

# Cyclodextrin and polysaccharide-based nanogels: entrapment of two hydrophobic molecules, benzophenone and tamoxifen.

*Samia Daoud-Mahammed<sup>1</sup>, Patrick Couvreur<sup>1</sup>, Kawthar Bouchemal<sup>1</sup>, Monique Chéron<sup>2</sup>, Geneviève Lebas<sup>1</sup>, Catherine Amiel<sup>3</sup>, Ruxandra Gref<sup>1\*</sup>*

<sup>1</sup> Université Paris-Sud, UMR CNRS 8612, Châtenay-Malabry, France

<sup>2</sup> Université Paris VI, BioMoCeTi, UMR CNRS 7033, Paris, France

<sup>3</sup> Université Paris Est, ICMPE-SPC, UMR 7181, Thiais, France

samia.daoud@u-psud.fr;   patrick.couvreur@u-psud.fr;   kawthar.bouchemal@u-psud.fr;  
monique.cheron@u-psud.fr;   genevieve.lebas@u-psud.fr;   catherine.amiel@glvt.fr;  
ruxandra.gref@u-psud.fr.

\* to whom correspondence should be addressed: Dr Ruxandra Gref. UMR CNRS 8612 - Faculté de Pharmacie - 5, rue Jean Baptiste Clément 92290 Châtenay-Malabry. tel: +33 146835909; fax: +33 146619334. [ruxandra.gref@u-psud.fr](mailto:ruxandra.gref@u-psud.fr).

DOI : 10.1021/bm801206f

**ABSTRACT**

The entrapment of two hydrophobic molecules, benzophenone and tamoxifen, into self-assembling cyclodextrin (CD) based nanogels has been studied. These nanogels formed spontaneously upon the association of a hydrophobically modified dextran (MD) and a cyclodextrin polymer (p $\beta$ CD). The interactions of benzophenone and tamoxifen with MD and p $\beta$ CD were investigated using phase solubility studies, circular dichroism and Isothermal Titration microCalorimetry. Both hydrophobic molecules were included into the CD cavities of the p $\beta$ CD and were also solubilized by MD into its hydrophobic microdomains. We took advantage of these interactions to form benzophenone- and tamoxifen-loaded nanogels. The highest benzophenone loadings were obtained by solubilising it in both p $\beta$ CD and MD solutions before mixing them to form nanogels. These studies open new possibilities of applications of the nanogels, mainly in the cosmetic field, as sun screen carriers prepared by a simple “green” technology.

**KEYWORDS:** nanogels – cyclodextrins – inclusion complexes – circular dichroism – isothermal titration microcalorimetry - drug loading.

## MANUSCRIPT TEXT

### Introduction

For many years, cyclodextrins (CDs) have been recognized in the cosmetic and pharmaceutical fields as promising carriers for the formulation of poorly water-soluble molecules<sup>1, 2</sup>. CDs are cyclic oligosaccharides consisting of six to eight D-glucopyranose units linked through  $\alpha$ -1,4-glycosidic bonds. They have the shape of a truncated cone with a hydrophobic internal cavity surrounded by an external hydrophilic surface. This particular structure enables CDs to accommodate into their cavity a wide variety of hydrophobic molecules such as lipophilic drugs, modifying thereby their physico-chemical properties<sup>3, 4</sup>. The inclusion of the “guest” drug into the “host” CD can improve its apparent solubility, physical and chemical stability, dissolution and bioavailability<sup>3</sup>, thus making CDs very attractive in drug formulation. However, CDs do not represent carrier systems capable of targeting the drug to the diseased cells and tissues.

Therefore, to overcome this drawback, research work has been oriented towards the development of colloidal drug carrier systems containing CDs, suitable for drug targeting<sup>5, 6</sup>. Among them, CD-containing nanoparticles have been of growing interest<sup>7</sup>. This concept combines the relative advantages of CDs and nanoparticles into a single system. Principally, two approaches have been proposed. In the first one, drug/CD water-soluble complexes were entrapped into poly(isobutylcyanoacrylate) nanospheres during the process of their anionic polymerization<sup>8</sup>. High drug loadings were thus obtained in comparison to the nanospheres encapsulating the free drug<sup>9, 10</sup>. In a second approach, non polymeric nanoparticles, either nanospheres or nanocapsules, were obtained by nanoprecipitation of amphiphilic CDs bearing aliphatic chains on their primary and/or secondary face, in the presence or not of surfactants<sup>11-16</sup>. High drug loadings could be achieved when CD amphiphilic nanoparticles were prepared from preformed drug/CD inclusion complexes<sup>17, 18</sup>. However, organic solvents and/or surfactants were needed to produce these CD nanoparticles.

Recently, our group has proposed a mild procedure to obtain colloidal nanoassemblies (nanogels) containing CDs in the absence of organic solvents<sup>19</sup>. As shown by electron microscopy after freeze fracture<sup>19</sup>, spherical shaped nanogels of 100-200 nm formed spontaneously in aqueous medium upon the association of two hydrosoluble polymers: a hydrophobically modified dextran by grafting alkyl side chains (MD) and a poly- $\beta$ -cyclodextrin polymer (p $\beta$ CD). Some alkyl chains form inclusion complexes with some CD cavities, leaving also CDs available to include hydrophobic molecules.

However, the possible applications of these nanogels in cosmetic and medical fields have not been investigated yet.

The present study aimed (i) to investigate the interaction of two hydrophobic molecules, benzophenone (BZ), widely used as sun screen in the cosmetic field, and the anticancer agent tamoxifen (TM), with both associative polymers (MD and p $\beta$ CD) and (ii) to take advantage of these interactions to formulate BZ and TM loaded MD-p $\beta$ CD nanoassemblies.

## Experimental section

### Materials

$\beta$ -cyclodextrin ( $\beta$ -CD) was purchased from Roquette (France).

Benzophenone (BZ) and tamoxifen (TM) were supplied from Sigma (France). Their purity was > 97% and > 99%, respectively.

Water was purified by reverse osmosis (Milli-Q, Millipore®, USA).

p $\beta$ CD was prepared by crosslinking  $\beta$ -CD under strongly alkaline conditions with epichlorohydrin (EP)<sup>20</sup>. Briefly, 100 g of anhydrous  $\beta$ -CD were dissolved in 160 mL NaOH 33% w/w solution under mechanical stirring overnight. Then, 81.52 g of EP (molar ratio  $\beta$ -CD/ EP = 10) was rapidly added to the solution heated to 30°C. In order to obtain a high molecular weight polymer, the reaction was stopped in the vicinity of the gelation point by addition of acetone. The obtained aqueous phase was heated to 50°C overnight, neutralized with 6N HCl and ultrafiltered using membranes with a cut-off of 100,000 g/mol. The  $\beta$ -CD polymer was finally recovered by freeze-drying. The  $\beta$ -CD content, as determined by <sup>1</sup>H NMR spectroscopy, was 70% w/w. The average molar mass of p $\beta$ -CD polymer was  $7 \times 10^5$  g/mol, as determined by size exclusion chromatography using pullulan standards.

Dextran bearing hydrophobic lauryl side chains (MD) was synthesized as previously described<sup>21, 22</sup>. Briefly, 4 g of dextran (40 000 g/mol) solubilized in 100 mL of dimethyl formamide containing 1 g of lithium chloride, were reacted for 3 h at 80°C with 0.43 mL of lauroyl chloride and 0.031 mL of pyridine. The MD was isolated by precipitation in isopropyl alcohol. It was further solubilized in distilled water, purified by dialysis for 48 h and finally freeze-dried. The substitution **degree** of MD was 4 % of glucose units, according to the <sup>1</sup>H NMR spectra.

