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Miscellaneous nanoaggregates made of β-CD esters synthesised by an enzymatic pathway

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Abstract

Various β-cyclodextrin (β-CD) fatty esters with different chain lengths (C4–C14) were synthesised by transesterification of β-cyclodextrin by vinyl fatty ester using thermolysin in DMSO. For each cyclodextrin derivatives, two batches of synthesis were realized. The ability of these derivatives to form nano-organized systems was investigated through the solvent displacement technique. During the formulation step, the effects of the initial concentration of β-CD fatty esters in the organic phase and that of the final volume of the aqueous non-solvent phase were studied. Except for the β-CD C4 ester, the transesterified β-CD derivatives led to measurable nanoparticles. Cryo-electron microscopy images showed a significant morphological variability. Spherical, rod-like or more irregularly-shaped nano-objects were observed with either matricial or lamellar structures. A statistical analysis by a two-way ANOVA was computed for each class of β-cyclodextrin esters in order to determine the effects of batch and formulation on the final size of nanoparticles.

Keywords: Thermolysin transesterification; β-cyclodextrin fatty esters; Nanosystems; Quasi-elastic light scattering; Cryo-transmission electron microscopy; Variance analysis

1. Introduction

α-, β- and γ-cyclodextrins are cyclic oligosaccharides made of six, seven and eight α-1.4-glycosidic-linked glycosyl residues, respectively (Biwer et al., 2002). Cyclodextrins have been widely used as pharmaceutical excipients to formulate drugs with poor bioavailability. Indeed, owing to their particular structure, which consists of a hydrophilic external surface and a more hydrophobic cavity lined with protons, cyclodextrins are capable of forming inclusion complexes with a variety of guest molecules, improving the solubility and/or stability of the guest compounds. Numerous chemical modifications have been carried out on cyclodextrins by grafting substituents to different positions (primary face, secondary face or both faces) in order to obtain amphiphilic derivatives able to form supramolecular aggregates in the form of nanoparticles considered as potential drug carriers (Davis and Brewster, 2004). Among the modifications realized, a series of molecules have been obtained by grafting fatty acids of different chain lengths on the hydroxyl groups of the secondary face. Two different synthetic ways have been investigated. The first approach involves a three-step chemical modification: (i) protection of primary hydroxyl groups, (ii) acylation of secondary face, (iii) deprotection of primary face (Zhang et al., 1991). The second approach involves the use of a thermolysin as a biocatalyser of transesterification of cyclodextrin by a vinyl fatty ester donor (Pedersen et al., 2005). Recently, we have shown that the formulation of β-CD capric ester derivative (β-CD C10) yielded hybrid structures presenting a crystal-like core surrounded by onion-like concentric bilayers, whereas β-CD caproic ester (β-CD C6) formed supramolecular assemblies presenting a homogeneous matrix (Choisnard et al., 2006).

From these results, numerous cyclodextrin derivatives have been synthesized by grafting alkyl chains of variable lengths

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(C4–C14) on the β-cyclodextrin. The products were tested for their ability to nano-aggregate. The morphological properties of some newly obtained cyclodextrin aggregates were investigated by cryo-transmission electron microscopy (cryo-TEM) and quasi-elastic light scattering (QELS). The results were statistically analyzed.

2. Materials and Methods

2.1. Materials

Thermolysin (EC 3.4.24.27), a protease Type X isolated from *Bacillus thermoproteolyticus rokko*, anhydrous DMSO (99%), vinyl capric ester (95%) and celite were obtained from Sigma Aldrich (Sigma Aldrich, L'Isle D'Abeau Chesnes, France). Vinyl caproic ester (99%), vinyl caprylic ester (>99%), vinyl myristic ester (>99%) and vinyl butyric ester (>97%) were purchased from TCI Europe nv (Interchim, Montluçon, France). Vinyl Lauric ester (>99%) was received from Fluka (Sigma Aldrich, L'Isle D'Abeau Chesnes, France). β-cyclodextrin (Kleptose®) was a gift of Roquette (Roquette, Lestrem, France). Anhydrous acetone was obtained from VWR (VWR International, Lyon, France). Water was freshly deionized in our laboratory.

