ESRF	**Experiment title:** Nanostructure and ordering properties of colloidal carriers made of self-assembled bio-transesterified cyclodextrins.	**Experiment number:** 02-01-723
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Report:

1. Scientific context of the experiments

One of the major challenges in the emerging field of nanobiotechnology consists in designing drug vectors able to target tissues specifically and effectively. In this context, colloidal carriers for drug delivery have received considerable interest. Nanoparticles have been prepared from various materials such as natural and synthetic polymers, proteins and lipids.

Original nanoparticles were designed using self-assembling amphiphilic cyclodextrins (CDs) : ring-shaped oligosaccharides with 6, 7 or 8 glucose units (α-, β- an γ-CDs, respectively), acylated on the secondary face (**Figure 1**). The morphology and structure of β-CD nanoparticles were recently studied using cryo-electron microscopy (cryo-TEM). Depending on the chemical structure of the modified CDs and the conditions of nanoprecipitation, different objects were observed : i) spherical particles with a matrix structure when hexanoyl chains were substituted ; ii) multilamellar particles with a semi-crystalline core surrounded by a shell of concentric layers when decanoyl chains were substituted.

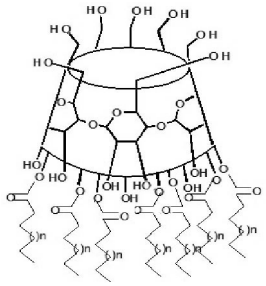

Figure 1. *Scheme of an acylated β-cyclodextrin.*

When amphiphilic CD nanoparticles prepared only by chemical methods were first studied, one limiting problem for structural analysis was the very low quantity of material available. Thanks to the new enzymatic procedure, a larger quantity of various acylated CDs could be produced for this project. It thus became possible to investigate the effect of a larger number of experimental parameters : nature of material (α, β or γ-CD), substitution degree, length of the alkyl chains, etc.

2. Experimental method

• Samples :

α, β and γ-CDs were used and the acylation on their secondary face was performed both chemically and enzymatically. The substitution degree and length of the alkyl chains (from 4 to 14) was varied. The nanoparticle suspensions were prepared using a nanoprecipitation technique and, for some samples, a guest molecule (phospholipids, drug) was added during the preparation, in order to evaluate the encapsulation properties and structural stability of amphiphilic CDs.

• Small- and wide-angle X-ray scattering :

SAXS and WAXS experiments were carried out on the BM02 (D2AM) beamline. Series of glass capillaries and cells with mica windows were attached to an automatic sample changer.

Scattering patterns were recorded at 16 keV (λ=0.776875 Å) with a CCD detector placed at distances of 20 and 155 cm in order to investigate *Q*-ranges corresponding to the ultrastructure and colloidal properties of the nanoparticles, respectively. 100 and 200 s exposure times were used. The diffraction patterns were calibrated using a silver behenate standard. WAXS and SAXS patterns were collected from about 100 suspensions. In addition, 10 specimens, selected according to their WAXS patterns, were put in a heating sample changer. The capillaries were sealed with wax to limit water evaporation (**Figure 2**) and heated by steps of 10°C, at a rate of 2.5°C/min, from 30 to 98°C. Every 10°C, they were kept at a constant temperature during 12 min while diffraction patterns were recorded for all specimens, during 50 s exposures.

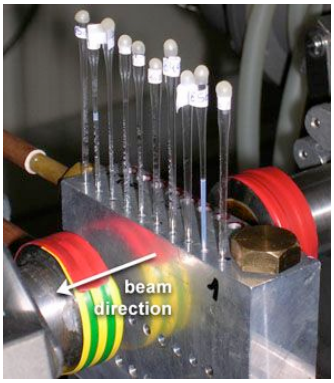

Figure 2. *Heating sample changer containing 10 capillaries sealed with wax.*

3. WAXS experiments

The majority of samples containing CDs only (about 50% of our specimens) yielded WAXS powder patterns containing up to 5 diffraction rings. The exceptions were the samples with short alkyl chains only containing 4 and 6 carbons, for which no ring pattern was observed. For the diagrams containing between 3 and 5 rings, the series of ratios between the diameter of the rings and that of the 100 reflection suggested a hexagonal structure. Diagrams with 4 and 5 sharp diffraction rings were only obtained with β-CD-C8 (**Figure 3a**) and β-CD-C10 samples. Samples made of α- and γ-CDs, whose WAXS diagrams only contained 1 or 2 rings, appeared to be less organized.

