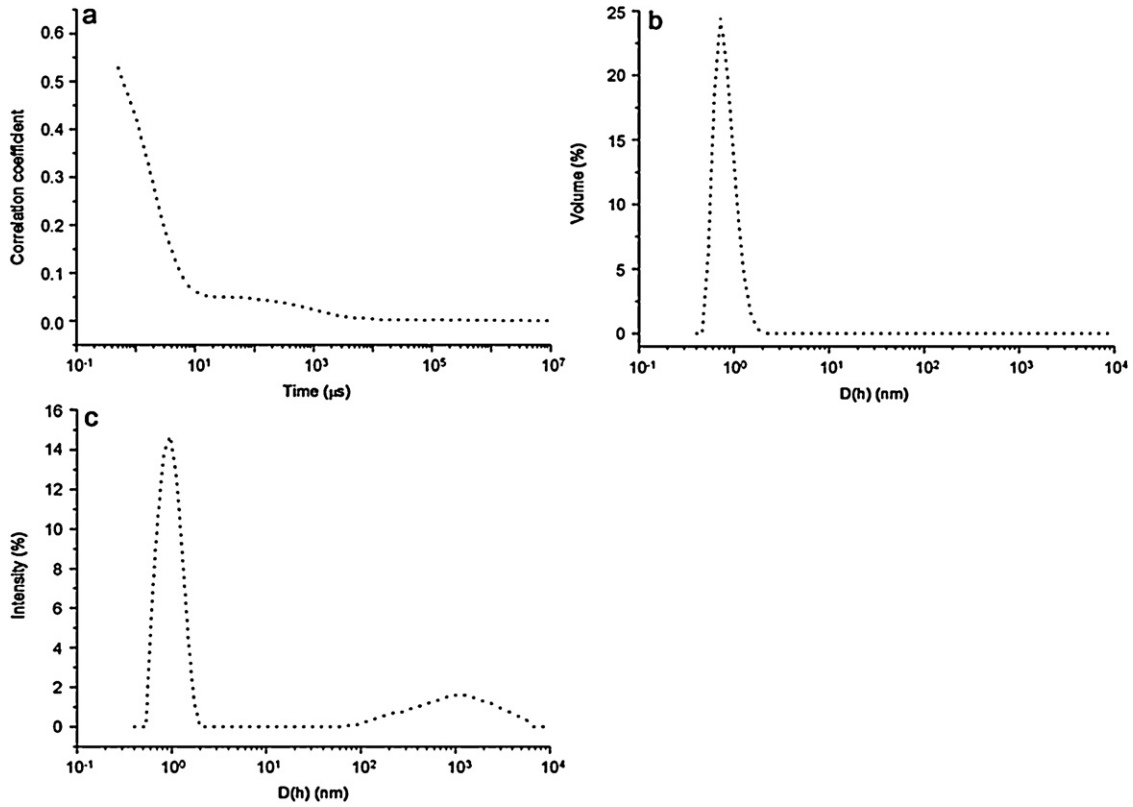

Fig. 6. DLS results of 50 mg/ml sugammadex unfiltered solution: (a) the correlation data; (b) the volume distribution; (c) the intensity distribution.

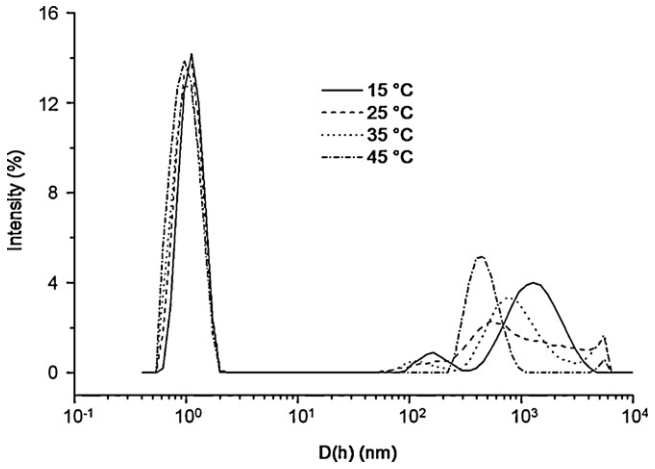

Fig. 7. Intensity distributions of 100 mg/ml sugammadex unfiltered solutions at different temperatures.

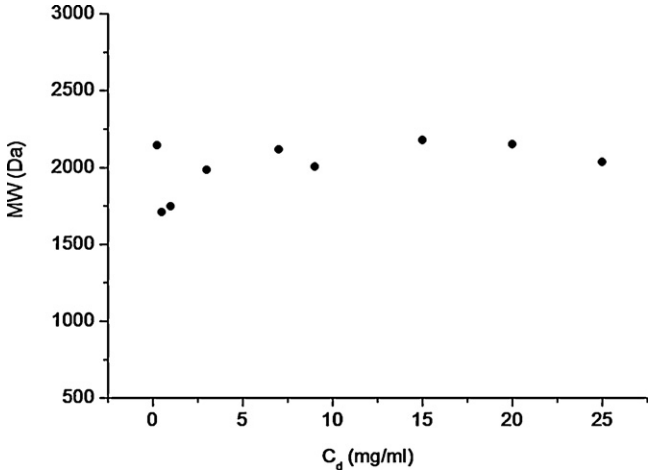

Fig. 8. Plot of molecular weight at different sugammadex concentrations determined using SE-AUC.

Fig. 8 shows the MW at each concentration, as determined with the non-interacting discrete species model, plotted against the concentration. As can be seen there is no increase in MW at higher concentrations indicating that in this concentration range no self-association of sugammadex occurs. These results are corroborated by more sophisticated global data analysis implemented in the SEDPHAT software. The data could be fitted to a model describing non-interacting discrete species on samples ranging from 3 to 9 mg/ml (RMSD < 0.004). Applying models which describe monomer–dimer or monomer–multimer associations resulted in worse fits (RMSDs of >0.15 and >0.01, respectively), indicating that these models are not suitable to describe the data.

4. Conclusions

Aqueous solutions of the γCD derivative sugammadex, the first selective relaxant binding agent, were comprehensively studied with regard to self-association phenomena. The sugammadex molecules with large van der Waals surface and presence of electronegative atoms such as sulfur and oxygen, that are able to participate in hydrogen bonding, could in theory be subject to self-association. However, the application of three methods based on different physicochemical principles yielded consistent results showing no indication of self-association in the studied solutions (0.25–100 mg/ml). Possibly, the massive motifs attached to the primary rim of γCD core cause steric hindrance for intermolecular hydrogen bonding of secondary hydroxyls. Moreover, negative charges of carboxylic groups attached to the edges of sugammadex "tails" must play a critical role in repulsion of molecules from each other making "solute–solute" interaction unfavorable. The present work demonstrates that sugammadex does not self-assemble in aqueous solutions, such as drug product, to form aggregates at pharmaceutically relevant concentrations. Therefore, it is unlikely that such self-association complexes of sugammadex are detrimental to the safety profile or therapeutic benefit of the product.

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