Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Controlled Release 111 (2006) 316–324
journal of controlled release
www.elsevier.com/locate/jconrel

New self-assembled nanogels based on host–guest interactions: Characterization and drug loading

Ruxandra Gref [a,*], Catherine Amiel [b], Karine Molinard [a], Samia Daoud-Mahammed [a], Bernard Sébille [b], Brigitte Gillet [c], Jean-Claude Beloeil [c], Catherine Ringard [a], Véronique Rosilio [a], Jaques Poupaert [d], Patrick Couvreur [a]

[a] *Université Paris Sud, UMR CNRS 8612, Châtenay Malabry, France*
[b] *Laboratoire de recherche sur les polymères, CNRS, Thiais, France*
[c] *Université Paris Sud, RMN Biologique, Gif sur Yvette, France*
[d] *Unité de chimie pharmaceutique et de radiopharmacie, Bruxelles, Belgium*

Received 1 September 2005; accepted 22 December 2005
Available online 28 February 2006

Abstract

We show here, for the first time, that two neutral polymers may completely associate together in water to spontaneously form supramolecular nanoassemblies (nanogels) of spherical shape. The cohesion of these stable structures of about 200 nm is based upon a "lock and key" mechanism: inclusion complexes are formed between the hydrophobic alkyl chains grafted on a polysaccharide (dextran) and the molecular cavities contained in a poly-cyclodextrin polymer. Production yields reached 95%. It was established that all the alkyl chains were included within the cyclodextrins' cavities in these nanoassemblies. The multivalent character of the interactions between the two polymers ensures the stability of the nanoassemblies. Moreover, empty cyclodextrin units remained accessible for the inclusion of compounds of interest such as benzophenon or tamoxifen.

Keywords: Nanogels; Self-assembly; Drug; Cyclodextrins; Inclusion complex

1. Introduction

The use of nanotechnologies for the treatment of diseases with a vital prognostic (i.e. cancer, auto-immune diseases, viral and bacterial infections etc.) has led to new therapeutic strategies [1]. Thus, liposomes or polymer nanoparticles allow the efficient targeting of the diseased cells and tissues, as well as the controlled release of their drug payload in these specific biological targets [2]. Nowadays, they are being developed for a wide range of biomedical and biotechnological applications, including drug delivery, enzyme immobilization and DNA transfection [3,4]. In the field of cosmetics, other applications of nanotechnologies are related to the protection of compounds sensitive to light, oxidation or moisture (i.e. vitamins or antioxidants) [5,6]. However, the commercialization of nano-devices helpful in pharmacology or cosmetics is limited by the fact that their preparation needs to employ large amounts of potentially toxic organic solvents and surfactants, which is often not acceptable, at least for parenteral administration. For example, polymer based nanoparticles may be prepared by the well-known solvent evaporation method in which the droplets of a nanoemulsion are composed of a volatile organic solvent in which the polymer is solubilized [7]. The preparation of liposomes starts usually by dissolving the phospholipids into an organic solvent too, before preparing an emulsion [8]. Traces of solvents are very difficult to eliminate even using very sophisticated and time consuming methods such as ultradialysis, ultracentrifugation or ultrafiltration. In general, the current available nanotechnologies are not able to meet the severe

* Corresponding author. School of Pharmacy, UMR CNRS 8612, 5 Rue J.B. Clément, 92290 Châtenay Malabry, France. Tel.: +33 1 46 83 59 09; fax: +33 1 46 61 93 34.
E-mail address: ruxandra.gref@cep.u-psud.fr (R. Gref).

0168-3659/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2005.12.025

requirements enacted by the public health agencies for medicines or cosmetics.

Therefore, there is an urgent need to develop new concepts and ideas to overcome these technological issues by proposing preparation procedures avoiding the use of organic solvents and surfactants. In this view, we show here that spherical supramolecular nanoassemblies (nanogels) may be obtained in pure water just by mixing two neutral polymers which instantaneously associate together.

Colloidal systems generally result from the association of amphiphilic polymers in water [9,10], from the complexation of oppositely charged polyions [11] or from hydrogen-bonding interactions [12]. Host–guest interactions leading to inclusion complexes between hydrophobic molecules and the apolar concavities of cyclodextrins (CDs) have not so far been described for colloid formation. Since these non-covalent interactions are relatively weak, it is not obvious that they could produce stable colloidal dispersions. Indeed, only aqueous phases with increased viscosities or gels were so far obtained based on the inclusion of hydrophobic side chains into the Cds on a polymer [13,14]. However, based on this "lock and key" mechanism, we now show that it is feasible to generate stable spherical nanoassemblies in aqueous medium devoid of polymer, able to entrap molecules of interest [15].

2. Materials and methods

2.1. Materials

β-cyclodextrin polymers (pβ-CD) were prepared by cross-linking β-cyclodextrin (β-CD) with epichlorohydrin (EP), under strongly alkaline conditions [16]. Briefly, 100 g of anhydrous β-CD were dissolved in 160 mL NaOH 33% w/w solution under mechanical stirring overnight. Then, 81.52 g of EP (molar ratio β-CD/EP = 10) was rapidly added to the solution heated to 30 °C. In order to obtain polymers with high molar masses (*M*), the reaction was stopped in the vicinity of the gelation point by addition of acetone. The obtained aqueous phase was heated at 50 °C overnight, neutralized with 6 N HCl and ultrafiltered using membranes with a cut-off of 100,000 g/mol. The β-CD polymer was finally recovered by freeze-drying. The β-CD content, as determined by ^{1}H NMR spectroscopy, was 70% w/w. Two batches of pβ-CD polymers were thus obtained, with average molar masses of 10^6 and 2.6 10^6 g/mol, as determined by gel permeation chromatography. A pβ-CD with molar mass of 3 10^4 g/mol was also obtained, and purified as previously, except that it was not ultrafiltered. Polymer molecular weights were determined by size exclusion chromatography using pullulan standards.