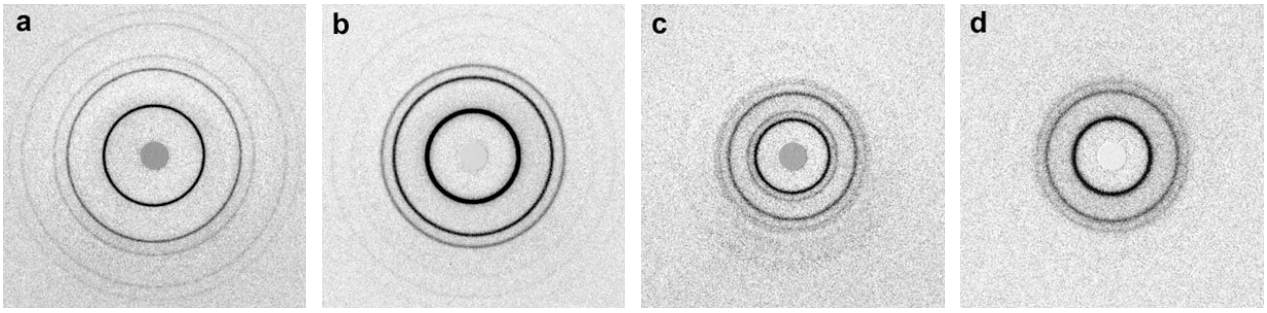

Figure 3. a) *WAXS pattern from β-CD-C8 particles suggesting a hexagonal unit cell with a=4.13 nm ;* ***b-d)*** *Patterns from samples prepared with phospholipids (PLs) :* ***b)*** *β-CD-C10 with 10 wt% PLs (a=4.26 nm) ;* ***c)*** *β-CD-C10 with 30 wt% PLs (a=5,66 nm);* ***d)*** *γ-CD-C10 with 30 wt% PLs (a=5.37 nm).*

β-CD-C10 samples prepared in the presence of phospholipids (PLs) exhibited different diffraction patterns depending on the initial amount of PLs in the formulation (**Figures 3b-d**). In particular, the diagram of the sample containing 30 wt% PL could be described as the superimposition of two sets of rings corresponding to hexagonal lattices (**Figure 3c**). Work is in progress to understand if this is due to the coexistence of two different types of particles. In addition, PLs seemed to have a structuring effect on the γ-CD-C10 sample (**Figure 3d** and **Figure 4**).

• Influence of the number of glucose units in the cyclodextrin ring :

For a given length of substituted alkyl chain, the unit cell parameters of the hexagonal lattice varied little depending on the number of glucose unit in the CD ring. As an example, **Figure 4** compares the meridional sections of WAXS patterns from α-, β- and γ-CD-C10 samples (without additive). The 100 ring does not significantly change. The γ-CD-C10 pattern only exhibits a strong 100 ring with a close additional weak ring which has not been explained so far.

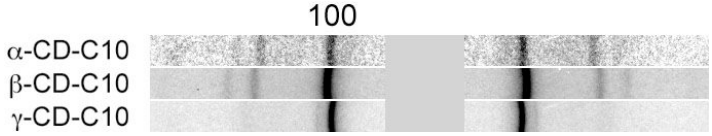

Figure 4. *Equatorial sections of the WAXS patterns showing the displacement of the 100 ring with the nature of the cyclodextrin.*

• Influence of the length of the substituted alkyl chain :

For β-CDs substituted with C8, C10, C12 and C14 alkyl chains (without additive), the patterns only differ by a scaling factor (**Figure 5a**). The longer the alkyl chain, the larger the hexagonal unit cell. The variation of the cell parameter (determined from the position of the 100 diffraction ring) with the length of the alkyl chain is linear (**Figure 5b**).

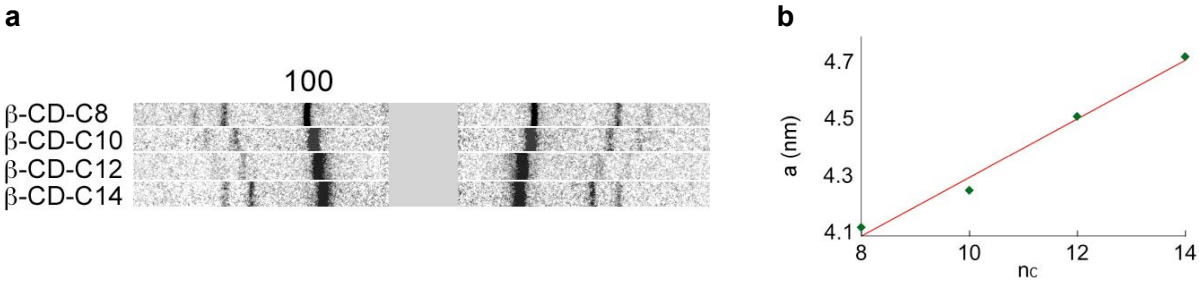

Figure 5. a) *Equatorial sections of a series of WAXS patterns showing the displacement of the diffraction rings with the length of the alkyl chain grafted onto β-CDs ;* ***b)*** *variation of the hexagonal unit cell parameter a as a function of the number of carbons n_C in the substituted alkyl chain.*