## Methods

### BZ and TM detection

BZ concentrations were determined by UV spectrophotometry at 260 nm (Perkin Elmer UV/VIS spectrophotometer, Germany).

TM concentrations were assessed using high-pressure liquid chromatography (HPLC)<sup>23</sup>. The equipment consisted of a Waters Model 600 E pump equipped with a Waters Model Whisp 712 automatic injector, a Waters Model variable UV detector and a Waters Model integrator. The analytical chromatography column was a Symmetry C<sub>18</sub> column (3.9 x 150 mm) while the mobile phase consisted of a mixture of 50 mM potassium phosphate buffer (pH 7) and acetonitrile (55:45 v/v). The injection volume was 50 µL and the flow rate of the mobile phase was 1 mL/min. TM was detected at 250 nm.

The presence of βCD, pβCD or MD did not affect the absorbances of both BZ and TM in water. This validated the entrapment and release experiments.

### Phase solubility studies

Phase solubility studies were performed according to Higuchi and Connors<sup>24</sup>. A series of native β-CD and pβCD aqueous solutions of increasing concentrations (0 - 75 g/L for pβCD and 0 - 15 g/L for β-CD) were introduced in vials containing an excess amount of BZ or TM. The suspensions, accurately sheltered from light, were shaken at a controlled temperature of 25 ± 0.5°C. Equilibrium was reached after four and five days for BZ and TM, respectively. After this time, the excess (not dissolved) amount of BZ and TM was constant. An aliquot of the samples was ultracentrifuged at 25°C for 45 minutes at 112 500 ×g (Beckman L7-55, USA). By this way, BZ- or TM-loaded β-CD and pβCD solutions were obtained. The concentrations of the solubilized molecules were measured by UV spectrophotometry or by HPLC as previously described. The phase solubility diagrams were obtained by plotting the mean solubilities of BZ or TM against β-CD concentrations. For pβCD polymer, the β-CD concentrations were calculated taking in account the β-CD content of the polymer estimated at 70 wt % by <sup>1</sup>H NMR spectroscopy.

The stability constants of the complexes formed ( $K_s$ ) were calculated from the regression curve of the initial straight part of the solubility diagrams, with the assumption of a 1:1 stoichiometry, according to Eq. 1:

$$K_s = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad \text{Eq. 1}$$

where  $S_0$  was BZ or TM solubility in water, in the absence of  $\beta$ -CD.

The solubilization of BZ or TM by the MD polymer was also studied using the same procedure (MD concentrations: 0 – 25 g/L).

### **Circular dichroism spectrometry**

Circular dichroism spectra of TM and BZ alone or in the presence of native  $\beta$ -CD or its polymer were obtained using a Dichro JASCO J-810 spectropolarimeter (Japan). The circular dichroism spectra were recorded in the 200-350 nm wavelength domain at room temperature. The p $\beta$ CD concentration was determined in order to achieve the same  $\beta$ -CD content either in the native  $\beta$ -CD solution or in the p $\beta$ CD one. Both native  $\beta$ -CD and p $\beta$ CD polymer had no circular dichroic signal in this wavelength domain for the concentrations used in the optical experiments.

### **Isothermal titration microcalorimetry (ITC) studies**

An isothermal calorimeter (ITC) (MicroCal Inc., USA) has been used for determining from a single titration curve simultaneously the enthalpy of the interaction between benzophenone and cyclodextrins in their native or polymerized form and the equilibrium constant corresponding to the formation of a complex between those species. The ITC instrument was periodically calibrated either electrically using an internal electric heater, or chemically by measuring the dilution enthalpy of methanol in water. This standard reaction was in excellent agreement (1-2%) with MicroCal constructor data.

In a typical experiment, a syringe filled with 283 $\mu$ L of  $\beta$ -cyclodextrin (10mM) or p $\beta$ -CD (concentration of  $\beta$ -CD cavities =10mM) aqueous solution, was used to titrate an aqueous solution of BZ into the calorimetric cell at 298.15 K. Aliquots of 10  $\mu$ L of titrant (cyclodextrins solution) were delivered over 25s, intervals between injections were 600s and agitation speed was 220 rpm. A background titration, consisting in injecting the same titrant solution in solely the milliQ water placed in the sample cell, was subtracted from each experimental titration to account for dilution effects.

Data consisting in series of heat flows as a function of time were collected automatically and when appropriate, the interaction process between the two species has been analysed by the one-site binding model proposed in the Windows-based Origin 7 software package supplied by MicroCal. Based on the concentrations of the titrant and the sample, the software used a nonlinear least-squares algorithm (minimization of Chi<sup>2</sup>) to fit the series of heat flows (enthalpograms) to an equilibrium binding

equation, providing best fit values of the stoichiometry, binding constant and change in enthalpy and entropy.

### Nanogels preparation and loading

Nanogel suspensions (polymer concentration  $C_p$  of 1 g/L and 2.5 g/L) were prepared by mixing a MD solution and a p $\beta$ CD solution, at room temperature under magnetic stirring. The concentrations of both solutions were the same and their respective volumes were varied in order to reach a polymer weight ratio MD/p $\beta$ CD of 50/50 and 33/67 (w/w).

The hydrophobic molecules (HM), BZ or TM, were incorporated into the MD-p $\beta$ CD nanoassemblies using two procedures: (i) HM-loaded nanogels (L-nanogels) were obtained by mixing an HM-free MD solution and an HM-loaded p $\beta$ CD solution. (ii) Bi-loaded nanogels (BL-nanogels) were prepared by mixing the two polymer solutions already loaded with HM. The HM-loaded polymer solutions were prepared as described in the section “phase solubility studies”.

The amount of HM entrapped into the nanoassemblies was evaluated by UV spectrophotometry or by HPLC analysis as previously described. Practically, this amount was determined by subtracting the amount of HM found in the dispersion medium after ultracentrifugation (112,504  $\times$ g, 30 min, 25°C) of the nanogels, from the total amount of HM initially brought in the nanogel suspension. The encapsulation efficiency was calculated according to Eq. 2:

$$\text{encapsulation efficiency \%} = \frac{\text{HM in the nanogels } (\mu\text{g})}{\text{HM in the suspension } (\mu\text{g})} \times 100 \quad \text{Eq.2}$$

Assuming that almost 95% of the polymers introduced form nanogels<sup>19</sup>, the amount “ $D$ ” of HM associated per 100 mg of dried polymer (loading) was calculated as follows:

$$D \% (w/w) = \frac{Q (mg)}{Pm (mg)} \times 100 \quad \text{Eq.3}$$

where,  $Q$  is the amount of HM effectively entrapped into the nanogels and  $Pm$  the total amount of the two polymers, p $\beta$ CD and MD, introduced in the sample preparation.

### Nanoassemblies size measurements

The mean diameter and the size distribution of the nanogels were determined by quasi-elastic light scattering (QELS) using a Coulter Nanosizer (Model N4MD, Coultronic, France). According to the need, samples were diluted with milliQ water in order to maintain the count per second between  $5 \times 10^4$

and  $1 \times 10^6$ . Each sample was measured three times for 1 min at  $20^\circ\text{C}$  and at an angle of  $90^\circ$ . Both unimodal and size distribution processor (SDP) analysis were performed.

### ***In vitro* release study**

The release experiments were carried out in water at  $37^\circ\text{C}$  on BZ-BL-nanogel suspensions ( $C_p = 2.5$  g/L and MD/p $\beta$ CD = 50/50 w/w). Freshly prepared BZ-BL-nanogel suspensions were diluted with the release medium. Then, the nanogel suspension was separated in 1mL aliquots and placed on a shaker Heildolph, Titramax 101 (Germany). At each given time-point, one of the aliquots was centrifuged ( $112,504 \times g$ , 30 min) to precipitate the nanogels and the free BZ in the supernatant was assessed by UV spectrophotometry.