To synthesize dextran bearing hydrophobic lauryl side chains (MD), 4 g of dextran (40,000 g/mol) were solubilized in 100 ml of dimethyl formamide containing 1 g of lithium chloride. Then, a well-defined amount of lauryl chloride (0.25 to 0.62 mL) and 0.031 mL of pyridine were added to the dextran solution. The reaction was carried out at 80 °C for 3 h. The obtained 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 yield of MD was determined according to the ^{1}H NMR spectra. It ranged from 2.8% to 5.8% of glucose units, according to the amount of lauryl chloride introduced in the reaction mixture.

Water was purified by reverse osmosis (Milli-Q, Millipore®, USA). All reagents were analytical grade. Benzophenon and tamoxifen were supplied from Sigma, France.

2.2. Methods

2.2.1. Surface tension measurements at the air/water interface

The interfacial behaviour of MD and pβ-CD solutions was studied as a function of their concentration. In this experiment, dextran grafted with C_{12} chains (percentage of substituted glucose units: 4%) and pβ-CD with an average molar weight of 2.5 10^6 g/mol were used. Both polymers were dissolved in tridistilled water under stirring for 24 h at room temperature. Surface tension γ was measured by the Wilhelmy plate method using a tensiometer (K10ST, Krüss, Germany). Measurements were performed in triplicate at 23 ± 2 °C in saturated vapour conditions. Small volumes (16 to 50 μL) of a pβ-CD solution (5 mg/mL) were injected into a MD solution (2 μg/mL in a total volume of 10 ml). The mixture was gently stirred for 2 min before γ measurement.

2.2.2. Nanoassemblies preparation and size measurement

Nanoassemblies were obtained by mixing at room temperature equal volumes of aqueous solutions of pβ-CD and of MD, at concentrations ranging from 1 to 10 mg/mL. Their mean diameter and the size distribution were determined at different time intervals 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 5×10^4 and 1×10^6. Each sample was measured three times for 1 min at 20 °C and at an angle of 90°. Both unimodal and size distribution processor analysis were performed. The experiments were made in triplicate.

Nanoassemblies suspensions were centrifuged (20,000 ×*g*, 30 min). Supernatants were collected and freeze-dried, in order to determine the weight of the polymers which did not form nanogels. The production yields were calculated from the mass ratio of polymers forming nanoassemblies and the polymers initially introduced in the fabrication procedure.

2.2.3. Microscopic observation of the nanoassemblies

Nanogel formation was visualized by dark-field microscopy using an Eclipse E60 Nikon microscope. For this, a 20 μL droplet of a MD solution was put on a microscopic lamella and covered with a cover slip. A second drop of 20 μL of pβ-CD was deposited in the vicinity of the cover slip. The solution penetrated by capillarity in the little space comprised between the two glasses and the MD and pβ-CD solution became in contact with each other. Apparition of white brilliant spots reflected nanogel formation.

The nanoassemblies suspensions obtained as described in the previous section were observed by transmission electron

microscopy (TEM) after freeze-fracture, in the absence of any additional substance such as glycerol. The cryotechnique procedure used involved three steps: i) high pressure freezing; ii) freeze fracture which includes fracturing, replication and cleaning of the replicas, and iii) TEM investigation of the replicas.

For this, a small amount of nanogel suspension was introduced into a 100 μm deep symmetric cup, made of copper and able to conduct heat rapidly away from the specimen. Then, the sample was frozen using a high pressure cooling device HPM 010 (Bal-Tec, Balzers Union): it was subjected to pressures of about 2100 bars by the injection of a little warm alcohol as a pressuring medium, a few milliseconds before the sample was cooled by two jets of liquid nitrogen. After the cryofixation has been performed, the sample was kept in liquid nitrogen in order to avoid any modification.

For fracturing, the samples were mounted on a cold table which was inserted inside the vacuum chamber of a Bal-Tec Model BAF 400 T apparatus, on a nitrogen-cooled support kept at 103 K. Once the vacuum was lower than 10^{-7} Torr, fracturing was achieved by displacing the single edge scalpel blade precooled at 83 K.

The replication of the fracture surface involved two steps. First, a thin layer (2 nm) of platinum was evaporated onto the specimen from a shadow angle of 45° to provide contrast enhancement of the topographic features of the surface. The second step consisted in depositing a thicker (20 nm) layer of carbon, which provided strength to the shadow cast. All film thickness determinations were monitored by a quartz crystal thickness gauge (4.96 MHz, quartz crystal holder QSK 301 and monitor QSG 060, Bal-Tec).

After complete deposition, the vacuum chamber was vented and the specimens were removed. The replicas of the surface were then floated off the specimen by submerging in successive baths of water, dimethylsulfoxide and acetone. They were finally collected onto naked 400 mesh grids which were subsequently mounted in a TEM apparatus for inspection. TEM observations were performed on a LEO 912 Omega high resolution microscope working at 120 kV.

2.2.4. NMR studies

The ^{1}H NMR spectra were performed at 25 °C on an Inova Varian spectrometer operating at frequencies of 400 MHz, using a 5-mm H X-probe. The spectra were recorded with a flip angle of 90°, a spectral width of 4000 Hz and 256 scans of 16 K points, a repartition time of 15 s, at 25, 30, 35 and 40 °C. The polymers and nanogel samples were prepared in D_2O.