• Thermal treatments :

Cyclodextrins without additive :

The particles made of pure amphiphilic cyclodextrins exhibited a surprisingly good thermal stability. For example, as seen in **Figure 6**, for β-CD-C8 and β-CD-C10 samples, no degradation or loss of structure was detected up to 98°C. Both samples showed a reversible expansion of the hexagonal unit cell parameter of about 0.1 nm (2.3%) but no phase transition.

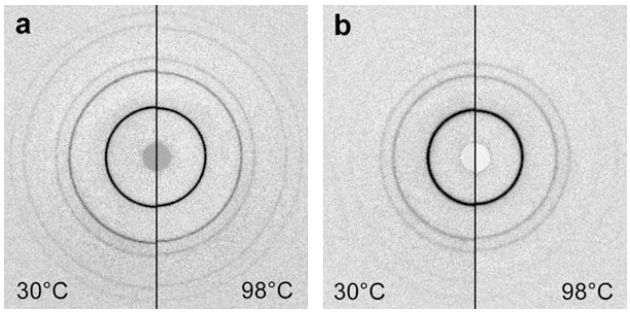

Figure 6. *Effect of temperature on samples of β-CD-C8 (**a**) and β-CD-C10 (**b**).*

Samples with phospholipids :

As shown in **Figure 8**, The samples prepared in the presence of PLs exhibited a more complex behavior upon heating. The β-CD-C10 sample containing the lowest amount of PLs (10 wt%) was stable up to 90°C. Then, a more complex diagram was recorded at 98°C (**Figure 8a**). This transition was reversible upon cooling. For the β-CD-C10 sample containing 30 wt% PLs, one of both superimposed hexagonal ring patterns (**Figure 3c**) rapidly disappeared upon heating and did not reappear upon cooling (**Figure 8b**). In addition, the variations in the ring pattern of the γ-CD-C10 sample with 30 wt% PLs suggested a significant but reversible contraction of the unit cell (**Figure 8c**).

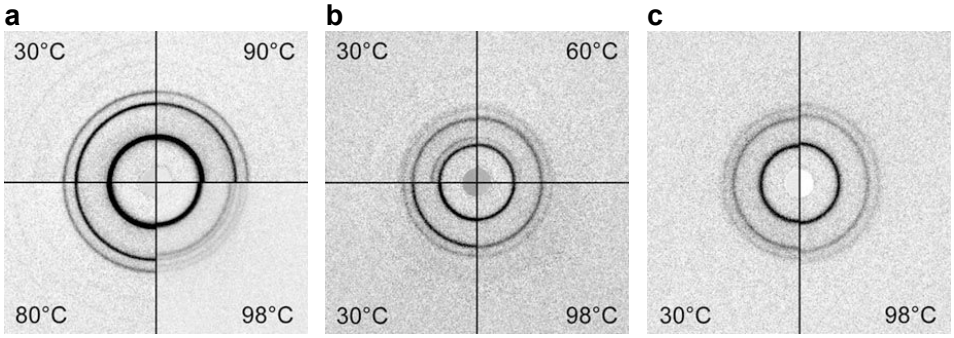

Figure 8. *Effect of temperature on samples containing phospholipids :* ***a)*** *β-CD-C10 / 10 wt% PLs ;* ***b)*** *β-CD-C10 / 30 wt% PLs ;* ***c)*** *γ-CD-C10 / 30 wt% PLs. In **a** and **b**, the variation of temperature during the thermal treatment reads clockwise from top-left.*

4. SAXS experiments

SAXS data were collected from dilute samples at a 16 keV energy and a 155 cm sample-to-detector distance allowing to cover a *Q*-range from 0.01 to 0.25 Å^{-1}. All scattering patterns were isotropic. For *Q*<0.04 Å^{-1}, most of the samples exhibited a monotonous Q^{-4} intensity decrease, revealing the presence of dense objects with sharp interfaces (**Figure 9a**). However, the size of these particles was probably larger than the smallest distance probed (~120 nm) and could thus not be measured. For *Q*>0.04 Å^{-1}, WAXS reflections were observed (*see* ***§3***).