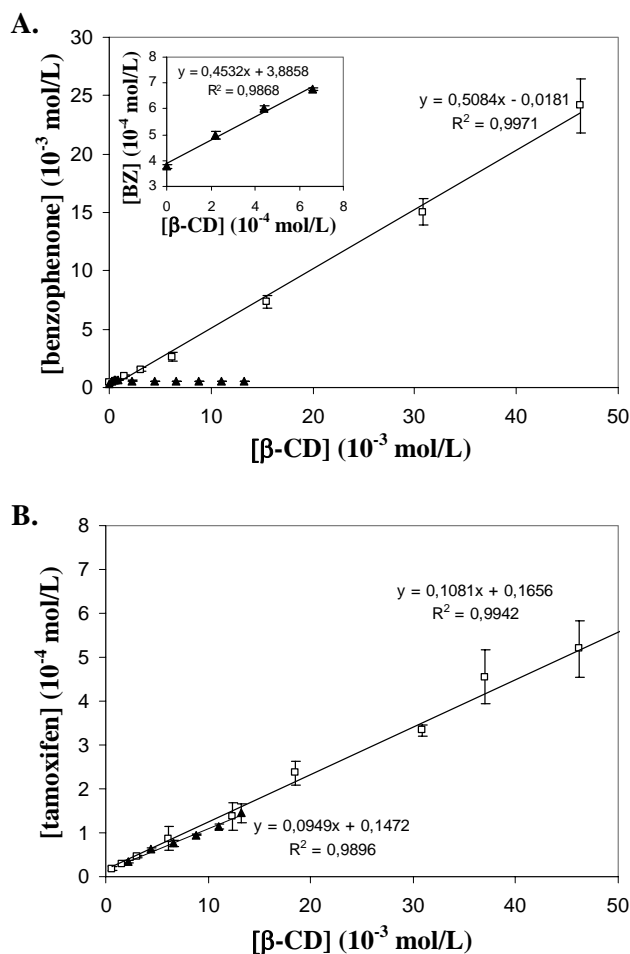
## **Results and discussion**

### ***BZ and TM interactions with $\beta$ -CD and its polymer***

Results in the literature showed that BZ and TM form inclusion complexes with native  $\beta$ -CD<sup>25-29</sup>. However, to our knowledge, the occurrence of complexation between the  $\beta$ -CD polymer and these hydrophobic molecules has not been evidenced yet. Therefore, BZ and TM interactions with both  $\beta$ -CD and its polymer were investigated here by solubility studies, circular dichroism and Isothermal Titration microCalorimetry (ITC).

### ***Phase solubility studies***

The interaction of  $\beta$ -CD and p $\beta$ CD with **BZ and TM** was evaluated using the solubility method, in water at  $25^\circ\text{C}$ . The equilibrium phase solubility diagrams obtained are presented in fig. 1.



**Figure 1.** Phase solubility diagrams of (A) BZ and (B) TM in the presence of (▲) native  $\beta$ -CD and (□) p $\beta$ CD in purified water at 25°C. Each value was the average of three independent experiments  $\pm$  SD.

Both BZ and TM have a very low solubility in water. BZ has a logP of 3.18, whereas TM has a logP of only 7.9. The apparent solubilities of these compounds were improved in the presence of  $\beta$ -CD and p $\beta$ -CD (Fig. 1A).

As it can be seen in fig. 1A, the BZ/ $\beta$ -CD solubility curve was a typical B<sub>S</sub>-type phase solubility diagram<sup>24</sup>. In the first portion of the diagram (inset to fig. 1A), the apparent solubility of BZ increased linearly by increasing  $\beta$ -CD concentration in the 0 – 0.75 g/L range (0 –  $6.6 \times 10^{-4}$  mol/L). This ascending portion was followed by a plateau region indicating the formation of insoluble BZ/ $\beta$ -CD complexes when  $\beta$ -CD concentration was increased above 0.75 g/L ( $6.6 \times 10^{-4}$  mol/L).

Conversely, in the presence of p $\beta$ CD, a continuous linear increase of BZ solubility was observed when the  $\beta$ -CD polymer concentration was increased. The phase solubility diagram was a typical A<sub>L</sub>-

type<sup>24</sup> indicating the formation of soluble BZ/ $\beta$ -CD complexes for the CD polymer. The apparent stability constant ( $K_S$ ) of the BZ/ $\beta$ -CD complexes, calculated from the initial straight part of the solubility diagrams, was found equal to 2070 M<sup>-1</sup> for BZ/ $\beta$ -CD complexes and was within the range reported in the literature<sup>26, 27</sup>. For BZ/p $\beta$ CD complexes,  $K_S$  was 2580 M<sup>-1</sup>, not significantly different from the BZ/ $\beta$ -CD one.

Fig. 1B shows that the apparent solubility of TM increased linearly by increasing the concentration of either  $\beta$ -CD or p $\beta$ CD. The diagrams were A<sub>L</sub>-type indicating the formation of soluble complexes for both the native  $\beta$ -CD and the CD polymer with  $K_S$  of 1230 M<sup>-1</sup> and 1400 M<sup>-1</sup> for the TM/ $\beta$ -CD and TM/p $\beta$ CD, respectively.

These results clearly demonstrate the advantage of using p $\beta$ CD polymers instead of the native  $\beta$ -CD to improve the apparent solubility of both BZ and TM. Indeed, although the stability constant of the complexes formed with the native  $\beta$ -CD and with the  $\beta$ -CD polymer were not significantly different, the solubilization of the hydrophobic molecules, especially BZ, was limited by the poor solubility of  $\beta$ -CD in water (18.5 g/L equivalent to 1.6×10<sup>-2</sup> mol/L). Conversely, the highly water-soluble  $\beta$ -CD polymer allowed a spectacular increase of the hydrophobic molecules' solubility. For example, a polymer concentration of 75 g/L ([ $\beta$ -CD] = 4.6×10<sup>-2</sup> mol/L) could enhance TM solubility by 65-fold, while a  $\beta$ -CD concentration of 15 g/L (1.3×10<sup>-2</sup> mol/L), near to saturation, allowed only an 18-fold increase of TM apparent solubility (fig. 1B). Moreover, in the case of BZ, the solubilization was stopped by the formation of insoluble BZ/ $\beta$ -CD complexes since the very low  $\beta$ -CD concentration of 0.75 g/L (6.6×10<sup>-4</sup> mol/L) (fig. 1A).

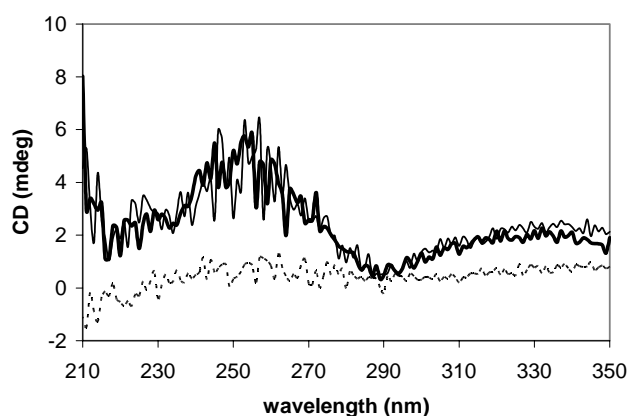
These solubility studies showed the presence of strong interactions between the two molecules studied and both the native  $\beta$ -CD and its polymer, suggesting the formation of complexes with the CDs. However, the guest molecules could be accommodated not only in the CD cavities but also externally, in confined spaces between CDs molecules in the p $\beta$ CD polymer. It has been shown that cyclodextrins and cyclodextrin complexes self-associate to form aggregates and that these aggregates act as solubilizers themselves<sup>30</sup>. Moreover, water-soluble polymers enhance the complexation efficiency by stabilizing these aggregates. Therefore, circular dichroism was used to study the location of BZ and TM.

