Monodisperse Nanoparticles from Self-Assembling Amphiphilic Cyclodextrins: Modulable Tools for the Encapsulation and Controlled Release of Pharmaceuticals

Alejandro Mendez-Ardoy,[1] Marta Gómez-García,[1] Annabelle Gèze,[2] Jean-Luc Putaux,[3] Denis Wouessidjewe,[2] Carmen Ortiz Mellet,[1] Jacques Defaye,[2,*] José M. García Fernández,[4] and Juan M. Benito[4,*]

[1]*Dpto de Química Orgánica, Facultad de Química, Universidad de Sevilla, E-41012 Sevilla, Spain*

[2]*Dépt de Pharmacochimie Moléculaire, Université de Grenoble/CNRS, UMR 5063/ICMG-FR 2607, F-38041 Grenoble, France*

[3]*Centre de Recherches sur les Macromolécules Végétales, CNRS, LP 5301, F-38041, Grenoble, France*

[4]*Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, E-41092 Sevilla, Spain*

Abstract: Selective chemical functionalization of cyclodextrins (CDs) is a readily amenable methodology to produce amphiphilic macromolecules endowed with modulable self-organizing capabilities. Herein, the synthesis of well-defined amphiphilic CD derivatives, with a "skirt-type" architecture, that incorporate long-chain fatty esters at the secondary hydroxyl rim and a variety of chemical functionalities (e. g. iodo, bromo, azido, cysteaminyl or isothiocyanato) at the primary hydroxyls rim is reported. Nanoprecipitation of the new CD facial amphiphiles, or binary mixtures of them, resulted in nanoparticles with average hydrodynamic diameters ranging from 100 to 240 nm that were stable in suspension for several months. The precise size, zeta potential and topology of the nanoparticles are intimately dependent on the functionalization pattern at the CD scaffold. Highly efficient molecular encapsulation capabilities of poorly bioavailable drugs such as diazepam (DZ) were demonstrated for certain derivatives, the drug release profile being dependent on the type of formulation (nanospheres or nanocapsules). The efficiency and versatility of the synthetic strategy, together with the possibility of exploiting the reactivity of the functional groups at the nanoparticle surface, offer excellent opportunities to further manipulate the carrier capabilities of this series of amphiphilic CDs from a bottom-up approach.

Keywords: Cyclodextrins, controlled drug release, drug delivery, facial amphiphiles, nanoparticles, nanospheres, nanocapsules, self-assembly.

1. INTRODUCTION

Cyclodextrins (CDs) are naturally occurring cyclic oligosaccharides composed by α-(1→4)-linked glucose units, arising from enzymatic degradation of starch, that hold a privileged position in supramolecular chemistry [1]. The most relevant members of this family are the commercially available hexa-, hepta- and octamers (α-, β-, and γCD, respectively). The three-dimensional structure resembles that of an empty truncated cone, with the glucopyranosyl α- and β-face pointing to the inner and outer space, respectively (Fig. **1**), and the hydroxyl groups flanking the wider (OH-2 and OH-3) and narrower rims (OH-6). As a consequence, the hydroxylated exterior of CDs is hydrophilic, while the interior, mostly coated by methinic groups, is rather hydrophobic. Such architecture provides CDs with a singular 'inner-outer' amphiphilic character and endows them with molecular inclusion capabilities, which have been extensively exploited by the pharmaceutical industry to improve bioavailability of poorly soluble or biodegradable drugs, to prevent undesired effects or to enhance permeability of biological membranes [2].

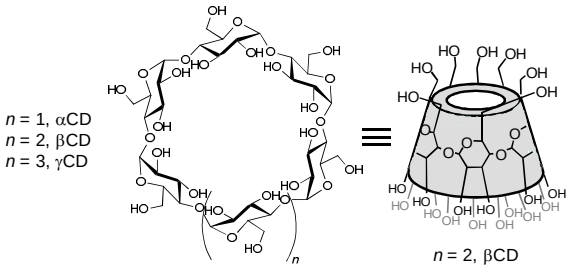

Fig. (1). Structure and schematic representation of native CDs.

A number of drug-CD complexes have been marketed (basically small drugs and drug-like molecules) [2-7] and applications have been extended to the agrochemical [8], cosmetic [9] and food industry [10,11]. A main limitation for these channels is that only the native α-, β- and γCD, to-

*Address correspondence to these authors at the Dépt. de Pharmacochimie Moléculaire, Institut de Chimie Moléculaire de Grenoble (CNRS – Univ. de Grenoble, UMR 5063, FR 2607), Bât. E André Rassat, BP 53, F-38041 Grenoble, France; Tel: +33 476635323; Fax: +33 476635313; E-mail: jacques.defaye@ujf-grenoble.fr;
Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, Américo Vespucio 49, Isla de la Cartuja, E-41092 Sevilla, Spain; Tel: +34 954479560; Fax: +34 954460565; E-mail: juanmab@iiq.csic.es.

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gether with a few synthetic derivatives, are available at the required scale to realistically consider bulk applications. Moreover, their molecular inclusion capabilities cannot be specifically tuned to each potential guest and, due to cavity size restrictions, the loading capacity is limited to one guest molecule per CD molecule in the best case. Even for suited CD:guest pairs, most of these complexes feature association constants (K_{as}) falling into the 10^2-10^4 M^{-1} range [12,13], which might be too low to prevent complex dissociation upon dilution.