2.2.5. Freeze-drying

Two milliliters of the nanogel suspensions were filled in 8 ml glass vials. The samples were slowly frozen at −20 °C in a conventional freezer for 24 h and then placed into the drying chamber of an alpha I/5 freeze-dryer (Christ, Germany), precooled to −20 °C. Drying was performed at a pressure of 0.05 mbar for 48 h. The freeze-dried samples were resuspended by adding 2 ml of milliQ water under manual shaking during 30 s and the nanogel size was evaluated by QELS as previously described.

2.2.6. Molecular modelling

Molecular modelling was employed to simulate the interaction between MD and pβ-CD in the presence of water molecules, using both molecular mechanics (MM2, MM3) and semi-quantum mechanics (AM1 and PM3). Two linear chains of pβ-CD (8 units) and MD (16 units) were inserted within a cylinder containing 720 water molecules. The system was energy-minimized and subjected to molecular dynamics (300 °K, step 1 fs, heating/cooling rate 1.000 kcal/atom/ps) for a trajectory of 95 ps. The nanoassembly was further modified by adding a benzophenon molecule in the fourth β-CD cavity and the system was subjected to molecular dynamics for an additional 25 ps (same conditions).

2.2.7. Drug entrapment

Benzophenon or tamoxifen was incorporated into the MD/pβ-CD nanoassemblies by two methods: i) by forming inclusion complexes with pβ-CD before mixing with MD or ii) by direct loading into the preformed nanoassemblies.

In the first case, MD and pβ-CD polymer solutions were prepared as previously (overnight stirring at room temperature), at concentration of 1 mg/mL. One milliliter of each suspension was incubated overnight at room temperature, under stirring, with 5 mg benzophenon or tamoxifen. Excess drug non-dissolved in the polymer solution was removed by centrifugation (20,000 × *g*, 30 min). The two MD and pβ-CD polymer solutions were mixed in equal volumes to produce drug-loaded nanogels.

In the second case, empty nanoassemblies were prepared as previously described, at a concentration of 1 mg/mL. One milliliter of suspension was incubated for 1 h with 1 mL of benzophenon solution (50 mg/mL in water).

In both cases, the size of the resulting nanogels was determined by QELS. The suspensions were centrifuged (20,000 × *g*, 30 min) and the amount of drug remaining in the supernatants was assessed by UV spectrophotometry. The amount of drug incorporated into the nanogels was determined as the difference between the total amount of drug introduced in the preparation procedure and the amount of non-entrapped drug, detected in the supernatant. Drug loading was calculated as the ratio between amount of entrapped drug and the total amount of polymers used for particle production.

3. Results and discussion

3.1. Conditions for nanogel preparation and stability studies

To prove the concept that two neutral polymers can associate to form nanogels spontaneously in water, we synthesized: i) a β-CD polymer here designated pβ-CD and ii) a hydrophobically modified polysaccharide, dextran grafted with alkyl moieties, designated MD. The physico-chemical properties of these macromolecules could be modulated by varying the molar mass ($3\ 10^4$ to $2.6\ 10^6$ g/mol), the number *N* of carbons in the alkyl

chains (8–16) and the percentage *Y* of glucose units substituted with alkyl chains (2.8–5.8%). Aqueous solutions of the two polymers were mixed at room temperature. Typically, the system separated in two phases, a highly viscous liquid phase and a supernatant. However, a very narrow domain was found where the system behaved in a completely different way: immediately after mixing the two polymers, suspensions of stable spherically shaped nanogels were obtained in a dispersing medium devoid of soluble polymer. This concept of pβ-CD/MD nanoassemblies is based upon the spontaneous inclusion of the dextran grafted alkyl chains into the β-CD cavities (Fig. 1). It allows a variety of hydrophobic molecules to be included in the remaining free β-CDs. Indeed, the synthesized pβ-CD copolymers had a remarkably enhanced solubility in water as compared to β-CD. Indeed, whereas at room temperature the solubility of β-CD is 17.5 g/l, the pβ-CD copolymers had solubilities higher than 100 g/l. This is certainly an advantage for a number of applications, among which the increase of the apparent solubility of water insoluble compounds able to form inclusion complexes with β-CD.

The feasibility and stability of such nanogels were found to depend upon: the percentage *Y* of MD glucose unit substitution, the polymer concentration *c*, the number *N* of carbons in the alkyl chains, the pβ-CD molar mass *M* and the weight ratio *R* between MD and pβ-CD polymers. The influences of all these parameters have been successively studied. Alkyl chains with more than 12 carbon atoms and *R* ratios comprised between 1 : 3 and 3 : 1 were required to produce stable nanogels (results not shown). Fig. 2 reflects the influence of *M*. Whereas nanogels of less than 200 nm were formed whatever the *M* studied (3 10^4 to 2.6 10^6 g/mol), the largest diameter variations during storage at room temperature were obtained with the lowest *M*. Therefore, high *M* is required to produce nanoassemblies with small size variations over time. The substitution yield *Y* played a main role, too, in the stability (results not shown). The use of MD with *Y* lower than 4% leads to a rapid aggregation and after one day, gel formation was observed. However, samples with $Y \geq 4\%$ did not aggregate and their size remained lower than 200 nm after one day storage.