2.2. Enzymatic reactions and products characterization

Thermolysin was immobilized on celite using a deposition technique according to the procedure previously reported (Choisnard et al., 2006). Before use, β-CD was dried under reduced pressure (1 mbar) for 48 h at 80 °C in presence of P_2O_5, the residual water content was not measured. Ten millilitres of dry β-CD (0.09 M) and vinyl fatty acid (1 M) in DMSO were placed in a capped glass vial containing 1.1 g of immobilized thermolysin. The heterogeneous medium was vigorously stirred (350 rpm) and the reaction was carried out at 45 °C for 72 h or until the vinyl fatty acid upper-phase was completely disappeared. At the end of the reaction, the immobilized thermolysin was removed from the liquid phase by centrifugation (1500 rpm; 15 min). The β-CD esters present in supernatant were precipitated by adding drop-wise a volume of 20 mL of MeOH/H_2O (35/65; v/v) and separated by centrifugation (2000 rpm; 15 min). The crude product was washed twice with 10 mL of DMSO/MeOH/H_2O (50/35/65; v/v/v) and purified by silica gel flash column chromatography using first CH_3Cl and then CH_3Cl/MeOH (10/90 or 50/50; v/v) as mobile phase. β-CD fatty ester was dried under reduced pressure and stored at room temperature. All the β-CD derivatives were analyzed by ^{1}H NMR, ^{13}C NMR and Heteronuclear Multiple-Quantum Coherence NMR (HMQC-NMR). The observations were in agreement with those previously published by Choisnard et al. (2006) and Pedersen et al. (2005). The β-CD trans-esterification occurred mainly at the C_2 position and the substitutions on C_3 and C_6 positions were considered marginal. Typical results of matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS) analyze of the derivatives are reported in Table 1.

Table 1
MALDI-MS analysis summary ($m/z + Na^+$) for β-CD butyric (β-CD C4), caproic (β-CD C6), caprylic (β-CD C8), capric (β-CD C10), lauric (β-CD C12) and myristic (β-CD C14) ester

	Number of acylations per β-cyclodextrin fatty ester molecule								
	2	3	4	5	6	7	8	9
β-CD C4	×	×	×	×	m/z = 1578 (1%)	m/z = 1648 (100%)	m/z = 1718 (29%)	×
β-CD C6	×	×	×	m/z = 1647 (1%)	m/z = 1745 (18%)	m/z = 1843 (100%)	m/z = 1943 (20%)	m/z = 2041 (1%)
β-CD C8	×	×	×	m/z = 1788 (5%)	m/z = 1915 (55%)	m/z = 2041 (100%)	m/z = 2167 (4%)	×
β-CD C10	×	×	×	×	m/z = 2083 (22%)	m/z = 2237 (100%)	m/z = 2391 (22%)	×
β-CD C12	m/z = 1522 (8%)	m/z = 1704 (25%)	m/z = 1887 (54%)	m/z = 2069 (100%)	m/z = 2251 (82%)	m/z = 2434 (29%)	m/z = 2616 (1%)	×
β-CD C14	×	×	×	×	m/z = 2419 (11%)	m/z = 2630 (100%)	m/z = 2840 (8%)	×

The relative intensity of peak signals is reported between parentheses.
×: compound not detected.

2.3. Nanoparticle preparation

The nanoparticle suspensions were prepared in triplicate using the solvent displacement technique (Skiba et al., 1996; Gèze et al., 2002). Ten milligrams (Formulation type I) or 20 mg (Formulation type II) of β-CD fatty ester were dissolved in 10 mL of acetone. The temperature of organic phase was adjusted to 25 °C with a double envelope thermostated water flow. This solution was slowly poured into 10 mL (Formulation type I) or 20 mL (Formulation type II) of stirred distilled water maintained at 25 °C using a double envelope system. The nanoparticles were formed immediately and the colloidal suspension was submitted to evaporation under reduced pressure to remove the organic solvent. The resulting colloidal suspension was filtered through a 0.8 μm membrane (Millex AA, Millipore, France) and stored in closed vials at room temperature.

2.4. Particle size measurements

The size of freshly made nanoparticles in water suspension was measured in triplicate by quasi-elastic light scattering (QELS) using a Zetasizer 3000 instrument (10 mW HeNe laser at 632.8 nm, K7132 correlator, Malvern instruments). Experimental conditions were the following: temperature 25 ± 0.1 °C, reference angle 90°, viscosity 0.899×10^{-3} Pa.s. *Z*-average hydrodynamic diameter (Dh) of the particles was calculated using a cumulant algorithm with Zetasizer 3000 software version V. 1.51. Dh values were derived from the measured translational diffusion coefficient of particles moving under Brownian motion. Sigmastat v3.5 (Systat Software) was used for the statistical analysis of the particle Z-average hydrodynamic diameter measurements.