To alleviate these drawbacks, synthetic chemists have created a number of selectively modified CD derivatives aimed at cooperatively interact with selected guests in order to attain improved guest complexation [14]. The traditional strategy towards this goal involves the covalent clustering of CD moieties into oligo- or polymeric species [3,15]. More recently, non-covalent self-assembling strategies, which generally involve amphiphilic CD derivatives, are taking over [16-18]. Self-assembling features may be theoretically tailored by modifying the amphiphilic CD structure to attain enhanced encapsulation or scheduled dissociation and release in response to external stimuli. Such pre-organized systems are expected to be more easily degraded in biological media and less prone to exert toxic or immunogenic responses *in vivo* that covalent conjugates. Depending on their decoration pattern, CD-based facial amphiphiles may spontaneously arrange into vesicles [19], nanoparticles [20], micellar systems [21] or even liquid crystals [22]. Since the first disclosure of amphiphilic CDs by Kawabata *et al.* [23], a number of promising applications for controlled drug release [17] and gene delivery [24,25], have been reported, illustrating the potential of CD-based self-assembling systems.

Adjustment of the self-organizing capabilities of amphiphilic CDs requires a precise control of the molecular topology, which critically depends on the availability of efficient functionalization methods of the CD scaffold. The development of strategies for the selective chemical functionalization of CDs has allowed the elaboration of nanoarchitectures engineered to perform a variety of tasks, e.g. targeting [26], sensing [27] or catalysis [28]. Nevertheless, the most relevant research in the field of CD-based nanoaggregates relay on synthetic methodologies which results in heterogeneous products [21]. Even relatively simple reactions, such as acylation, have been reported to lead to mixtures when applied to CDs [29,30], which could eventually hamper reproducibility of the aggregation and host encapsulation profiles from batch to batch.

We have recently reported a reliable and diversity-oriented approach for the synthesis of libraries of homogeneous polycationic amphiphilic CDs (paCDs) [31] that were shown to self-organize in the presence of plasmid DNA (pDNA), the mixed paCD-pDNA complexes (CDplexes) exhibiting remarkable gene delivery capabilities [32]. The synthetic strategy relies on the differential reactivity of primary vs secondary hydroxyls on the CD torus to homogeneously functionalize each rim with cationic elements and long fatty acyl tails, respectively. Herein, we describe the implementation of this synthetic scheme to elaborate a series of neutral facially-differentiated CD derivatives (Fig. **2**) and the preliminary assessment of their self-assembling features, their molecular encapsulation capabilities and the *in vitro* release profile of loaded nanospheres and nanocapsules using the non-polar anxiolytic drug diazepam as a model.

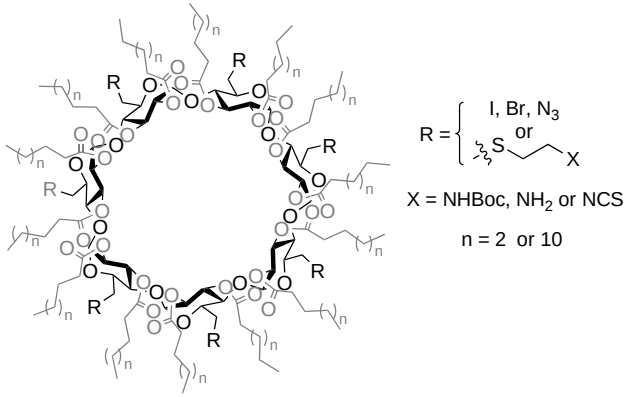


Fig. (2). General structure of the facially-differentiated CDs prepared in this work.

2. MATERIALS AND METHODS

2.1. Synthesis

Reagents and solvents were purchased from commercial sources and used without further purification. Optical rotations were measured at 20 ºC in 1-cm or 1-dm tubes on a Perkin-Elmer 141 MC polarimeter. IR spectra were recorded on a FTIR spectrometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 500 (125.7) and 400 (100.6) MHz. 2D COSY, 1D TOCSY, and HMQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with Kieselgel 30 F245 (E. Merck), with visualization by UV light and by charring with 10% H_2SO_4 in EtOH. Column chromatography was carried out on Silica Gel 60 (E. Merck, 230-400 mesh). Electrospray mass spectra (ESIMS) were obtained with a Bruker Esquire6000 instrument. MALDI-TOF mass spectra were acquired on a GSG System spectrometer operating in the positive-ion mode with an accelerating voltage of 28 keV. Samples were dissolved in water at millimolar concentration and mixed with a standard solution of 2,5-dihydroxybenzoic acid (DHB; 10 mg mL^{-1} in 10% aq EtOH, 2 μL) in 1:1 v/v relative proportions; 1 μL of the mixture was loaded onto the target plate, then allowed to air-dry at room temperature. Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain). Heptakis(6-deoxy-6-iodo)cyclomaltoheptaose (**1**), heptakis(6-bromo-6-deoxy)cyclomaltoheptaose (**2**), heptakis(6-bromo-2,3-di-*O*-hexanoyl)cyclomaltoheptaose (**5**), heptakis(6-bromo-6-deoxy-2,3-di-*O*-myristoyl)cyclomaltoheptaose (**6**), heptakis[6-(2-aminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltoheptaose (**11**), heptakis[6-(2-aminoethylthio)-2,3-di-*O*-myristoyl]cyclomaltoheptaose (**12**), and heptakis[2,3-di-*O*-hexanoyl-6-(2-isothiocyanatoethylthio)]cyclomaltoheptaose (**13**) were prepared according to the reported procedures [32,33]. Dimethylformamide, dichloromethane, tri-fluoroacetic acid and diazepam were named using the acronyms DMF, DCM, TFA, and DZ, respectively.