### Circular dichroism

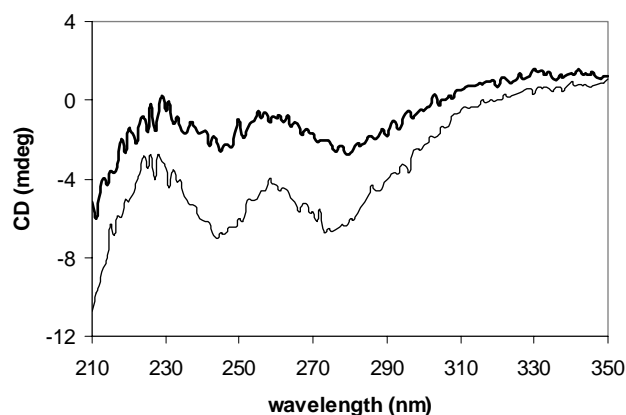
Circular dichroism spectroscopy is particularly useful to ascertain the existence of cyclodextrin inclusion compounds in aqueous solutions<sup>31-33</sup>. Indeed, it was reported that the inclusion of an achiral guest molecule into the asymmetric CD cavity induced extrinsic Cotton effects located in the absorption band of the guest molecule, measured by circular dichroism. On the contrary, an outer surface association between guest molecules and CDs causes only a modification of other spectral properties without inducing Cotton effect<sup>34</sup>.

Fig. 2 presents the circular dichroism spectra recorded for BZ and TM in the presence or in the absence of native  $\beta$ -CD or p $\beta$ CD. In order to compare the dichroic signal obtained when BZ and TM were complexed with CDs in their monomeric and polymerized forms, the p $\beta$ CD concentration was determined to achieve the same  $\beta$ -CD content either in  $\beta$ -CD or in p $\beta$ CD samples.

**A.**



**B.**



**Figure 2.** (A) Circular dichroism spectra of benzophenone: --- free-BZ, — BZ: $\beta$ -CD and — BZ:p $\beta$ CD; [CD] =  $6.5 \times 10^{-4}$  mol/L. (B) Circular dichroism spectra of tamoxifen: --- free TM, — TM: $\beta$ -CD and — TM:p $\beta$ CD; [CD] =  $7 \times 10^{-3}$  mol/L.

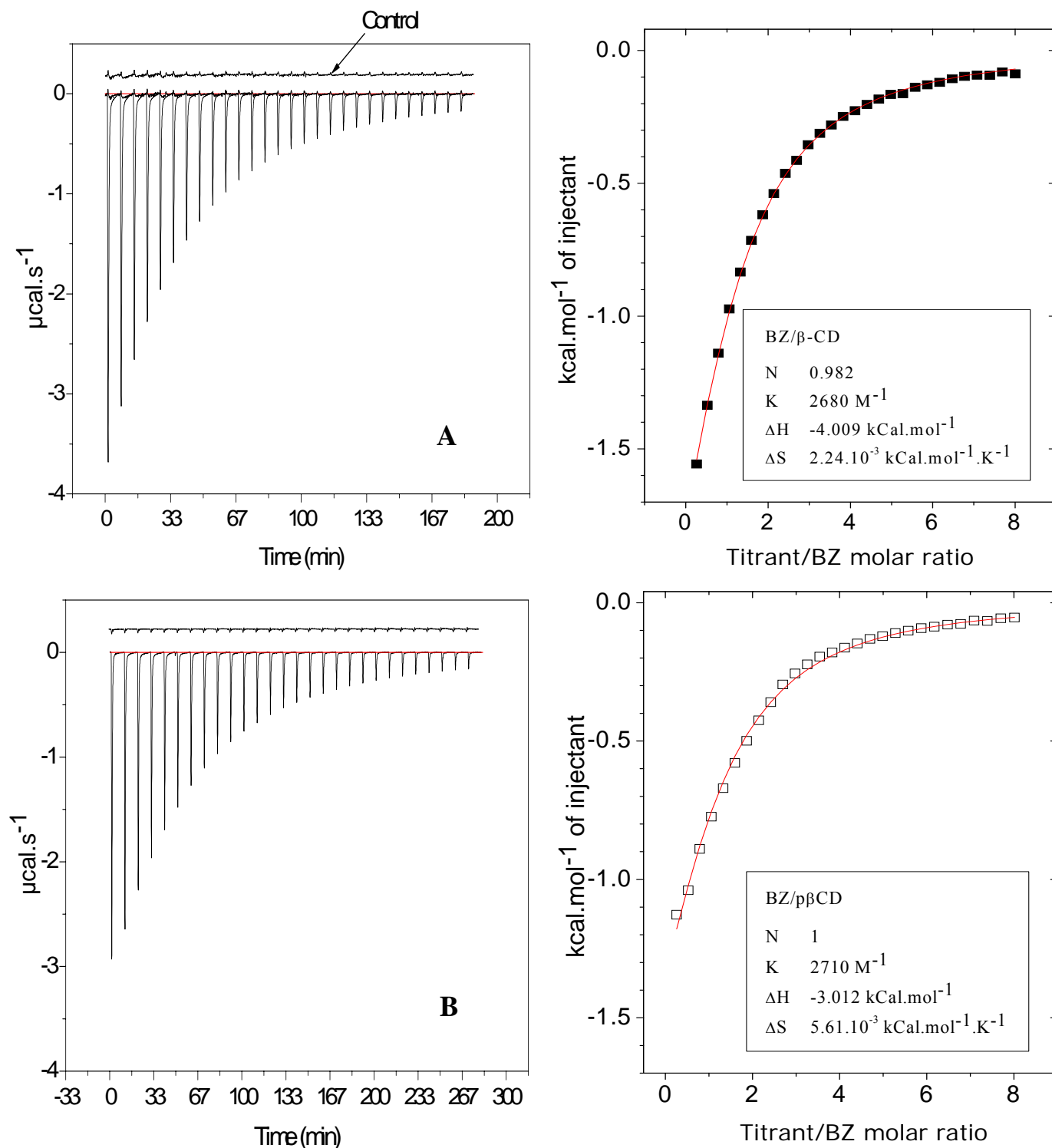
As shown in fig. 2A, BZ free did not produce dichroic signal. Its interaction with either the native  $\beta$ -CD or the p $\beta$ CD resulted in two positive induced circular dichroism (icd) bands observed at 257 nm and 330 nm. This could be attributed to the perturbation of the electronic transitions of the guest,  $\pi$ - $\pi^*$  for the band at 257 nm and n- $\pi^*$  of the carbonyl group for the band at 330 nm<sup>25, 26</sup>, caused by inclusion into the asymmetric cavity of the CD<sup>35, 36</sup>. Moreover, the icd spectra recorded in the presence of native  $\beta$ -CD and of the  $\beta$ -CD polymer were practically the same, indicating similar inclusion modes and orientation of BZ into the CD cavities.

No circular dichroic signal was observed for TM alone in solution (fig. 2B). In the presence of p $\beta$ CD and native  $\beta$ -CD, two negative icd bands were observed at 239 nm and 278 nm. This clearly demonstrated the inclusion of TM into the free  $\beta$ -CDs and the cross-linked CDs in the polymer. The intensity of the icd bands recorded for both BZ and TM increased by increasing the concentration of  $\beta$ -CD or its polymer in the samples, confirming therefore the inclusion of the HMs into  $\beta$ -CD cavities (data not shown).