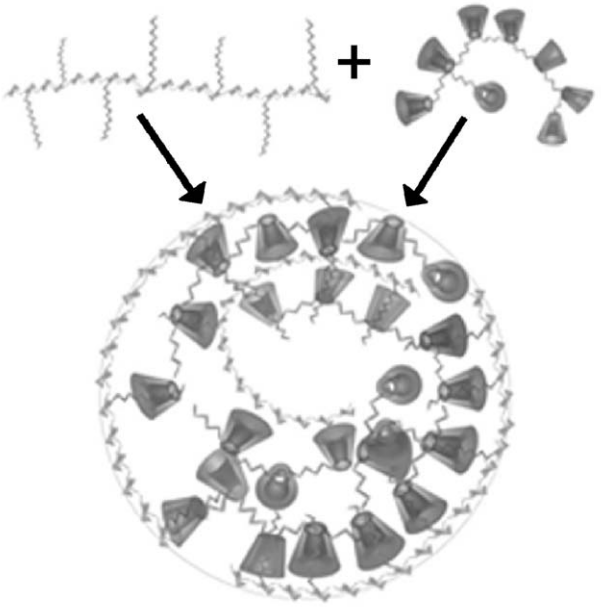

Fig. 1. Schematic representation of the formation of supramolecular nanoassemblies from two associative polymers: a linear MD and a cross-linked pβ-CD. Inclusion complexes are formed between β-CDs and alkyl chains, leaving also empty β-CD cavities.

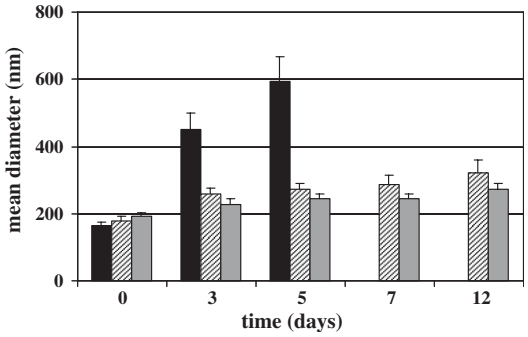

Fig. 2. Stability at room temperature of nanogels prepared by mixing equal volumes of two aqueous solutions (10 mg/mL) of pβ-CD and MD grafted with alkyl chains. Influence of the pβ-CD molar mass: 3 10^4 (■), 10^6 (▨) and 2.6 10^6 g/mol (□), in the case of dextran grafted with C_{12} chains, with a substitution yield *Y* of 4%.

All these studies were carried out with nanogels at concentrations of 10 mg/mL. The concentration of the nanogels was found to be a key point for the stability of the corresponding suspensions, as it reflects the probability that two particles came in contact to each other. This can lead to fusion, if free alkyl chains on one of them encounter free β-CD on the other. Fig. 3A shows the influence of the particle concentration. At low *c* (1 mg/mL) the suspensions were remarkably stable, whatever *Y* (4% or 5.8%). No significant diameter changes were observed over more than 20 days of storage. However, in the case of high *c* (10 mg/mL), a diameter increase was observed for $Y = 4\%$, whereas for $Y = 5.8\%$, a little gel formation was observed. In this last case nanoassemblies coexisted with the gel form which explains the little decrease in the main particle size, as observed in the first week of storage.

The influence of the temperature on the nanoassemblies' stability was also investigated (Fig. 3B). In the first 5 h, at room temperature, no size changes were observed (results not shown). This was also the case at body temperature (37 °C, Fig. 3B). However, at 60 °C, the diameter, initially below 200 nm, increased rapidly, reaching 400 nm after 1 1/2 h of storage. This could be explained by the lower stability of the complexes at this temperature. The higher the temperature, the higher the number of non-complexed alkyl chains and the lower their affinity for the β-CDs. The nanoassemblies loose their structure based on C_{12}:CD complex formation, fuse together and finally form a gel deposit. Therefore, it appears that there is a temperature limit above which the nanogels are not anymore stable.

In conclusion, very stable nanogel suspensions (at temperatures ≤ 37 °C) could be obtained under the following experimental conditions: $Y \geq 4\%$, $c < 10$ g/L, pβ-CD molecular

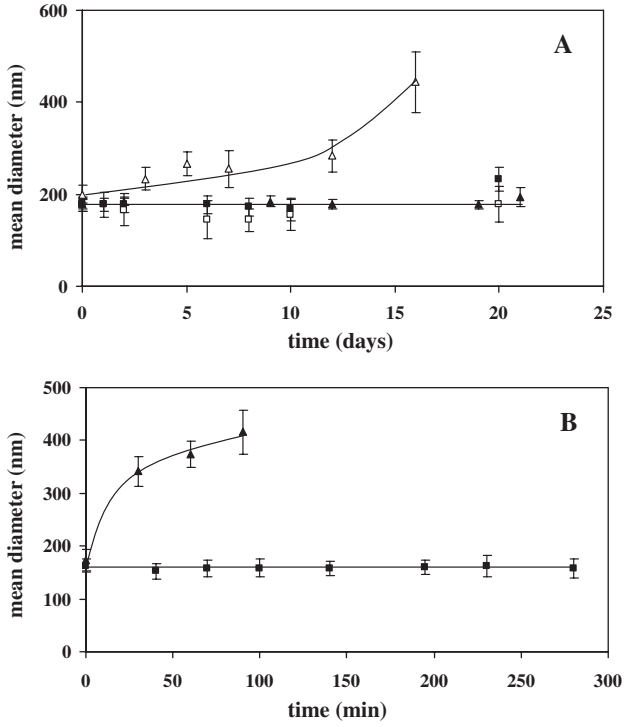

Fig. 3. Stability of nanogel suspensions prepared by mixing equal volumes of two aqueous solutions of pβ-CD (2.6 10^6 g/mol) and MD grafted with alkyl chains. A: Influence of polymer concentration (10 mg/mL, open symbols, or 1 mg/mL, filled symbols) and of MD substitution yield (4%, triangles, or 5.8%, squares). B: Influence of the temperature: 37 (■) and 60 °C (▲) in the case of MD grafted with C_{12} chains, substitution yield of 4% and equal pβ-CD and MD concentrations (10 mg/mL).