2.5. Cryo-transmission electron microscopy

The cryo-transmission electron microscopy was performed on aqueous suspensions of nanoparticles resulting from Formulation type I only. One millilitre of colloidal suspension was centrifuged (10 000 rpm, 10 min, 25 °C) in order to reach a concentration of 5 mg/mL. According to the method described elsewhere (Durrieu et al., 2004), specimens for cryo-TEM were prepared by quench-freezing thin liquid films of 5 mg/mL (w/v) β-CD fatty ester suspensions into liquefied ethane (−171 °C). Once transferred in a Gatan 626 cryoholder cooled down with liquid nitrogen, the specimens were observed at low temperature (−180 °C), using a Philips CM200 "cryo" microscope operating at 80 kV. The low dose procedure (Philips) was used to prevent any detrimental radiation damage in the areas of interest before actual image recording on Kodak SO163 films. The cryo-TEM experiments were performed twice for each sample, using two different magnifications. Depending on the sample and the particle concentration in the images, an average of 50–100 particles were observed.

3. Results

The ability of β-CD fatty esters to self-assemble into nanoparticles was evaluated using the well-known solvent displacement

Table 2
Z-average hydrodynamic diameters and polydispersity index (PI) of nanoaggregates obtained with β-CD fatty derivatives using the Formulation I and II

Formulation I	β-CD C4		β-CD C6		β-CD C8		β-CD C10		β-CD C12		β-CD C14	
Batch of synthesis	A	B	A	B	A	B	A	B	A	B	A	B
Z-average hydrodynamic diameter ± S.D. ($n = 3$)	–	–	229 ± 24	223 ± 19	223 ± 3	216 ± 6	118 ± 6	102 ± 14	113 ± 11	170 ± 12	147 ± 1	134 ± 8
Polydispersity index ± S.D. ($n = 3$)	–	–	0.10 ± 0.04	0.08 ± 0.03	0.18 ± 0.07	0.05 ± 0.04	0.08 ± 0.02	0.09 ± 0.01	0.06 ± 0.04	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.02
Formulation II	β-CD C4		β-CD C6		β-CD C8		β-CD C10		β-CD C12		β-CD C14	
Batch of synthesis	A	B	A	B	A	B	A	B	A	B	A	B
Z-average Hydrodynamic diameter ± S.D. ($n = 3$)	–	–	175 ± 2	201 ± 6	90 ± 2	87 ± 1	99 ± 1	95 ± 1	90 ± 1	120 ± 4	122 ± 8	120 ± 6
Polydispersity index ± S.D. ($n = 3$)	–	–	0.09 ± 0.01	0.04 ± 0.02	0.05 ± 0.04	0.08 ± 0.01	0.06 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.03

technique. The organic phase concentration of β-CD fatty esters involved in Formulations I and II were 1 and 2 mg/mL, respectively. In both formulations, the volume of the final non-solvent aqueous phase was adjusted to obtain a β-CD fatty ester concentration of about 1 mg/mL. Firstly, the presence of organized nanoparticles was confirmed using QELS experiment (Table 2). All the *z*-average measurements were obtained with a percent of merit varying between 60 and 70%. The smallest nanoparticle sizes were measured between 87 and 90 nm when β-CD C8 was involved in Formulation II. The largest nanoparticles