### ***Isothermal titration microcalorimetry***

At the present time, Isothermal titration microcalorimetry (ITC) is the most modern and sensitive method available for the determination of thermodynamics of the host (CD)-guest interaction<sup>37-44</sup>. ITC shows whether an association process occurs and allows the evaluation of the stability constant (K), the enthalpy ( $\Delta H$ ) and the entropy ( $\Delta S$ ) of the interaction from which the Gibbs free energy ( $\Delta G$ ) of the process can be derived<sup>40, 45</sup>. In our work, ITC has been used in addition to phase solubility studies and circular dichroism experiments to gain information on the inclusion ability of p $\beta$ CD toward hydrophobic molecules, in comparison with that of native  $\beta$ -CD. It is noteworthy to point that, unfortunately in the case of tamoxifen, ITC experiments were not possible due to the very poor aqueous solubility of the drug.

Typical ITC data corresponding to the binding interaction of BZ and  $\beta$ -CD - in its native form or polymerized one – are presented in Fig. 3.



**Figure 3.** Typical ITC data corresponding to the binding interaction of benzophenone (0.25mM) with  $\beta$ -cyclodextrin (10 mM) (■) and poly- $\beta$ -cyclodextrin (10 mM) (□) in milliQ water at 25°C. Left panels show exothermic heat flows which are released upon successive injection of 10 $\mu$ L aliquots of  $\beta$ -CD (A) and p $\beta$ CD (B) into benzophenone. Right panels show integrated heat data, giving a differential binding curve which was fit to a standard single-site binding model yielding the following parameters: N, K,  $\Delta$ H and  $\Delta$ S.

The integration of the exothermic heat flows which were released upon successive injection of 10  $\mu\text{L}$  aliquots of CDs into BZ solution leads to a differential binding curve which was fitted to a standard single-site binding model leading to the direct determination of  $K$ ,  $\Delta H$  and  $\Delta S$  changes of the interaction. From these experimentally determined parameters,  $\Delta G$  was calculated using Eqs. 4 and 5<sup>46</sup>.

$$\Delta G = -RT \ln K \quad \text{Eq.4}$$

where  $R$  is the gas constant ( $8.314 \text{ J.K}^{-1}.\text{mol}^{-1}$ ) and  $T$  is the absolute temperature of the interaction in degrees Kelvin.

$$\Delta G = \Delta H - T\Delta S \quad \text{Eq.5}$$

The stability constants and the thermodynamic parameters of the association between BZ and  $\beta$ -CD or p $\beta$ CD are presented in table 1.

**Table 1.** Complexes stability constants ( $K$ ), and thermodynamic parameters in  $\text{kJ.mol}^{-1}$  for inclusion complex formation of benzophenone with  $\beta$ -CD and p $\beta$ -CD at 298 K (25°C).

CD derivative	$K$ $\text{M}^{-1}$	$\Delta H$ $\text{kJ.mol}^{-1}$	$T\Delta S^a$ $\text{kJ.mol}^{-1}$	$\Delta G^a$ $\text{kJ.mol}^{-1}$	$N^b$
$\beta$ -CD	2680	-16.77	2.77	-19.55	0.98
p $\beta$ -CD	2710	-12.60	6.97	-19.58	1

<sup>a</sup>  $\Delta G = -RT \ln K$ ,  $T\Delta S = \Delta H - \Delta G$ . <sup>b</sup>  $N$  (N CD : 1 BZ)

As it can be seen, the stability constants  $K$  were in the same order of magnitude for BZ/ $\beta$ -CD ( $2680 \text{ M}^{-1}$ ) and BZ/p $\beta$ CD ( $2710 \text{ M}^{-1}$ ) complexes. These values were totally in accordance with the ones obtained from phase solubility experiments ( $2070 \text{ M}^{-1}$  and  $2580 \text{ M}^{-1}$  for  $\beta$ -CD and p $\beta$ CD, respectively). The stoichiometries of the complexes  $N$  were also quite similar: 0.98 (which is consistent with a 1:1 stoichiometry) and 1 for  $\beta$ -CD and p $\beta$ CD, respectively, indicating that the polymerization of  $\beta$ -CD did not affect the inclusion ability of CD cavities in the polymer.

From a thermodynamic point of view, both BZ/ $\beta$ -CD and BZ/p $\beta$ CD interactions were exclusively exothermic  $\Delta H < 0$  with a minor positive entropic contribution  $\Delta S > 0$  and mostly enthalpy driven

( $|\Delta H| > |T\Delta S|$ ). Moreover, the formation of BZ/ $\beta$ -CD and BZ/p $\beta$ CD complexes was spontaneous in both cases as evidenced by the negative value of the Gibbs free energy  $\Delta G$ .

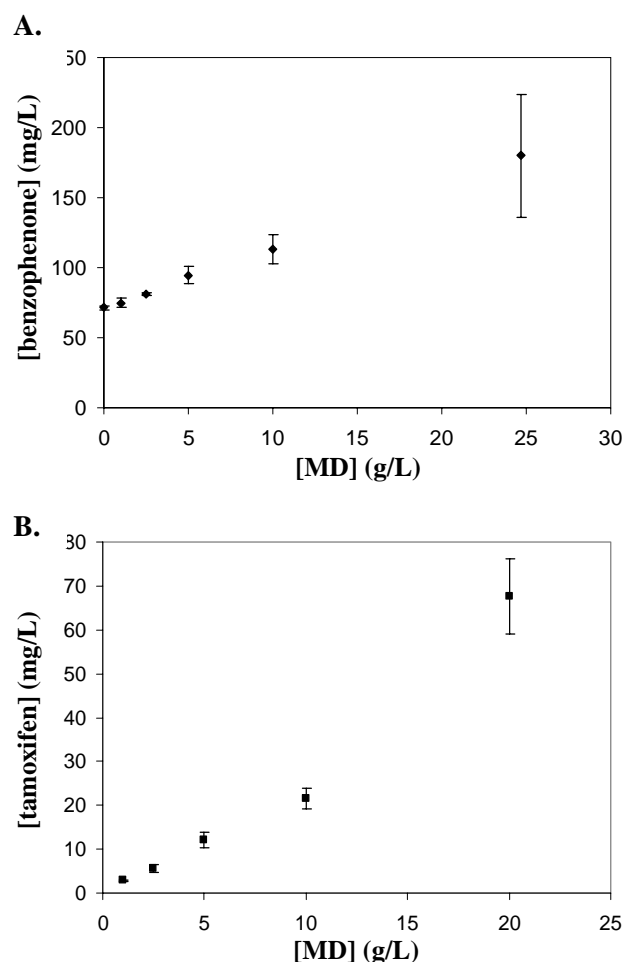
The changes of the thermodynamic parameters upon inclusion into CDs are the result of several effects: the penetration of the hydrophobic part of the guest molecule into the CD cavity, the dehydration of the guest, the formation of hydrogen bonds, the release of water molecules originally included in the CD cavity and conformational changes of CD upon the inclusion of the guest molecule. Among the several possible weak interactions involved in the complexation of guests with CDs, Van der Waals forces and hydrophobic interactions – related to the size/shape matching between the guest and the CD cavity - and other intermolecular interactions such as hydrogen bonding and electrostatic forces have been reported<sup>47, 48</sup>.