weight higher than 10^6 g/mol, $N=12$ and for a weight ratio R between MD and pβ-CD equal to 1. For example, with $c=1$ g/L and $Y=4\%$, the mean diameter was about 170 nm and remained unchanged over one month storage. Interestingly, nanogels with the same diameters were obtained whatever was the manner used to mix the two MD and pβ-CD polymer solutions (order of introduction of the two solutions, mode of mixing, temperature, etc…). In particular, no stirring was necessary, the simple diffusion of one solution into the other was sufficient to form the nanogels. Polydispersity indices were lower than 0.2, showing that the nanogels were monodisperse. This has been confirmed by electron microscopy observations (Fig. 4A). Furthermore, nanoassemblies could also be directly freeze-dried and reconstituted in water without any change in size, which provides an opportunity for unlimited storage (results not shown). Production yields reached 95%, showing the remarkable efficiency of the polymer association. The ease of production and storage is certainly an advantage for future applications. Another benefit is the nanogel stability upon dilution in water and the rapidity of nanogel formation, as revealed by dark-field microscopy. This technique allowed a direct observation of the formation process and showed that the nanogel is shaped up in less than 1 s after mixing the two polymer solutions (Fig. 4B).

The most stable nanogels were obtained with high (>10^6 g/mol) molecular weight pβ-CD polymers. These pβ-CD polymer solutions were completely clear and size measurements by light scattering were impossible because of the poor correlation function. However, by freeze fracture studies we could detect some tiny aggregates of less than 15 nm (results not shown). Only when MD was added, milky solutions formed and size measurements were possible. It can be hypothesized that the pβ-CD polymer solution consists of preformed small aggregates which are cross-linked by MD. Possibly, free CDs inside the cross-linked p-βCD polymer are less accessible to the alkyl side chains linked on a high molecular weight dextran, but more accessible to small sized drug molecules.

The amphiphilic MD polymer has a critical aggregation concentration (cac) of about 0.2 mg/L, as determined by surface tension measurements (see Section 3.3). This means that the stable nanogels described above were prepared using MD solutions (concentrations 1 mg/L) where the MD macromolecules were associated under the form of micelles. Therefore, MD micelles might dissociate so that the resulting MD chains form cross-links with the p-βCD polymer.

3.2. NMR studies

A further insight into the mechanism of nanogel formation and their supramolecular architecture was gained by ^{1}H NMR spectroscopy. It is well established that alkyl chains form inclusion complexes with CDs [17,18]. To evidence the role of C_{12}:CD complexes in the nanogel formation, in a first step, β-CD was added to MD dextran solution. In the case of the uncomplexed MD, the A and B signals corresponded to the CH_3 and adjacent CH_2 protons in the alkyl chains of (Fig. 5A I).

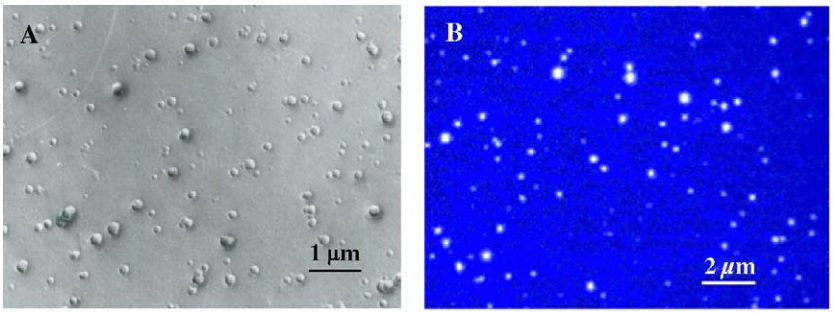

Fig. 4. Photomicrographs of a nanogel suspension taken by transmission electron microscopy after freeze-fracture (A). Dark-field microscopy was used to investigate nanogel formation (B). The polymers used were pβ-CD (10^6 g/mol) and MD grafted with C_{12} chains, $Y=4\%$. For nanogel preparation, 1 mL of MD solution in water (1 mg/mL) was mixed at room temperature with 1 mL of pβ-CD solution in water (1 mg/mL).

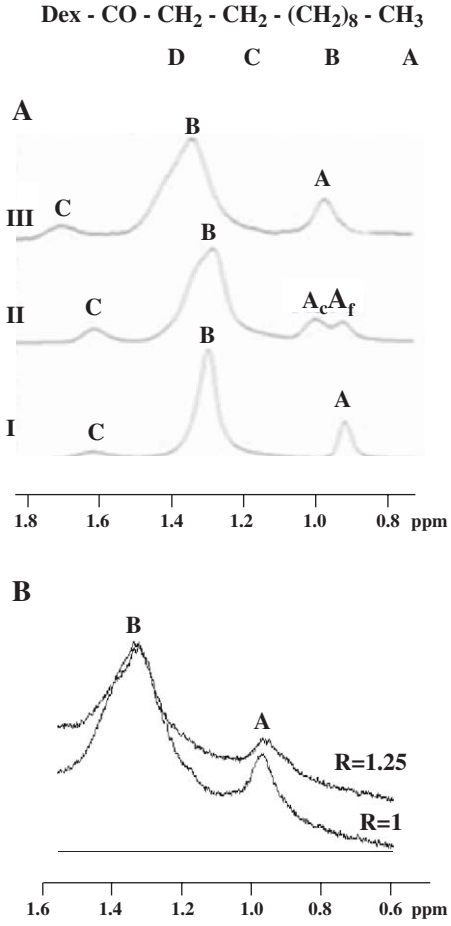

Fig. 5. ^{1}H NMR spectra recorded at 400 MHz, at 25 °C, in D_2O. The polymers used were pβ-CD 10^6 g/mol and MD grafted with lauryl chains, Y=4%. A: (I) MD (5 mg/ml); (II) mixture of MD and β-CD (300 µl MD solution at 5 mg/ml and 434 µl β-CD solution at 17 mg/ml) and (III) nanogel suspension (MD+pβ-CD). For nanogel preparation, 1 mL of MD solution in water (5 mg/ml) was mixed at room temperature with 1 mL of pβ-CD solution in water (5 mg/ml). Enlargement of the 0.8 to 1.8 ppm region. B: ^{1}H NMR spectra of mixtures of MD (5 mg/ml) and pβ-CD (5 mg/ml) with R=1 and 1.25. Enlargement of the 0.6 to 1.6 region.