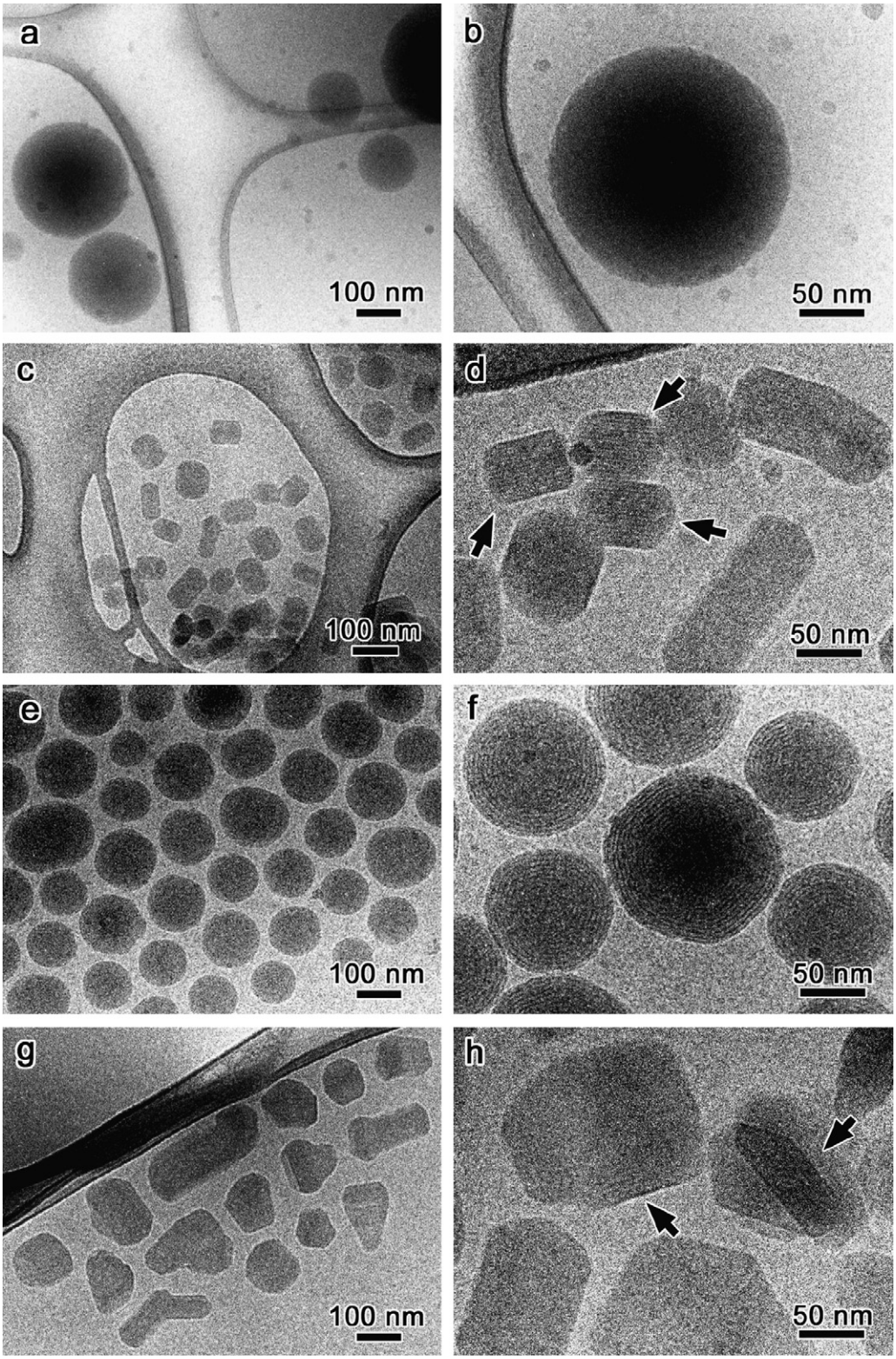

Fig. 1. Cryo-TEM images of β-CD nanoparticles embedded in vitreous ice, recorded at two different magnifications: (a,b) β-CD C6; (c,d) β-CD C8; (e,f) β-CD C10; (g,h) β-CD C14. The black arrows in d and h indicate parallel fringes that can be seen on selected particles.