In the case of BZ and CDs association, the main driving forces of the binding are Van der Waals because  $|\Delta H| > |T\Delta S|$ <sup>48</sup>. However, some variations were observed in the enthalpy and entropy changes suggesting that the mechanism of binding was slightly different in the case of  $\beta$ -CD or p $\beta$ CD. Indeed,  $\Delta H$  took the value of  $-16.77 \text{ kJ}\cdot\text{mol}^{-1}$  and  $-12.60 \text{ kJ}\cdot\text{mol}^{-1}$  for BZ/ $\beta$ -CD and BZ/p $\beta$ CD complexes, respectively while  $T\Delta S$  increased from  $2.77 \text{ kJ}\cdot\text{mol}^{-1}$  to  $6.97 \text{ kJ}\cdot\text{mol}^{-1}$  for BZ/ $\beta$ -CD and BZ/p $\beta$ CD complexes, respectively. Large positive entropy changes usually arise from the significantly important translational and conformational freedoms of host and guest upon complexation. Because the more hydrophilic environment of the CD cavity in the p $\beta$ CD polymer<sup>49</sup>: the reorganization of surface/cavity neighbouring water molecules that were released upon BZ inclusion could be higher in the case of p $\beta$ CD polymer than in native  $\beta$ -CD resulting in a more positive entropy changes. Furthermore, the desolvation upon guest inclusion and the induced dehydration from peripheral hydroxyl groups in the bridges between CDs in the polymer appear to be jointly responsible for an entropic gain.

### ***BZ and TM interactions with the hydrophobically modified dextran***

Many studies in the literature have reported the ability of hydrophobically modified polymers to improve the solubility of poorly water-soluble drugs<sup>50-53</sup>. Drug solubilization may result from interactions between the insoluble compound and the microdomains made upon the assembly of the hydrophobic moieties grafted onto the hydrophilic polymer backbone. The dextran polymer used in this study bears dodecyl groups. Precedent work has demonstrated that these hydrophobic groups lead to the formation of polymeric micelles<sup>54</sup>. Therefore, it was needed to evaluate also the potential of MD to

improve the apparent solubility of both BZ and TM. Fig. 4 presents the effect of MD on the apparent solubility of BZ and TM.



**Figure 4.** (A) Benzophenone and (B) tamoxifen solubilization by MD in purified water at 25°C ( $0 < [\text{MD}] < 25$  g/L). Each value was the average of three independent experiments  $\pm$  SD.

As it can be seen, the amount solubilized of both molecules increased in a linear manner by increasing the MD concentration, clearly indicating the existence of interactions between the hydrophobic molecules and the hydrophobically modified polymer (MD acts as a surfactant). Noteworthy is the previous observation that the critical aggregation concentration (CAC) of MD (in the same range of substitution yield as here), which corresponded to the onset of the formation of hydrophobic microdomains, was about 0.2 g/L, as determined by surface tension measurements<sup>19</sup>. Above this concentration, the hydrophobic dodecyl moieties of MD associate together, leading to the formation of polymeric micelle-like aggregates. Therefore, the observed increase in BZ and TM solubilities could be reasonably attributed to their solubilization into the hydrophobic microdomains of the amphiphilic MD.

It is noteworthy to point out that the solubilization of both BZ and TM was more efficient in the presence of  $\beta$ -CD polymer than in the presence of MD. Indeed, the concentration of BZ was about  $244.4 \pm 18.2$  mg/L in the presence of 5 g/L p $\beta$ CD and only about  $94.7 \pm 6.2$  mg/L (2.5-fold lower) in the presence of the same concentration of MD. We further took advantage of the ability of both BZ and TM to complex with p $\beta$ CD and to interact with MD, to entrap them into the associative MD-p $\beta$ CD nanogels.

### ***BZ and TM entrapment into MD-p $\beta$ CD nanogels***

As detailed in the Materials and methods section, two procedures were used to incorporate the hydrophobic molecules (HM) into the associative MD-p $\beta$ CD nanogels. In the first one, HM loaded-nanogels (L-nanogels) were prepared by forming HM: p $\beta$ CD inclusion complexes and mixing the obtained solution with a MD solution. Using this procedure, TM and BZ entrapment was tested into nanogels prepared with Cp of 1 g/L or of 2.5 g/L, and MD/p $\beta$ CD ratio of 50/50 (w/w) or of 33/67 (w/w). These two ratios, corresponding to a stoichiometry of 1 C<sub>12</sub>: 3 CD and 1 C<sub>12</sub>: 9 CD, respectively, were chosen in order to have an excess of CD cavities with regard to alkyl chains.

For TM, drug loadings of about 0.5 % (w/w) of the dried polymer mixture were achieved in nanogels of about 200 nm in diameter. Unfortunately, the suspensions were very instable: in the first five minutes after their formation, the nanogels fused together and finally formed a gel deposit. Possibly, TM induced nanogel fusion since nanogel particles were negatively charged<sup>19</sup>, whereas TM was positively charged at the pH of the experiment.

For BZ, nanogels sizing 100 nm to 200 nm were obtained whatever the polymer concentration and the polymer ratio used. In the case of the 1 g/L preparations, these nanogels were stable, with a mean diameter lower than 200 nm over more than 15 days storage at 4°C (data not shown). Table 2 presents the features of BZ entrapment into the L- nanogels. In all experiments the molar ratio BZ:CD was 0.51.

**Table 2.** Characteristics of BZ entrapment into L- nanogels<sup>a</sup>.

Cp <sup>b</sup> (g/L)	MD/pβCD ratio (w/w)	entrapment efficiency (%)	BZ loading (% w/w)	C <sub>12</sub> : CD ratio (w/w)
1	50/50	16.3 ± 0.7	0.8 ± 0.1	1/3
	33/67	14.8 ± 2.1	1.0 ± 0.1	1/9
2.5	50/50	28.0 ± 1.7	0.9 ± 0.1	1/3
	33/67	21.7 ± 2.8	0.9 ± 0.1	1/9

<sup>a</sup>. Loaded nanogels (L-nanogels) were prepared by mixing a solution of preformed BZ:pβCD complexes with a BZ-free MD solution. Each value was the average of three independent experiments ± SD. <sup>b</sup> Cp: polymer concentration.

The first observation is that the BZ encapsulation efficiency increased by increasing the polymer concentration. Indeed, in the 1 g/L nanogels, BZ was incorporated with encapsulation efficiencies of 15-16% whatever the ratio of the two polymers, whereas higher encapsulation efficiencies were found in the case of the 2.5 g/L nanogels: around 28 % for the 50/50 (w/w) ratio and around 22 % for the 33/67 (w/w) ratio. These findings could be related to the preparation procedure of the nanoassemblies. Indeed, they were obtained from a solution of BZ:pβCD complexes. In this solution, the BZ content is the sum of the BZ complexed into the CD cavities and of BZ free in solution (which was present at a concentration equal to its aqueous solubility). Therefore, the proportion of the complexed BZ rose as the concentration of pβCD increased, leading to a better entrapment. However, as we have previously reported that the most stable nanogels were obtained for concentrations less than or equal to 2.5 g/L<sup>55</sup>, the highest polymer concentration here tested for loading experiments was 2.5 g/L.

The second observation is that, whatever the polymer concentrations and ratios tested, the BZ loading, here defined as the amount of BZ associated per 100 mg of dried polymer, was less than or equal to 1 % (w/w). One possible explanation for this low BZ loading arises from the method used for nanogel preparation. Indeed, BZ was entrapped into the MD-pβCD nanogels through its inclusion into the CD cavities of pβCD. When the MD solution was added to the BZ-pβCD solution, the BZ:CD complexes were diluted and could dissociate before the assembly of the two polymers occurred.

Thus, in order to limit the effect of the dilution and to improve BZ incorporation, a second method of preparation has been proposed. Bi-loaded (BL) nanogels were indeed prepared by mixing a BZ-pβCD

solution and a BZ-MD solution as previously explained (see Materials and methods section). Table 3 summarizes the features of BZ entrapment into the BL-nanogels.