When β-CD was added to the MD solution, a soluble complex was formed without nanogel formation. The A and B signals were broadened because less shielded signals appeared (Fig. 5A II). These new peaks correspond to the interaction between the alkyl chains and the β-CD cavities. In particular, the A signal was composed of two peaks: A_f, at the same chemical shift as CH_3 in the MD spectrum (0.92 ppm) and A_c, at 1.0 ppm.

When the temperature was increased, it was observed that: (i) the chemical shift of A_f remained constant, whereas the A_c signal was shielded and (ii) the relative proportion of A_f increased (results not shown). These variations could be well fitted using a model taking into account two types of MD alkyl chains. The chains characterized by the A_f are free in solution, whereas the remaining ones do interact with β-CD and their A_c signal characterises their inclusion.

In a second step, nanogels were prepared from MD and pβ-CD mixtures with polymer weight ratios R varying from 0.5 to 1.25 and the NMR spectra were registered. When R was equal to 1, the A signal was composed of a single peak at 0.96 ppm (Fig. 5A III), contrarily to the MD/β-CD mixtures where two distinct peaks were evidenced (Fig. 5A II). This single A peak corresponded to the A_c form, showing that all the MD alkyl chains were interacting with the pβ-CD cavities. The same observations were drawn from all spectra of polymer mixtures when R was less than or equal to 1. However, for R=1.25, the A signal was broader than for R=1 (Fig. 5B), and a slightly shielded signal appeared, showing the apparition of some not included alkyl chains. A possible explanation is that when increasing R, and thus the ratio C_{12}:CD, not all of the hydrophobic chains are able to form inclusion complexes with the pβ-CD cavities, because of sterical constraints within the nanoassemblies. On the contrary, when the number of the C_{12} chains was low compared to the available β-CDs, the probability that the alkyl chains find a vicinal β-CD to get included was high.

In conclusion, it was shown that the alkyl chains on MD were clearly interacting with β-CDs in their monomeric form as well as in their polymerized form (pβ-CD), and that the complex with pβ-CD was more stable than the complex with β-CD. Moreover, if $R \leq 1$, all the alkyl chains were interacting with pβ-CD cavities. This may be the principal reason for the remarkable stability of the nanogels within the narrow domain of experimental conditions previously defined. In this case, sufficient physical cross-links between the chains were formed by means of inclusion complexes, thus stabilizing the supramolecular nanoassemblies. In these conditions, no bridging between the nanogels occurred, as there were no free alkyl chains left.

3.3. Surface tension

Polymer association was also demonstrated by measuring the surface tension, γ, of pβ-CD and MD polymer solutions and their mixtures (Fig. 6). At low MD concentration, the measured

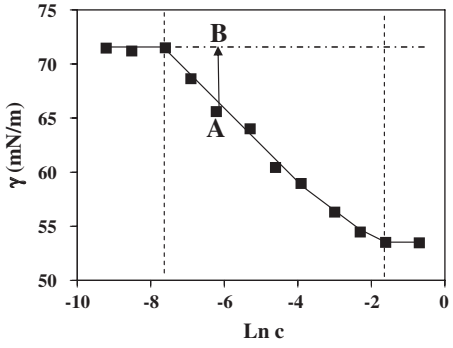

Fig. 6. Interfacial behaviour of MD (M 40,000 g/mol) as a function of its concentration. Dextran was grafted with C_{12} chains (percentage of substituted glucose units: 4%). Polymers were dissolved in tridistilled water under stirring for 24 h at room temperature. Surface tension γ was measured by the Wilhelmy plate method using a tensiometer (K10ST, Krüss, Germany) as a function of the concentration logarithm (in g/L). Measurements were performed in triplicate at 23±2 °C in saturated vapour conditions. Small volumes (16 to 50 µl) of a pβ-CD solution (5 mg/ml) were injected into a MD solution (2 µg/ml in a total volume of 10 ml). The mixture was gently stirred for 2 min before γ was measured. An increase in the surface tension value was observed from A (MD solution) to B (mixture of MD and pβ-CD), tending to the value of pure water.

γ was close to the one of water, as not enough amphiphilic MD molecules reached at the air/water interface (Fig. 6, region I). When the MD concentration was increased, more and more amphiphilic MD polymer adsorbed at the air/water interface, and this lowered the surface tension (Fig. 6, region II) until the interface got saturated and γ reached stable values (Fig. 6, region III). At this point, the critical aggregation concentration (cac) could be estimated at 0.2 mg/L. Contrastingly to MD, pβ-CD did not show any surface activity. When pβ-CD was injected into the MD solution, γ increased and tended to that of pure water (Fig. 6, A to B). This can be explained by the adsorbed MD macromolecules progressively leaving the interface, as a consequence of the sequestration of the MD alkyl chains into the β-CDs of the pβ-CD macromolecules. Although these alkyl chains were oriented towards air and not towards water, and the non-surface-active pβ-CD was unable to reach the interface, MD depletion from the interface took place until the surface was bare. It is probably sufficient for a few alkyl chains immersed in the water phase to be captured by the β-CD cavities to produce a chain reaction leading to a complete depletion of the interface, by a "gear-wheel"-like process. It is reasonable to assume that the non-surface-active supramolecular complex formed between MD and pβ-CD is the precursor for nanogel formation. When β-CD was injected in the MD solution instead of the pβ-CD polymer, it failed to deplete MD from the interface, although, as shown previously by ^{1}H NMR, alkyl chains on MD interacted with β-CD in solution (Fig. 5A). Thus, β-CD molecules in solution were unable to capture alkyl chains exposed to air. This clearly shows that pβ-CD polymer is crucial for the spontaneous interlocking of the two polymer chains.