Heptakis(6-deoxy-2,3-di-*O*-hexanoyl-6-iodo)cyclomaltoheptaose (**3**). To a solution of heptakis(6-deoxy-6-

iodo)cyclomaltoheptaose [33] (**1**, 2.0 g, 1.04 mmol) in dry DMF (20 mL) under Ar at 0 ºC, DMAP (5.4 g, 44.0 mmol, 3 equiv) was added. Hexanoic anhydride (13.6 mL, 58.8 mmol, 4.0 equiv) was dropwise added, the reaction mixture was stirred at room temperature for 45 min, and MeOH (25 mL) was added. After 1 h, the solution was poured into ice-water (50 mL) and extracted with CH_2Cl_2 (4 x 50 mL). The organic phase was washed with dilute H_2SO_4 (2 x 50 mL), water and aqueous saturated $NaHCO_3$ (4 x 50 mL), dried (Na_2SO_4), concentrated and purified by column chromatography (1:12 → 1:10 EtOAc-petroleum ether) to give **3**. Yield: 2.32 g (68%); R_f = 0.40 (1: 5 EtOAc-petroleum ether); $[\alpha]_D$ = + 63.8 (*c* 1.0 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.32 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.14 (d, 7 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.79 (dd, 7 H, H-2), 3.73 (m, 14 H, H-5, H-6a), 3.60 (dd, 7 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6b}$ = 5.5 Hz, H-6b), 3.58 (t, 7 H, $J_{4,5}$ = 9.0 Hz, H-4), 2.39-2.16 (m, 28 H, CH_2CO), 1.56 (m, 28 H, C*H*$_2$CH$_2$CO), 1.29 (m, 28 H, CH$_3$C*H*$_2$), 1.23 (m, 28 H, CH$_3$CH$_2$C*H*$_2$), 0.89, 0.87 (2 t, 42 H, $^3J_{H,H}$ = 6.7 Hz, CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 173.2, 171.7 (CO), 96.5 (C-1), 80.5 (C-4), 70.1 (C-2), 69.9 (C-5), 69.8 (C-3), 34.0, 33.8 (*C*H$_2$CO), 31.4, 31.3 (CH$_3$CH$_2$*C*H$_2$), 24.4, 24.3 (*C*H$_2$CH$_2$CO), 22.4, 22.3 (CH$_3$*C*H$_2$), 13.9 (CH_3), 8.6 (C-6); MALDI-TOFMS: *m/z* 3300.5 $[M + Na]^+$. Anal. Calcd for $C_{126}H_{203}I_7O_{42}$: C 46.16, H 6.24. Found: C 45.87, H 5.95.

Heptakis(6-deoxy-6-iodo-2,3-di-*O*-myristoyl)cyclomaltoheptaose (**4**). To a solution of heptakis(6-deoxy-6-iodo)cyclomaltoheptaose [33] (**1**, 0.1 g, 53 μmol) in dry DMF (5 mL) under Ar at 0 ºC, DMAP (0.27 g, 2.2 mmol, 3 equiv) was added. Myristoyl anhydride (1.29 g, 2.94 mmol, 4 equiv) was dropwise added; the reaction mixture was stirred at room temperature for 16 h and then filtered. The resulting solid was extensively washed with water and MeOH. A mixture of MeOH-DCM (95:5, 50 mL) (25 mL) was added and the solution was refluxed for 1 h, decanted and purified by column chromatography (petroleum ether → 1:15 EtOAc-petroleum ether). Yield: 0.13 g (52%); R_f = 0.49 (1:8 EtOAc-petroleum ether); $[\alpha]_D$ = +49.2 (*c* 1.0 in DCM). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.33 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.0 Hz, H-3), 5.15 (d, 7 H, $J_{1,2}$ = 3.0 Hz, H-1), 4.80 (dd, 7 H, H-2), 3.74 (m, 14 H, $J_{4,5} = J_{5,6a}$ = 9.0 Hz, H-5, H-6a), 3.64 (bd, 7 H, $J_{6a,6b}$ = 11.8 Hz, H-6b), 3.58 (t, 7 H, H-4), 2.41-2.11 (m, 28 H, CH_2CO), 1.58 (m, 28 H, C*H*$_2$CH$_2$CO), 1.27 (m, 280 H, CH_2), 0.87 (t, 42 H, $^3J_{H,H}$ = 7.0 Hz, CH_3). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 173.3, 171.7 (CO), 96.4 (C-1), 80.4 (C-4), 70.2 (C-2), 69.9 (C-5), 69.7 (C-3), 34.1, 33.9 (*C*H$_2$CO), 32.0 (*C*H$_2$CH$_2$CH$_3$), 29.9-29.2 (CH_2), 24.9, 24.8 (*C*H$_2$CH$_2$CO), 22.7 (*C*H$_2$CH$_3$), 14.1 (CH_3), 8.6 (C-6). MALDITOF-MS: *m/z* 4842.6 $[M + H]^+$. Anal. Calcd for $C_{238}H_{427}I_7O_{42}$: C 58.95, H 8.88. Found: C 58.76, H 8.57.

Heptakis(6-azido-6-deoxy-2,3-di-*O*-hexanoyl)cyclomaltoheptaose (**7**). To a solution of either **3** or **5** (0.2 mmol) in dry DMF (20 mL), sodium azide (0.18 g, 2.8 mmol, 2 equiv) was added. The mixture was stirred at 80 ºC for 16 h and then the solvent was removed under reduced pressure. The resulting residue was extracted with DCM (40 mL) and washed with water (2 x 20 mL). The organic phase was dried (Na_2SO_4) and concentrated and the resulting syrup was purified by column chromatography (1:9 EtOAc-petroleum ether). Yield: 0.46 g (85%); R_f = 0.42 (1:9 EtOAc-petroleum ether); $[\alpha]_D$ = +115.0 (*c* 1.0 in DCM). IR: υ_{max} = 2117 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.30 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, H-3), 5.06 (d, 7 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.80 (dd, 7 H, H-2), 4.00 (ddd, 7 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6b}$ = 4.9 Hz, $J_{5,6a}$ = 2.7 Hz, H-5), 3.73 (t, 7 H, H-4), 3.71 (dd, 7 H, $J_{6a,6b}$ = 13.6 Hz, H-6a), 3.63 (dd, 7 H, H-6b), 2.40-2.16 (m, 28 H, CH_2CO), 1.59 (m, 28 H, C*H*$_2$CH$_2$CO), 1.30 (m, 56 H, C*H*$_2$CH$_3$, C*H*$_2$CH$_2$CH$_3$), 0.91, 0.89 (2 t, 42 H, $3J_{H,H}$ = 7.3 Hz, CH_3). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 173.2, 171.7 (CO), 96.4 (C-1), 76.7 (C-4), 70.8 (C-5), 70.1 (C-2, C-3), 51.6 (C-6), 34.0, 33.8 (*C*H$_2$CO), 31.4, 31.2 (*C*H$_2$CH$_2$CH$_3$), 24.6, 24.3 (*C*H$_2$CH$_2$CO), 22.3 (*C*H$_2$CH$_3$), 13.8 (CH_3). ESIMS: *m/z* 2706.3 $[M + Na]^+$. Anal. Calcd for $C_{126}H_{203}N_{21}O_{42}$: C 56.38, H 7.62, N 10.96. Found: C 56.33, H 7.23, N 10.93.