**Table 3.** Characteristics of BZ entrapment into BL- nanogels<sup>a</sup>.

Cp <sup>b</sup> (g/L)	MD/pβCD ratio (w/w)	entrapment efficiency (%)	BZ loading (% w/w)	C <sub>12</sub> : CD ratio (w/w)
1	50/50	26.6 ± 5.9	2.9 ± 0.8	1/3
	33/67	22.0 ± 2.1	2.6 ± 0.3	1/9
2.5	50/50	40.7 ± 7.0	2.5 ± 0.6	1/3
	33/67	42.5 ± 5.0	2.9 ± 0.3	1/9

<sup>b</sup> Cp: polymer concentration. <sup>a</sup> Bi-loaded nanogels (BL-nanogels) were prepared by mixing a solution of preformed BZ:pβCD complexes with a BZ-loaded MD solution. Each value was the average of three independent experiments ± SD.

As expected, BZ entrapment was significantly enhanced. In the BL-nanogels, BZ loadings were about 2.5 % (w/w) of dried polymer, approximately three times higher than in the nanogels obtained using the first preparation procedure (0.9 % (w/w)) (table 2). Both MD and pβCD solutions were saturated in BZ. Thus, during the formation process, BZ remained in its complexed form into the CD cavities, since no dilution of the BZ:CD complexes occurred.

Interestingly, the comparison of the nanogel sizes evidenced an increase depending on the presence or not of BZ and the way it was incorporated into the nanogels (table 4).

**Table 4.** Z-average mean diameter of MD-p $\beta$ CD nanogels loaded or not with BZ.

Cp <sup>a</sup> (g/L)	MD/p $\beta$ CD ratio (w/w)	mean diameter (nm)		
		unloaded nanogels	L- nanogels <sup>b</sup>	BL-nanogels <sup>c</sup>
1	50/50	110 $\pm$ 20	135 $\pm$ 19	152 $\pm$ 23
	33/67	106 $\pm$ 15	141 $\pm$ 24	160 $\pm$ 20
2.5	50/50	136 $\pm$ 24	173 $\pm$ 30	192 $\pm$ 42
	33/67	140 $\pm$ 17	180 $\pm$ 28	188 $\pm$ 35

<sup>a</sup> Cp: polymer concentration. <sup>b</sup> Loaded nanogels (L-nanogels) were prepared by mixing a solution of preformed BZ:p $\beta$ CD complexes with a BZ-free MD solution. <sup>c</sup> Bi-loaded nanogels (BL-nanogels) were prepared by mixing a solution of preformed BZ:p $\beta$ CD complexes with a BZ-loaded MD solution. Each value was the average of three measurements  $\pm$  SD.

The higher the BZ loading, the higher the nanogel size. For instance, in the case of nanogels prepared with Cp = 2.5 g/L and MD/p $\beta$ CD = 50/50 (w/w), the mean diameter increased from 136 nm for unloaded nanogels to 192 nm for BZ-BL-nanogels, thus suggesting that, although the effective BZ loading was max. 2.5 % (w/w), the conformation of the nanoassemblies was influenced by the presence of BZ.

The BZ-BL-nanogel suspensions were stable: no release was observed upon storage at room temperature or at 37°C. However, upon dilution in purified water, a fast BZ release occurred in the first 15 minutes. Then, a plateau was reached, i.e. no release was observed within the 24 h of the experiment, suggesting that quickly, partition equilibrium was obtained between BZ in the supernatant and in the polymeric nanogel phase. For example, for a 2/3 dilution corresponding to a final polymer concentration of 1.7 g/L, 54 % of the total amount of BZ entrapped in the nanogels was released. Successive dilutions of the BL-nanogel suspensions allowed finally extracting BZ from the nanogels (> 90 % for a 1/10 dilution). Previously, we showed that BZ was more efficiently solubilized by the  $\beta$ -CD polymer than by MD. Therefore, we presume that in the nanogels, BZ would be located mainly into the CD cavities of p $\beta$ CD. As a consequence, BZ release from the associative nanogels could be probably provided by the dissociation of BZ:CD complexes due to the dilution, as it has been suggested previously for the release of progesterone from nanoparticles made of pre-formed drug: amphiphilic CD inclusion complexes<sup>17</sup>. In conclusion, the release mechanism appears to be mainly driven by the dissociation of the complexes

CD-BZ. Studies are underway to incorporate the BZ-loaded nanogels in cosmetic formulations, to determine the nanogels' stability and the anti-UV properties of encapsulated BZ.

### **Conclusion**

We took advantage of the high interactions of two hydrophobic molecules, benzophenone and tamoxifen, with both the  $\beta$ -CD polymer and the hydrophobically modified dextran, to incorporate them into MD-p $\beta$ CD associative nanogels. Optimization studies performed with BZ enabled to design nanogels displaying a loading of 2.5 % (w/w) also underlining the impact of the loading technique used on the entrapment efficiency into the associative nanogels. Release was governed mainly by the dissociation of the complexes  $\beta$ -CD-molecule of interest. These studies open new possibilities of applications of the nanogels, mainly in the cosmetic field, as sun screen carriers prepared by a simple "green" technology.

## REFERENCES

- (1) Duchêne, D.; Glomot, F.; Vaution, C. In *Cyclodextrins and their industrial uses*, Duchêne, D., Ed. Editions de Santé: Paris, 1987; pp 211-257.
- (2) Uekama, K. *J. Incl. Phenom. Macrocyclic Chem.*, **2002**, *44*, 3-7.
- (3) Uekama, K.; Otagiri, M. *Crit. Rev. Ther. Drug Carrier Syst.*, **1987**, *3*, 1-40.
- (4) Loftsson, T.; Brewster, M. E. *J. Pharm. Sci.*, **1996**, *85*, 1017-25.
- (5) McCormack, B.; Gregoriadis, G. *J. Drug Targeting*, **1994**, *2*, 449-454.
- (6) Duchêne, D.; Wouessidjewe, D.; Ponchel, G. *J. Control. Rel.*, **1999**, *62*, 263-268.
- (7) Duchêne, D.; Ponchel, G.; Wouessidjewe, D. *Adv. Drug Deliv. Rev.*, **1999**, *36*, 29-40.
- (8) Silveira, A. M.; Ponchel, G.; Puisieux, F.; Duchêne, D. *Pharm. Res.*, **1998**, *15*, 1051-1055.
- (9) Silveira, A. M. Formulation et caractérisation de nanoparticules combinées de poly(cyanoacrylate d'isobutyle) et de cyclodextrines destinées à l'administration de principes actifs faiblement solubles dans l'eau. PhD thesis, University of Paris XI, 1998.
- (10) Boudad, H.; Legrand, P.; Lebas, G.; Cheron, M.; Duchêne, D.; Ponchel, G. *Int. J. Pharm.*, **2001**, *218*, 113-124.
- (11) Skiba, M.; Wouessidjewe, D.; Fessi, H.; Devissaguet, J. P.; Duchêne, D.; Puisieux, F. Préparation et applications de nouveaux systèmes colloïdaux nanovésiculaires dispersibles à base de cyclodextrines, sous forme de nanocapsules. PCT Appl Fr 93/00593, 1993.
- (12) Skiba, M.; Wouessidjewe, D.; Coleman, A. W.; Fessi, H.; Devissaguet, J. P.; Duchêne, D.; Puisieux, F. Préparation et utilisations de nouveaux systèmes colloïdaux dispersibles à base de cyclodextrines, sous forme de nanosphères. PCT Appl Fr 93/00594, 1993.
- (13) Skiba, M.; Duchêne, D.; Puisieux, F.; Wouessidjewe, D. *Int. J. Pharm.*, **1996**, *129*, 113-121.
- (14) Lemos-Senna, E.; Wouessidjewe, D.; S., L.; Duchêne, D. *Pharm. Dev. Technol.*, **1998**, *3*, 1-10.
- (15) Memisoglu, E.; Bochot, A.; Sen, M.; Charon, D.; Duchene, D.; Hincal, A. A. *J. Pharm. Sci.*, **2002**, *91*, 1214-24.
- (16) Memisoglu, E.; Bochot, A.; Ozalp, M.; Sen, M.; Duchene, D.; Hincal, A. A. *Pharm. Res.*, **2003**, *20*, 117-25.
- (17) Memisoglu, E.; Bochot, A.; Sen, M.; Duchene, D.; Hincal, A. A. *Int. J. Pharm.*, **2003**, *251*, 143-53.
- (18) Memisoglu-Bilensoy, E.; Vural, I.; Bochot, A.; Renoir, J. M.; Duchene, D.; Hincal, A. A. *J. Control Rel.*, **2005**, *104*, 489-96.
- (19) Gref, R.; Amiel, C.; Molinard, K.; Daoud-Mahammed, S.; Sebille, B.; Gillet, B.; Beloeil, J. C.; Ringard, C.; Rosilio, V.; Poupaert, J.; Couvreur, P. *J. Control Rel.*, **2006**, *111*, 316-24.
- (20) Renard, E.; Deratani, A.; Volet, G.; Sebille, B. *Eur. Polym. J.*, **1997**, *33*, 49-57.
- (21) Arranz, F.; Sanchez-Chaves, M. *Polymer*, **1988**, *29*, 507-512.
- (22) Amiel, C.; Moine, L.; Sandier, A.; Brown, W.; David, C.; Hauss, F.; Renard, E.; Gosselet, M.; Sebille, B. In *Stimuli-Responsive Water Soluble and Amphiphilic Polymers*, McCormick, C. L., Ed. American Chemical Society: Washington, DC, 2001; Vol. 780, pp 58-81.