3.4. Molecular modelling

Molecular modelling studies brought several elements of information which complement the NMR studies and the surface tension investigations. The stability of the system (via molecular dynamics) and the presence of hydrophobic interactions which led to tethering of the alkyl chains inside the β-CD cavities could thus be evidenced. Thus, molecular modelling was employed to simulate the interaction between MD and pβ-CD in the presence of water molecules. In our simulations, when a segment of pβ-CD (containing two linear chains of 8 units, see experimental section) was put into contact with MD (featured by a 16-unit chain), MD tightly interlaced around pβ-CD, which tended to adopt a bent helical structure (Fig. 7). It should be noted that MD established an extensive network of hydrogen bonds with pβ-CD (not illustrated). This network was found to be stable (a trajectory of 400 ps at 300 K was acquired).

It has been previously shown by molecular modelling that β-CD could easily accommodate up to 12 hydrogen-bonded water molecules within its so-called hydrophobic core [19]. In our simulations, molecular dynamics clearly demonstrated the displacement of water molecules from β-CD cavities as a result of alkyl chain inclusion. The resulting "free" water molecules are no longer at a distance compatible with the establishment of hydrogen bonds. As their number increases, it can be inferred that polymer organization in nanoassemblies is essentially entropy-driven.

3.5. Drug encapsulation

As already shown by ^{1}H NMR, in the optimal conditions for nanoassemblies formation, at $R=1$, all alkyl chains were involved in the complex formation which generated nanogels. This leaves about half of the β-CDs empty. Thus, these remaining free cavities could further be used to entrap lipophilic molecules of appropriate size. Since benzophenon is known to form inclusion complexes with β-CD [20,21], it has been used as a drug model molecule. Indeed, association constants of benzophenon with free β-CDs and their polymeric form (pβ-CD) were found around 2000 L/mol. Benzophenon was

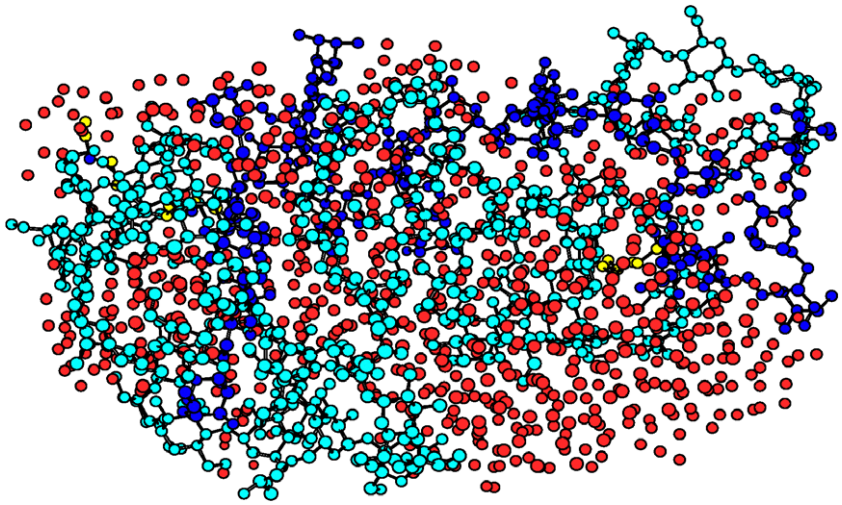

Fig. 7. Molecular modelling of the supramolecular nanoassemblies using both molecular mechanics (MM2, MM3) and semi-quantum mechanics (AM1 and PM3). Two linear chains of p-βCD (8 units, turquoise) and MD (16 units, the dextran chain is blue, C12 side-chains are yellow) were inserted within a cylinder containing 720 water molecules (red). The system was energy-minimized and subjected to molecular dynamics (300 K, step 1 fs, heating/cooling rate 1.000 kcal/atom/ps) for a trajectory of 95 ps.

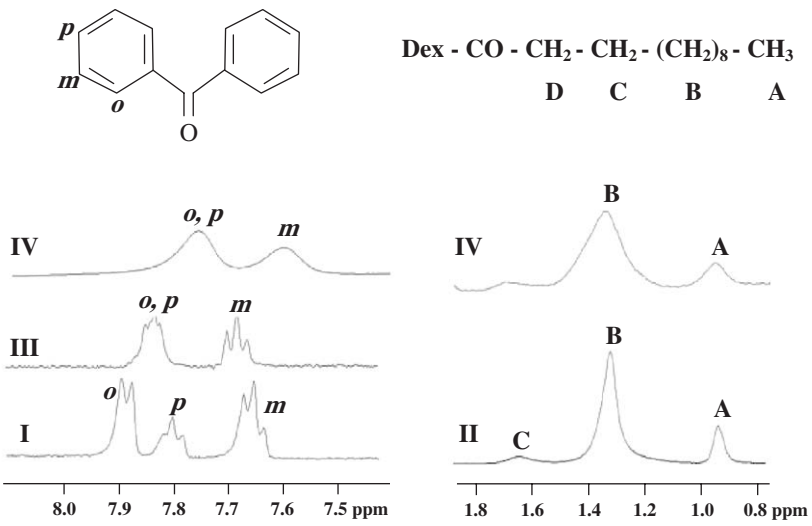

Fig. 8. ^{1}H NMR spectra recorded at 400 MHz, at 25 °C. Regions between 0.8 and 1.8 ppm and between 7.5 and 8 ppm of D_2O solutions: (I) benzophenon; (II) MD (5 mg/ml); (III) complexes between benzophenon and pβ-CD and (IV) nanogel suspension (5 mg/ml) containing benzophenon (2 wt.%). The polymers used were pβ-CD 10^6 g/mol and MD grafted with lauryl chains, Y=4%.