Heptakis(6-azido-6-deoxy-2,3-di-*O*-myristoyl)cyclomaltoheptaose (**8**). To a solution of either **4** or **6** (0.15 mmol) in dry DMF (20 mL), sodium azide (0.14 g, 2.1 mmol, 2 equiv) was added. The mixture was stirred at 80 ºC for 16 h and then the solvent was removed under reduced pressure. The resulting residue was extracted with DCM (40 mL) and washed with water (2 x 20 mL). The organic phase was dried (Na_2SO_4) and concentrated and the resulting syrup was purified by column chromatography (1:15 EtOAc-petroleum ether). Yield: 0.55 g (81%); R_f = 0.40 (1:8 EtOAc-petroleum ether); $[\alpha]_D$ = +66.1 (*c* 1.0 in DCM). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.28 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 5.02 (d, 7 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.77 (dd, 7 H, H-2), 3.98 (m, 7 H, H-5), 3.71 (t, 7 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.68 (m, 7 H, H-6a), 3.60 (dd, 7 H, $J_{6a,6b}$ = 13.5 Hz, $J_{5,6b}$ = 4.5 Hz, H-6b), 2.32, 2.21 (m, 28 H, CH_2CO), 1.53 (m, 28 H, C*H*$_2$CH$_2$CO), 1.25 (m, 280 H, CH_2), 0.86 (t, 42 H, $^3J_{H,H}$ = 7.1 Hz, CH_3). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 173.2, 171.8 (CO), 96.4 (C-1), 76.7 (C-4), 70.8 (C-5), 70.2 (C-2), 70.1 (C-3), 51.6 (C-6), 34.1, 33.9 (*C*H$_2$CO), 32.0 (*C*H$_2$CH$_2$CH$_3$), 29.9-29.2 (CH_2), 24.9, 24.8 (*C*H$_2$CH$_2$CO), 22.7 (*C*H$_2$CH$_3$), 14.0 (CH_3). MALDI-TOFMS: *m/z* 4523.7 $[M + H]^+$. Anal. Calcd for $C_{238}H_{427}N_{27}O_{42}$: C 67.18, H 10.11, N 6.91. Found: C 66.82, H 9.84, N 6.66.

Heptakis[6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltoheptaose (**9**). To a suspension of **5** (0.29 g, 0.1 mmol) and Cs_2CO_3 (0.33 g, 1 mmol) in dry DMF (3 mL), *tert*-butyl *N*-(2-mercaptoethyl)carbamate (164 μL, 1 mmol, 1.4 equiv) was added. The suspension was stirred under Ar atmosphere at 70 ºC for 48 h. The reaction mixture was then cooled to rt, poured into ice-water (30 mL), and stirred overnight. The resulting solid was filtered and washed with water, and the residue was purified by column chromatography (1:3 EtOAc-petroleum ether). Yield: 0.25 g (70%). Analytical and spectroscopic data were identical to those reported [32].

Heptakis[6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-myristoyl]cyclomaltoheptaose (**10**). To a suspension of **6** (0.45 g, 0.1 mmol) and Cs_2CO_3 (0.33 g, 1 mmol) in dry DMF (3 mL), *tert*-butyl *N*-(2-mercaptoethyl) carbamate (165 μL, 1 mmol, 1.4 equiv) was added. The suspension was stirred under Ar atmosphere at 70 ºC for 48 h. The reaction mixture was then cooled to rt, poured into ice-water (30 mL), and stirred overnight. The resulting solid was filtered and washed with water, and the residue was purified by column chromatography (1:2 EtOAc-petroleum ether). Yield: 0.37 g

(72%). Analytical and spectroscopic data were identical to those reported [32].

Heptakis[6-(2-isothiocyanatoethylthio)-2,3-di-*O*-myristoyl]cyclomaltoheptaose (**14**). To a solution of heptaamine **12** [32] (102 mg, 21.2 μmol) in a mixture of DCM (1.6 mL) and water (1.6 mL), $CaCO_3$ (59 mg, 0.59 mmol, 4 equiv) and $CSCl_2$ (23 μL, 0.30 mmol, 2 equiv) were added. The reaction mixture was vigorously stirred for 16 h and then concentrated. The residue was dissolved in DCM (15 mL) and washed with water (6 mL). The organic phase was decanted, dried (Na_2SO_4) and concentrated. The residue was subjected to column chromatography (1:6 EtOAc-petroleum ether). Yield: 35 mg (35%); R_f = 0.38 (1:4 EtOAc-petroleum ether); $[\alpha]_D$ = +72.3 (*c* 0.6 in $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.27 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.05 (d, 7 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.77 (dd, 7 H, H-2), 4.14 (dbd, 7 H, $J_{5,6b}$ = 2.5 Hz, H-5), 3.82 (t, 7 H, $J_{4,5}$ = 8.7 Hz, H-4), 3.78 (t, 14 H, $^3J_{H,H}$ = 6.5 Hz, CH_2N_{cyst}), 3.17 (bd, 7 H, $J_{6a,6b}$ = 13.1 Hz, H-6a), 3.07 (dd, 7 H, $J_{5,6b}$ = 2.5 Hz, H-6b), 3.00, 2.92 (2 dt, 14 H, $^2J_{H,H}$ = 13.0 Hz, $^3J_{H,H}$ = 6.0 Hz, CH_2S_{cyst}), 2.38-2.17 (m, 28 H, CH_2CO), 1.54 (m, 28 H, C*H*$_2$CH$_2$CO), 1.27 (m, 280 H, CH_2), 0.86 (t, 42 H, $^3J_{H,H}$ = 7.0 Hz, CH_3). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 173.4, 171.8 (CO), 132.3 (NCS), 96.7 (C-1), 78.5 (C-4), 71.6 (C-5), 70.3 (C-2), 70.2 (C-3), 45.6 (CH_2N_{cyst}), 34.1 (C-6, *C*H$_2$CO, CH_2S_{cyst}), 33.8 (*C*H$_2$CO), 32.0 (*C*H$_2$CH$_2$CH$_3$), 30.0-29.3 (CH_2), 24.9, 24.8 (*C*H$_2$CH$_2$CO), 22.7 (*C*H$_2$CH$_3$), 14.1 (CH_3). MALDITOF-MS: *m/z* 4779.7 $[M + Na]^+$. Anal. Calcd for $C_{259}H_{455}N_7O_{42}S_{14}$: C 64.97, H 9.58, N 2.05. Found: C 64.87, H 9.53, N 1.96.