- (23) Waters-Corporation. *Waters column*, **1996**, 6, 6-14.
- (24) Higuchi, T.; Connors, K. A. *Adv. Anal. Chem. Instrum.*, **1965**, 4, 117-212.
- (25) Matsuura, N.; Takenaka, S.; Tokura, N. *J. Chem. Soc. Perkin Trans.*, **1977**, 2, 1419-1421.
- (26) Monti, S.; Flamigni, L.; Martelli, A.; Bortolus, P. *J. Phys. Chem.*, **1988**, 92, 4447-4451.
- (27) Yu, S. C.; Bochot, A.; Chéron, M.; Seiller, M.; Grossiord, J. L.; Lebas, G.; Duchêne, D. *STP Pharm. Sciences*, **1999**, 9, 273-277.
- (28) Armstrong, R. D.; Ward, T. J.; Pattabiraman, N.; Benz, C. *J. Chromatogr.*, **1987**, 414, 192-196.
- (29) Gerloczy, A.; Vikmon, M.; Szejtli, J.; Redenti, E.; Ventura, P. In Mutual solubility enhancement by binary multicomponent complexation of clomiphene and tamoxifen, 9<sup>th</sup> International Symposium on Cyclodextrins, Santiago de Compostela, May 31/June 3, 1998; Kluwer Academic Press: Santiago de Compostela, 1998; pp 277-280.
- (30) Duan, M. S.; Zhao, N.; Ossurardottir, I. B.; Thorsteinsson, T.; Loftsson, T. *Int. J. Pharm.*, **2005**, 297, 213-222.
- (31) Saito, Y.; Ueda, H.; Abe, M.; Sato, T.; Christian, S. D. *Colloids Surf. A: Physicochem. Eng. Aspects*, **1998**, 135, 103-108.
- (32) Scalia, S.; Villani, S.; Scatturin, A.; Vandelli, M. A.; Forni, F. *Int. J. Pharm.*, **1998**, 175, 205-213.
- (33) Marconi, G.; Monti, S.; Manoli, F.; Degli Esposti, A.; Mayer, B. *Chem. Phys. Lett.*, **2004**, 383, 566-571.
- (34) Setnicka, V.; Urbanova, M.; Kral, V.; Volka, K. *Spectrochim. Acta Part A: Mol. Biol. Spectrosc.*, **2002**, 58, 2983-2989.
- (35) Hirayama, F.; Uekama, K. In *Cyclodextrins and Their Industrial Uses*, Duchêne, D., Ed. Editions de Santé: Paris, 1987; pp 131-172.
- (36) Ventura, C. A.; Puglisi, G.; Zappla, M.; Mazzone, G. *Int. J. Pharm.*, **1998**, 160, 163-172.
- (37) Bouchemal, K. *Drug Discovery Today*. **2008**, 13(21-22) 960-972.
- (38) Tong, W. Q.; Lach, J. L.; Chin, T. F.; Guillory, K. *J. Pharm. Biomed. Anal.*, **1991**, 9, 1139-1146.
- (39) De Sousa, F. B.; Denadai, A. M. L.; Lula, I. S.; Lopes, J. F.; Dos Santos, H. F.; De Almeida, W. B.; Sinisterra, R. D. *Int. J. Pharm.*, **2008**, 353, 160-169.
- (40) Denadai, M. L.; Teixeira, K. I.; Santoro, M. M.; Pimenta, A. M. C.; Cortes, M. E.; Sinisterra, R. D. *Carbohydrate Res.*, **2007**, 342, 2286-2296.
- (41) Charlot, A.; Heyraud, A.; Guenot, P.; Rinaudo, M.; Auzély-Velty, R. *Biomacromol.*, **2006**, 7, 907-913.
- (42) Castronuovo, G.; Elia, V.; Velleca, F.; Viscardi, G. *Thermochimica Acta*, **1997**, 292, 31-37.
- (43) Gomez, C. G.; Chambat, G.; Heyraud, A.; Villar, M.; Auzély-Velty, R. *Polymer*, **2006**, 47, 8509-8516.
- (44) Ollila, F.; Pentikäinen, O. T.; Forss, S.; Johnson, M.; Slotte, J. P. *Langmuir*, **2001**, 17, 7107-7111.
- (45) Rekharsky, M. V.; Inoue, Y. In *Cyclodextrins and Their Complexes*, Dodziuk, H., Ed. Wiley-VCH Verlag GmbH&Co KGaA: Weinheim, 2006; pp 199-230.
- (46) Cooper, A. *Curr. Opin. Chem. Biol.*, **1999**, 3, 557-563.
- (47) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.*, **1998**, 98, 1875-1917.

- (48) Inoue, Y.; Hakushi, T.; Tong, L.; Shen, B.; Jin, D. *J. Am. Chem. Soc.*, **1993**, *115*, 475-481.
- (49) Harries, D.; Rau, D. C.; Parsegian, A. *J. Am. Chem. Soc.*, **2005**, *127*, 2184-2190.
- (50) Miwa, A.; Ishibe, A.; Nakano, M.; Yamahira, T.; Itai, S.; Jinno, S.; Kawahara, H. *Pharm. Res.*, **1998**, *15*, 1844-50.
- (51) Francis, M. F.; Lavoie, L.; Winnik, F. M.; Leroux, J. C. *Eur. J. Pharm. Biopharm.*, **2003**, *56*, 337-46.
- (52) Zhang, C.; Qineng, P.; Zhang, H. *Colloids Surf. B Biointerfaces*, **2004**, *39*, 69-75.
- (53) Weiping, S.; Changqing, Y.; Yanjing, C.; Zhiguo, Z.; Xiangzheng, K. *Colloids Surf. B Biointerfaces*, **2006**, *48*, 13-6.
- (54) Wintgens, V.; Amiel, C. *J. Photochem. Photobiol., A Chem.*, **2004**, *168*, 217-226.
- (55) Daoud-Mahammed, S.; Couvreur, P.; Gref, R. *Int. J. Pharm.*, **2007**, *332*, 185-191.