had a size between 223 and 229 nm when using β-CD C6 in the conditions of Formulation I. Depending on cryo-TEM images of nanoparticles made of β-CD C6, β-CD C8, β-CD C10 and β-CD C14 derivatives and embedded in a thin film of vitreous ice are shown in Fig. 1. In all suspensions, no significant aggregation was observed, although the nanoparticles were prepared without any stabilizing agent. The average size of the nanoparticles measured from the images was in good agreement with that measured by QELS (Gèze et al., 2004). The shape of the nanoparticles clearly depends on the β-CD ester used in the formulation. The β-CD C6 particles were spherical (Fig. 1a) with a homogeneous contrast and without any visual evidence of inner organization at the magnification that was used for imaging (Fig. 1b). The β-CD C8 particles were slightly smaller (Fig. 1c). They looked mostly rectangular in projection, suggesting a barrel-like shape. At a higher magnification, parallel fringes could be seen on selected particles (Fig. 1d). They always ran parallel to the longer axis of the particles and might correspond to the projection of a specific inner molecular organization or the particle seen along favorable projection axes. β-CD C10 formed larger and spherical particles (Fig. 1e). As shown in Fig. 1f and as already reported (Choisnard et al., 2006), the nanospheres exhibited an original multilamellar structure, with concentric bilayers surrounding a crystal-like core. By analogy with the structure of multilamellar phospholipid particles (Roux et al., 2004), we proposed a tentative model describing the organization of amphiphilic β-CD C10 in alternating hydrophilic and hydrophobic bilayers formed by the cyclodextrins and alkyl chains, respectively (Choisnard et al., 2006). Both β-CD C12 (not shown) and β-CD C14 particles exhibited irregularly facetted shapes (Fig. 1g). This faceting and the diffraction contrast that were often observed at low magnification in some regions of selected particles also strongly suggested an organized structure. Indeed, parallel fringes could sometimes be observed (Fig. 1h), but additional imaging at a higher magnification would be necessary to determine the nature of the molecular organization in β-CD C14 particles. Moreover, X-ray diffraction analyses of the different samples are in progress in order to collect additional data and provide more detailed information on the inner structure of the various nanoparticles.

4. Discussion

One can note that the transesterification using thermolysin was successfully applied to vinyl fatty esters from C4 to C14 chain length. As already observed by Pedersen et al. (2005), the derivatives were a mixture of various acylated species (Table 1). For example, the MALDI-MS spectrum of β-CD C12 ester present a rate of molecular substitution from 2 to 8, which is quite similar to the result previously reported by Pedersen et al. (2005). Moreover, in other classes of β-CD esters, it was observed that the more intense signal was attributed to the seven substituted species.

Both formulations were carried out to investigate the influence of aqueous to organic phase ratio and the initial concentration of β-CD fatty ester solubilised in organic phase on the final nanoparticle size (Table 2). All the classes of β-CD fatty esters except the β-CD butyric ester derivative (β-CD C4) lead to supramolecular nanoaggregates presenting different morphologies and mean range between 90 and 230 nm. Note that the preparation yields were almost near a hundred per cent. In the special case of β-CD C4, the following phenomena were observed during the nanoprecipitation procedure: after pouring the organic solution of cyclodextrin into the aqueous phase, nanospheres did not spontaneously form as generally expected. After removing the organic phase by evaporation under reduced pressure, it was observed a loss of β-CD C4 material sticking on the inner wall of the vessel, while the other part remained in the aqueous phase. It is likely that β-CD C4 self-assembles in water in the form of nanostructures presenting low light scattering intensities which could not be detected by the Zetasizer 3000 apparatus. The presence of micelles was hypothesized. It was then assumed that the alkyl chain length and the number of acylation per β-CD molecule may play a key role on the ability of β-CD fatty esters to self-organize. These relationships are still under investigation. However, our results show that esters of β-CD bearing alkyl chains from six carbons to longer aliphatic chains lead to nanoparticles with a size range around or up to a hundred nm.

Before going thoroughly into statistic analysis of nanoparticle size, the normality of the crude data distribution of *Z*-average hydrodynamic diameter values was checked using a

Table 3
Z-average hydrodynamic diameter two-way analysis of variance (ANOVA) procedure

β-CD fatty ester species	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*	*p*-value	Conclusion ($\alpha = 1\%$)
β-CD C6	Batch	1	331	331	1.343	0.280	NS
	Formulation	1	4294	4294	17.438	0.003	S
β-CD C8	batch	1	79	79	6.866	0.031	NS
	Formulation	1	51326	51326	4457.632	<0.001	S
β-CD C10	batch	1	506	506	8.785	0.018	NS
	Formulation	1	299	299	5.194	0.052	NS
β-CD C12	batch	1	5764	5764	81.857	<0.001	S
	Formulation	1	4070	4070	57.800	<0.001	S
β-CD C14	batch	1	171	171	4.266	0.073	NS
	Formulation	1	1058	1058	26.401	<0.001	S

NS: not significant test for difference, S: significant test for difference ($\alpha = 1\%$).

Shapiro–Wilk W test (p-value < 0.05).The two-way analysis of variance (ANOVA) procedure reported in Table 3 summarizes the comparison of the mean value of nanoparticle Z-average hydrodynamic diameters prepared from two batches of enzymatic synthesis, across both types of Formulations (I and II) which were made in triplicate (Table 2).