2.2. Nanoparticle Formation and Characterization

2.2.1. Preparation of Homogeneous and Mixed Unloaded Nanospheres

The nanoparticle suspensions were prepared in triplicate using the nanoprecipitation technique [34,35] taking advantage of the spontaneous self-assembling capabilities of amphiphilic βCD derivatives when dispersed into an aqueous phase. Briefly, the corresponding amphiphilic βCD was dissolved in anhydrous acetone to a final concentration of 1 mg·mL^{-1} and this solution was incorporated into an equal volume of distilled water with magnetic stirring (500 rpm) at rt. Nanoparticles were formed spontaneously and the organic solvent was removed under reduced pressure at 35 ºC. The aqueous suspension was filtered through 0.8 μm membrane (Millex AA, Millipore, France), characterized, and stored in closed vials.

2.2.2. Preparation of Homogeneous Unloaded Nanocapsules

The corresponding amphiphilic βCD was dissolved in anhydrous acetone (2 mg·mL^{-1}) containing a small amount (100μL) of capric/caprylic triglycerides (Miglyol® 812), a non-ionic hydrophobic surfactant (Montane® 80, 2 mg·mL^{-1}) and an aqueous phase with a non-ionic hydrophilic surfactant (Montanox® 80, 2 mg·mL^{-1}). This solution was incorporated into distilled water (twice the volume of acetone) under magnetic stirring (500 rpm) at rt. Nanoparticles were formed spontaneously and organic solvent was removed under reduced pressure at 35 ºC. The nanoparticle suspensions were prepared in triplicate and were stored in closed vials at +6 °C.

2.2.3. Preparation of Homogeneous DZ-Loaded Nanospheres and Nanocapsules

The nanoparticle suspensions were prepared following the above described procedures respectively, from acetone solutions containing both, the corresponding βCD derivative and DZ (0.5 mg·mL^{-1} for nanospheres and 1 mg·mL^{-1} for nanocapsules). To formulate DZ loaded-nanospheres, polysorbate 80 (Montanox® 80) was added to the aqueous phase at a concentration of 1 mg·mL^{-1} before nanoprecipitation. The nanoparticle suspensions were prepared in triplicate and were stored in closed vials at rt. The recovery of DZ in the colloidal suspensions was assayed spectrophotometrically at 285 nm and 366 nm following solubilization of a known volume of the suspension in MeOH/H_2SO_4 (5 g·L^{-1}).

2.2.4. Size and Zeta Potential Measurements

A Zetasizer 3000 instrument (10mWHeNelaser at 632.8 nm) with a K7132 correlator was used at the fixed angle of 90º and at 25 ± 0.1 ºC for size analysis and zeta potential (ζ) determination (Malvern Instruments, Orsay, France). The average hydrodynamic diameter Dh (Z average mean) and polydispersity index (PI) were calculated in intensity using the Cumulant analysis mode. Dh values were derived from the measured translational diffusion coefficient of the particles moving under Brownian motion. The values of merit for size analysis varied from 60 to 70%. ζ potential values were evaluated by laser Doppler anemometry. In both determinations, the colloidal suspensions were analyzed following appropriate dilution with NaCl (0.01% w/w).

2.2.5. Cryo-Transmission Electron Microscopy (Cryo-TEM).

Thin vitrified films of CD nanoparticle suspensions were prepared as previously described [36]. Droplets of 0.1% (w/v) suspensions were deposited on 'lacey' carbon films. After blotting the liquid in excess with filter paper, the grid was quench-frozen into liquefied ethane. The specimens were then mounted in a Gatan 626 cryo-holder, transferred into a Philips CM200 'Cryo' microscope and observed at low temperature (-180 ºC), under reduced illumination, at an accelerating voltage of 80 kV. Images were recorded on Kodak SO163 films.