Exceptions to these general features arose for γ-CD-C10 samples that exhibited oscillations in the lower *Q*-range, revealing the presence of nanoparticles with a low polydispersity. Because of the limited *Q*-range, SAXS could not unambiguously reveal the particle geometry but the data allowed an accurate determination of the size. Since dilute samples were used, the scattering curves were tentatively fitted using the form-factor of spheres (suggested by TEM images ; **Figure 10a**) with a Gaussian size distribution. A good agreement between calculated and experimental profiles was obtained and the diameter of the particles was extracted (**Figure 9b**). Depending on the samples, mean diameters ranging from 74 to 106 nm were found. The presence of guest molecules only had little effect on the particle size (95.4 nm with additive and 94 without, for γ-CD-C10 nanoparticles).

Some of the β-CD-C10 samples also exhibited a smooth oscillation. Since a rod-like shape was suggested by TEM images (**Figure 10c**) from these samples, the SAXS curves were fitted using the form factor of rods and a relatively good agreement was achieved (**Figure 10c**). The comparison of particle sizes for spheres and rods measured from TEM images and SAXS profiles showed a very good agreement (**Figures 9b** and **10a**, **Figures 9c** and **10c**).

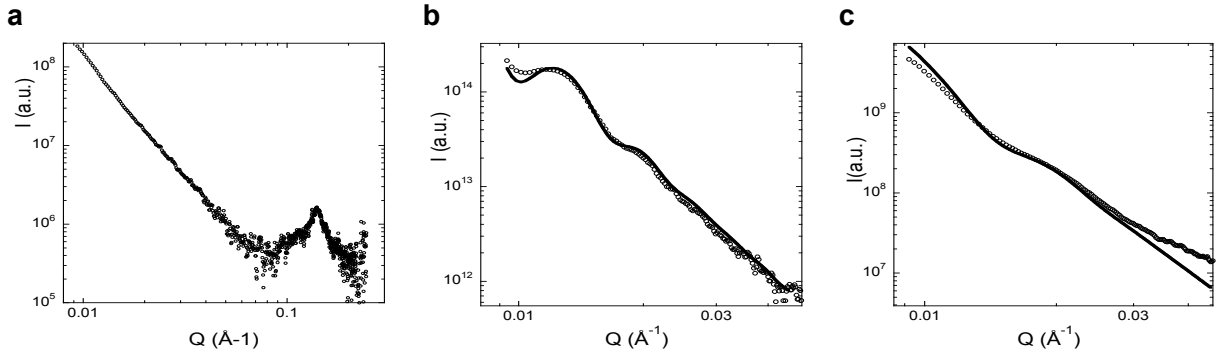

Figure 9*. SAXS profiles from different nanoparticle suspensions :* ***a)*** *β-CD-C10 ;* ***b)*** *γ-CD-C10 (symbols) and fit using the form factor of spheres (line ;* D_{sphere} *= 95 nm) ;* ***c)*** *β-CD-C10 (symbols) and fit using the form factor of cylinders (line ;* D_{rod} *= 56 nm).*

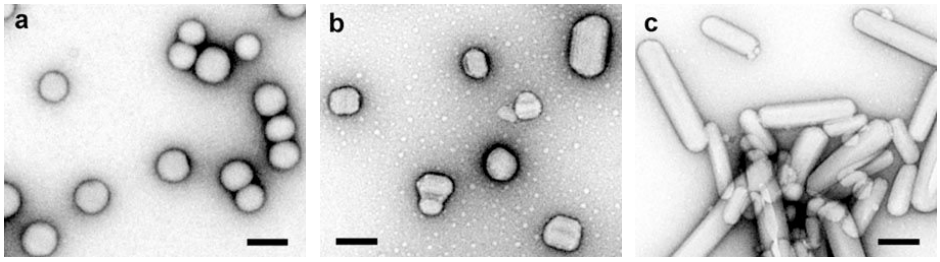

Figure 10. *TEM images of negatively stained amphiphilic CD nanoparticles :* ***a)*** *γ-CD-C10 (spheres) ;* ***b)*** *β-CD-C12 (facetted particles) ;* ***c)*** *β-CD-C10 with PLs (rods). Scale bars : 100 nm.*

5. Conclusion and perspectives

Work is in progress to validate the suggested hexagonal unit cells and propose models to describe the structure of particles. Two types of experiments should to be conducted in order to complement this datasets : i) A lower *Q*-range should be investigated by SAXS by working at a lower energy (e.g. 8 keV), in order to quantify the size of large objects, to better discriminate between spherical and cylindrical shapes and to study the self-ordering properties in more concentrated suspensions ; ii) Suspensions in sealed capillaries should be heated at temperatures higher than 100°C to study the structural changes that were observed for some samples, in particular those containing phospholipids. The reversibility of the structural modifications also has to be monitored.