As reported in Table 3, for β-CD C6, β-CD C8, β-CD C10 and β-CD C14, the difference in the Z-average hydrodynamic diameter values for the two batches of synthesis of the same class of β-CD fatty ester is not large enough to exclude the possibility that the difference is just due to random sampling variability. The difference is not statistically significant ($p > 1\%$). Consequently, the z-average values obtained from two different batches of synthesis for a same β-CD fatty ester derivative could be averaged.

However, for β-CD C12, the difference in the Z-average hydrodynamic diameter values between the two batches of synthesis is larger than would be expected by random error. In this case, there is a statistically significant difference ($p < 1\%$). For this reason, it was decided to avoid the evaluation of the real effect of the β-CD C12 derivative on the formulation. This result may be correlated to the low esterification rate which was observed for this derivative in MALDI-MS analysis (Table 1). Indeed, the most intense signal was obtained for the five acylated species, whereas the most intense signal in the case of the others β-CD fatty esters was at seven acylations. Further new β-CD C12 synthesis will be carried out in the future to clarify this point. The statistical analysis of the effect of formulation type was performed only on the derivatives which presented no statistical difference between batch to batch synthesis.

Based on a similar statistical approach, it can be concluded that the difference in the Z-average hydrodynamic diameter values between both formulations were statistically significant ($p < 1\%$) for the β-CD C6, β-CD C8, β-CD C10 and β-CD C14 samples. The nanoparticles prepared according to Formulation II were smaller than the nanoparticles obtained according to Formulation I. These results agree with the observations previously made regarding the influence of aqueous to organic phase ratio on the size of spherical nanoaggregates (Choisnard et al., 2005). In order to decrease the nanosphere size, the nanoprecipitation should be achieved with a large amount of water and small amount of organic solvent. The smallest nanoparticles were obtained with β-CD C8 using the Formulation II (90 nm). The largest nanoparticles were obtained with β-CD C6 using Formulation I (220 nm). Except for the β-CD C12 derivative which presents a batch effect, all the other averaged sizes are summarized in Fig. 2. Whatever the nature of the formulation investigated, the β-CD C6 formed nanoparticles with the largest Z-average hydrodynamic diameter values. It is likely that the grafting short acyl chain length, that is, hexanoyl chains yields a β-CD fatty esters with a molecule geometry which is favorable to the self-assembly into large size nanoparticles.

In conclusion, the efficient one-step enzymatic synthesis using thermolysin as biocatalyser yielded β-CD fatty esters bearing alkyl chain lengths from C4 to C14. Ongoing studies of the enzymatic synthesis and purification steps are being carried out. The MALDI-MS characterization of the derivatives will be advantageously completed using chromatographic analysis. Various architectures made of organized modified cyclodextrin molecules were obtained for β-CD C6, β-CD C8, β-CD C10, β-CD C12 and β-CD C14 derivatives. For a better understanding of the ultrastructure of the nanoparticles, further high-resolution cryo-TEM imaging combined with X-ray diffraction experiments will be performed.

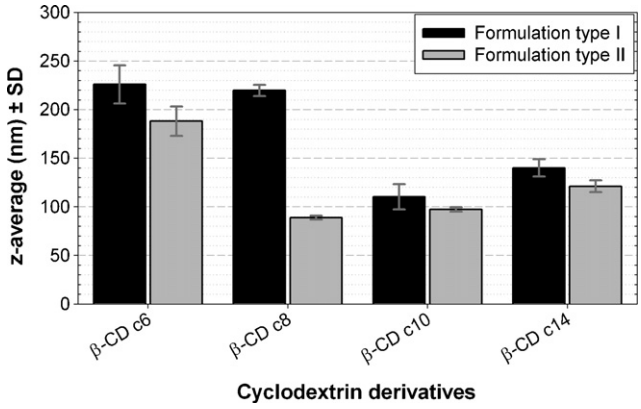

Fig. 2. Z-average hydrodynamic diameter values of nanoparticles achieved with β-CD C6, β-CD C8, β-CD C10 and β-CD C14 derivatives. S.D.: standard deviation.

The variety of particles shown in this work may offer the opportunity to host drug molecules at different levels within the nanostructure, that is, surface adsorption, matrix entrapment, interaction with the cavity or with the aliphatic chains. Consequently, interesting features in terms of drug protection and drug release from the nanocarriers could reasonably be expected.

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