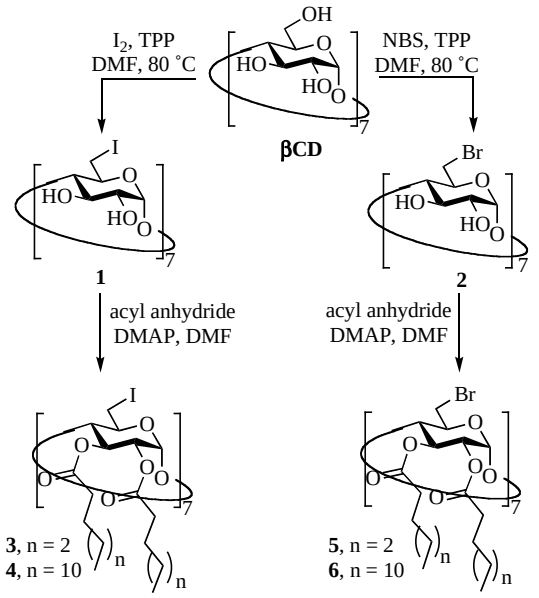
2.2.6. In Vitro Release Kinetics

In vitro release kinetics studies were performed in triplicate, by dialysis (cutoff of 8kDa, Spectra/Por® Dialysis, Fisher Bioblock, France), in phosphate buffer saline medium (0.1M, pH 7.4, 37°C) using a paddle apparatus (Sotax AT6, Sotax, Switzerland). The rotating paddle was set at 50 rpm. All the drug release experiments were performed to ensure that the maximum concentration corresponding to 100% DZ release did not exceed 25% of the saturating DZ concentration in PBS 0.1M at 37 °C (experimentally determined at 0.045 g/L). Drug quantification over time in the release medium was assayed spectrophotometrically at 280 nm in PBS 0.1M.

3. RESULTS AND DISCUSSION

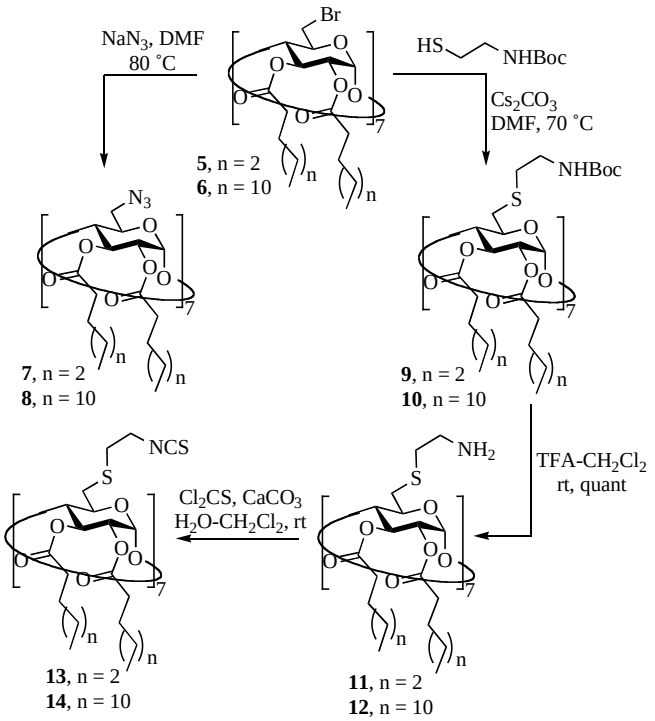
3.1. Synthesis

While functionalization at the primary hydroxyl positions of CDs is relatively straightforward taking advantage of the differential reactivity of primary vs. secondary hydroxyls, particularly by using the per-C6-halogenation methodology reported by Gadelle and Defaye [33], subsequent acylation of the secondary hydroxyls with fatty acid chains is nontrivial [37]. We recently found that the use of the acyl anhydride/4-(*N*,*N*-dimethylamino)pyridine (DMAP) system efficiently furnished homogeneous per-(O2,O3)-esters. Tetradecahexanoates **3** and **5** were prepared accordingly in multigram scale from per-C6-iodo (**1**) and per-C6-bromo (**2**) βCD, respectively, in 68-72% yield after column chromatography purification (Scheme **1**). The corresponding tetradecamyristoylates **4** (52%) and **6** (67%) were synthesized analogously, the slightly lower yields obtained being attributed to difficulties in the separation of the pure compounds from the excess of acylating reagent rather than to lower reactivity. The homogeneity of compounds **3**-**6** was demonstrated by NMR, ESI-MS and combustion analysis (see experimental section).



Scheme 1. Synthesis of face-differentiated CDs **3**-**6**.

Compounds **3**-**6** were thus obtained in only two steps from commercial βCD. They are themselves interesting facially-differentiated CDs with self-organizing capabilities (see hereinafter). Most interestingly, they are also excellent precursors for further chemical elaboration since the halogen groups can be displaced by a variety of nucleophiles. For instance, the reaction of the per-C6-bromo βCD derivatives **5** and **6** with sodium azide furnished tetradecahexanoate **7** [38] and tetradecamyristoylate **8** [38], respectively, with excellent yields (85 and 81%, respectively) (Scheme **2**). Cesium carbonate-promoted nucleophilic displacement of bromine in **5** and **6** by *N*-*tert*-butoxycarbonyl (Boc)-cysteamine afforded **9** [32] and **10** [32], respectively, in 70 and 72% yields. Analogous results were obtained using the corresponding iodides **3** and **4** as starting materials instead.



Scheme 2. Synthesis of face-differentiated CDs **7**-**14**.

Further on, derivatives **9** and **10** were transformed into the hepta-isothiocyanates **13** and **14** following the previously described tandem acid-catalysed carbamate cleavage—thiophosgene-mediated isothiocyanation protocol [32]. The functional groups at the primary face of the CD platform, i.e. azide and isothiocyanate, were purposely selected to be compatible with "click" type conjugation by either the copper(I)-catalyzed azide-alkyne cycloaddition [38] or the thiourea-forming reaction [39], opening the possibility for surface modification of nanoparticles following self-assembly.

3.2. Self-Assembling Capabilities

The ability of the new hexanoylated and myristoylated CDs to self-assemble into submicronic particles in aqueous media was subsequently evaluated in the conditions of nanoprecipitation [34,35]. Dispersion of acetone solutions of the neutral CDs into bulk water instantaneously formed nanospheres in all cases. Dynamic light scattering (DLS) monitoring of nanoparticle size revealed unimodal distributions, with little differences in the average hydrodynamic diameter between hexanoylated and myristoylated compounds. The particle size was more sensitive to the nature of the functional group at the primary rim of the CD (Table **1**). Thus, halogenated derivatives yielded larger nanospheres, while the less bulky azide and isothiocyanate substituents gave rise to the smallest aggregates. The ζ potentials, slightly negative for all formulations, were also affected by the nature of the functional groups at the primary rim, but to a lesser extent.

The low polydispersity index (PI) measured by DLS was further confirmed by transmission electron microscopy. Fig. (**3**) shows the cryo-TEM micrograph for the highly homogeneous and spherical nanoparticles obtained from compound

Table 1. Hydrodynamic Size, Polydispersity and ζ Potential of Homogeneous Blank Nanospheres

Compound	Acyl Chain	Functional Group	Z Average Mean ± SD (nm)	PI	ζ Potential ± SD (mV)
3	Hex	I	240 ± 54	0.08	-36 ± 4
5	Hex	Br	238 ± 29	0.02	-36 ± 4
7	Hex	N_3	181 ± 5	0.08	-34 ± 2
9	Hex	$S(CH_2)_2NHBoc$	197 ± 4	0.04	-25 ± 5
13	Hex	$S(CH_2)_2NCS$	123 ± 2	0.04	-43 ± 8
4	Myr	I	217 ± 17	0.04	-39 ± 8
6	Myr	Br	244 ± 13	0.13	-54 ± 2
8	Myr	N_3	170 ± 10	0.06	-33 ± 2
10	Myr	$S(CH_2)_2NHBoc$	211 ± 10	0.03	-24 ± 4
14	Myr	$S(CH_2)_2NCS$	111 ± 2	0.04	-42 ± 2

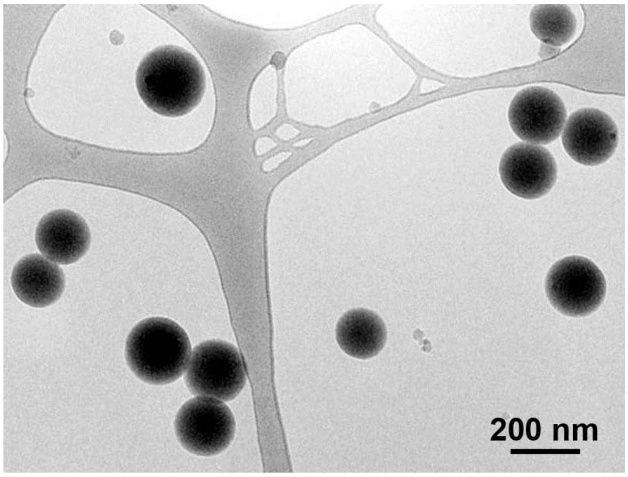


Fig. (3). Cryo-TEM micrograph of nanospheres prepared from **3**.

3. The diameter of nanoparticles observed by TEM (ca. 150 nm for **3**) is significantly smaller than that measured by DLS, the particle size discrepancy probably arising from the different operational principles, as observed previously [36].

When co-formulated with pharmaceutically-approved synthetic triglycerides, these face-differentiated CDs were able to form nanocapsules. In general, nanocapsules were larger in size but also very homogeneous, which might be interesting in order to increase their loading capacity. For instance, dispersion of a solution containing a mixture of CD derivative **7**, Montane® 80 and Miglyol® 812 into water containing a non-ionic surfactant (Montanox® 80) furnished stable nanocapsules with an average hydrodynamic diameter of 240 ± 10 nm (PI, 0.05). Remarkably, formulations of either nanospheres or nanocapsules were found highly stable in water. No significant changes in the measured properties were observed after 8-month storage at 6 ºC (data not shown).

Nanoprecipitation of equimolar mixtures of halogenated CDs **3** and **5** furnished nanospheres with similar size and ζ potential than those obtained from **3** and **5** independently (Table **2**, line 1). This is very sound since the self-aggregation behaviours of **3** and **5** were virtually identical (Table **1**). A similar situation was observed after co-formulation of **3** and **5** with derivative **7** (Table **2**, lines 2 and 3), thereby providing a way to access multifunctional

Table 2. Hydrodynamic Size, Polydispersity and ζ Potential of Mixed Blank Nanospheres

Compounds (ratio)	Functional Group	Z Average Mean ± SD (nm)	PI	ζ Potential ± SD (mV)
3 + **5** (1:1)	I + Br	225 ± 31	0.04	-40 ± 10
3 + **7** (1:1)	I + N_3	204[a]	0.02	n. d.
5 + **7** (1:1)	Br + N_3	200[a]	0.02	n. d.
3 + **11** (4:1)	I + $S(CH_2)_2NH_3^+$	98 ± 14	0.04	+30 ± 9
5 + **11** (4:1)	Br + $S(CH_2)_2NH_3^+$	122 ± 14	0.16	+35 ± 6
7 + **11** (4:1)	N_3 + $S(CH_2)_2NH_3^+$	105 ± 2	0.02	+35 ± 5
5 + **11** (7:3)	Br + $S(CH_2)_2NH_3^+$	117 ± 39	0.15	+39 ± 6

n. d. Not determined, [a] Results from one batch

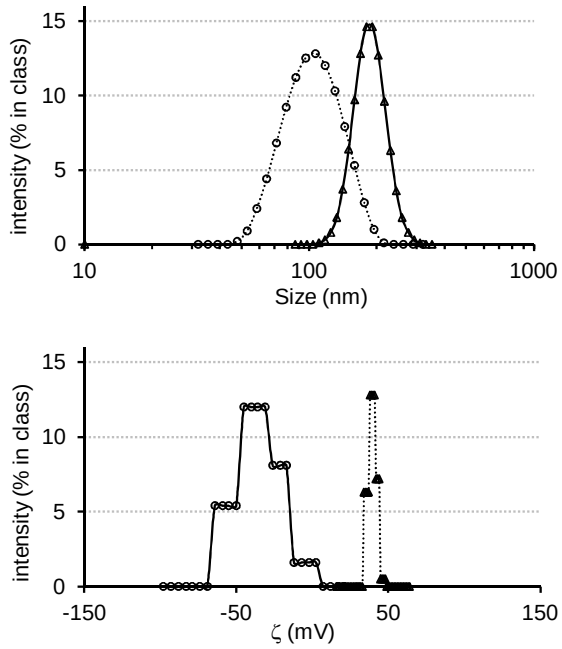


Fig. (4). Particle size (above) and ζ potential (below) distribution for **7** (solid line) and mixed **7:11** (4:1 ratio, scattered line) nanospheres obtained by DLS.

nanoparticles exposing different functional groups at the surface.

In contrast with the behaviour of neutral CD derivatives, the cationic compounds **11** and **12** were unable to form detectable nanoparticles (DLS) using the nanoprecipitation method. Apparently, disordered aggregation takes place producing a fast precipitation in spite of their expected higher solubility. The scenario is completely different when cationic CDs such as compound **11** are co-formulated with the neutral amphiphilic CDs **3**, **5** or **7**. Nanospheres turned much smaller (ca. 120 nm) even using small amounts of **11** (Table **2**, entries 4 to 6) and the ζ potential switched to positive. Increasing the cationic CD ratio resulted in slightly higher compaction, furnishing particles with smaller size and higher ζ potential (Table **2**, entry 7). Fig. (**4**) exemplifies the dramatic switch in size and ζ potential for nanoparticles obtained from CD **7** when co-formulated with **11**, illustrating the potential to modulate nanoparticle properties using relatively simple tools.

3.3. Drug Encapsulation and Release

To assess the potential of these CD derivatives as encapsulation and release systems, the anxiolytic drug diazepam (DZ) was chosen as a model guest. DZ is a benzodiazepine used for the treatment of nervous disorders and muscle spasms. The low solubility of diazepam in water is a limitation for certain administration routes (e.g. parenteral). To overcome this hurdle, a number of carrier systems have been developed [40-42], and this makes DZ an appropriate comparative model.

DZ-charged nanospheres and nanocapsules were prepared from hepta-azide **7** (**7**:DZ NS and **7**:DZ NC, respectively) as above described using a mixture of both components. The average particle size and ζ potential did not differ significantly from those measured for unloaded NS and NC. The DZ recovery in the aqueous suspension was determined spectrophotometrically and found to be 75% and 93% for NS and NC, respectively. The high solubility of DZ in Miglyol® 812 used in NC formulation probably explains the higher efficiency observed for the latter system and is consistent with the lipophilicity of the drug [43]. These results parallel those reported for other types of amphiphilic CD [44] and are remarkable considering that there is no need of performing the CD:drug complex [45].

Concerning the *in vitro* drug release from DZ-loaded nanoparticles, it was shown that DZ recovery gradually increased upon stirring according to a single-mode release (hyperbolic) profile in both NS and NC. However, the release kinetics were rather contrasting: while DZ is completely released from NS in approximately 6 h, in NC two days were required for complete DZ recovery (Fig. **5**). The rapid DZ release phase (during the first 4 hours) observed in the case of nanospheres strongly suggests that the drug is partly adsorbed at the surface of particles, this drug localization being responsible for this pronounced burst effect. The subsequent slower release phase (from time 4h) may be attributed to a fraction of DZ more deeply anchored within the

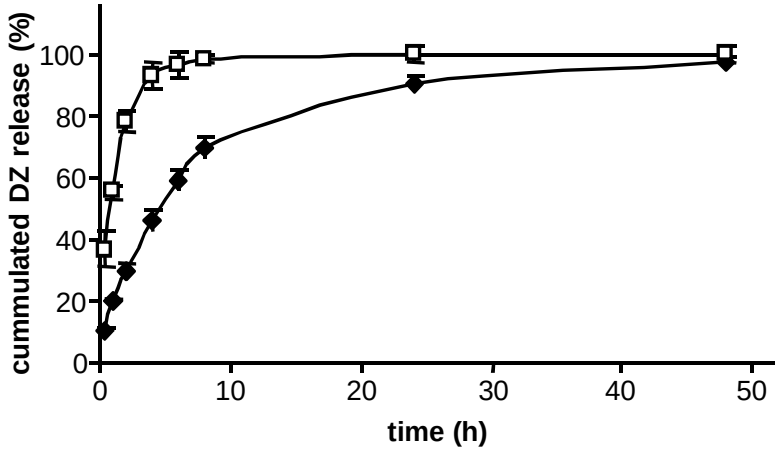


Fig. (5). *In vitro* release profiles of DZ from nanospheres (NS, □) and nanocapsules (NC, ♦) formulated with CD **7** in PBS (pH 7.4) at 37 ºC under sustained stirring (50 rpm).

nanosphere matrix. While DZ release from NS might solely depend on the interactions between DZ and the CD-nanoparticle, in NC the DZ partition between the oil phase at the inner particle and the external aqueous media may govern drug release. Similar behaviours have been already observed for the encapsulation of DZ and other non-polar drugs into amphiphilic CD-based nanosystems [43, 44, 46].

4. CONCLUSIONS

In summary, an efficient synthetic strategy has been implemented for the elaboration of face-differentiated CD derivatives displaying self-organizing capabilities. The methodology is very flexible and allows elaborating a broad variety of selectively decorated and monodisperse CD derivatives. The self-assembling features depend on their chemical functionalization pattern, highlighting the utmost relevance of molecular architecture on the supramolecular behaviour. The possibility to combine different amphiphilic CDs to modulate nanoparticle properties and the possibility to install reactive groups suitable for further surface manipulation are relevant features of this approach. Preliminary assessment of the drug encapsulation potential using diazepam-loaded nanospheres and nanocapsules prepared from the CD derivative **7** revealed highly efficient association capacities. In agreement with previous reports, the DZ-association efficiency and the release profiles were strongly dependent of the type of nanoparticle formulation. Confirmation of these results with additional CD derivatives and other drug candidates is presently being carried out in our laboratories.

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