successfully incorporated into nanogels of around 200 nm by two methods: i) by forming inclusion complexes with pβ-CD before mixing with MD or ii) by direct loading into preformed nanoassemblies. Loadings up to 5 wt.% of dry nanogels were achieved.

In order to study the location of benzophenon inside the nanoassemblies, the ^{1}H NMR spectra of the loaded suspensions have been recorded (Fig. 8). Solutions of benzophenon presented three peaks, at 7.88, 7.80 and 7.65 ppm, corresponding to the ortho, para and metha protons, respectively (Fig. 8 I). In the case of benzophenon–pβ-CD solutions, these peaks were shifted: the ortho signal was shielded and overlapped with the para signal which was less shielded, and the metha signal was shielded (Fig. 8 III). These spectra modifications clearly reflect the formation of inclusion complexes between benzophenon and the pβ-CD polymer. Moreover, the spectra obtained with benzophenon-loaded nanogels presented also important peak modifications comparatively to free benzophenon (Fig. 8 I and IV left). This strongly suggests that benzophenon in the nanoassemblies was mainly included in its β-CD-complexed form.

The analysis of the C_{12} region of the benzphenon-loaded nanogels (Fig. 8 IV) shows a broadening of both peaks A and B compared to MD free in solution (Fig. 8 II). This reveals the coexistence of the two C_{12} species, bound and unbound. Therefore, it was concluded that, as in the case of empty nanoassemblies, the cohesion of the benzophenone-loaded particles was also ensured by alkyl chain inclusion in the β-CD cavities.

The highly crystalline anticancer drug tamoxifen is known to be particularly difficult to be entrapped into submicronic particles [22]. We, however, took advantage of the ability of tamoxifen to form inclusion complexes with β-CD [23,24] to efficiently encapsulate it in the supramolecular nanoassemblies. Drug loadings up to 100 μg/mg of dried polymer were achieved in 200 nm nanoparticles. According to literature data, such loadings are highly sufficient to ensure a biological response in pathologies such as the experimental autoimmune uveitis when administered by the intravitreal route [25]. However, in this study, the therapeutical benefit was counterbalanced by a moderate inflammatory reaction, which might be attributed to the administration of nanoparticles prepared using organic solvents [25]. The system described here could overcome this inconvenience and further experiments will be performed with this type of nanocarrier. Moreover, it is expected that other hydrophobic molecules of interest such as drugs, dyes, aromas, or toxic compounds could be associated, in the same way.

4. Conclusion

The new supramolecular nanoassembly concept presented here avoids some of the inconveniences of the currently employed methods for nanoparticle manufacture, such as the use of surfactants and organic solvents, and therefore removes a technological blockage. Moreover, drug carriers using β-CDs are of major interest, since β-CDs are known to form inclusion complexes with a variety of hydrophobic drug molecules [26]. Such nanoassemblies may also find applications in the biomedical field, in chemistry (e.g. as microreactors for chiral induction) or other innovative technologies.

Acknowledgment

This work was supported by the French National Research Center. We thank N. Razzouk and K. Ethève for their assistance with the experimental part.

References

[1] R. Langer, Drug delivery. Drugs on target, Science 293 (2001) 58–59.
[2] M.J. Alonso, Nanomedicines for overcoming biological barriers, Biomed. Pharmacother. 58 (2004) 168–172.

[3] R. Gref, Y. Minamitake, M.T. Peracchia, V. Trubetskoy, V. Torchilin, R. Langer, Biodegradable long-circulating polymeric nanospheres, Science 263 (1994) 1600–1604.
[4] D.A. LaVan, D.M. Lynn, R. Langer, Moving smaller in drug discovery and delivery, Nat. Rev. Drug Discov. 1 (2002) 77–84.
[5] C. Song, S. Liu, A new healthy sunscreen system for human: solid lipid nanoparticles as carriers for 3,4,5-trimethoxybenzoylchitin and the improvement by adding Vitamin E, Int. J. Biol. Macromol. 36 (2005) 116–119.
[6] S.A. Wissing, R.H. Muller, Cosmetic applications for solid lipid nanoparticles (SLN), Int. J. Pharm. 254 (2003) 65–68.
[7] S. Vemuri, C.T. Rhodes, Preparation and characterization of liposomes as therapeutic delivery systems: a review, Pharm. Acta Helv. 70 (1995) 95–111.
[8] K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, W.E. Rudzinski, Biodegradable polymeric nanoparticles as drug delivery devices, J. Control. Release 70 (2001) 1–20.
[9] H. Ringsdorf, J. Simon, Molecular self-assembly. Snap-together vesicles, Nature 371 (1994) 284.
[10] P.G. De Gennes, Ultradivided matter, Nature 412 (2001) 385.
[11] A. Harada, K. Kataoka, Chain length recognition: core–shell supramolecular assembly from oppositely charged block copolymers, Science 283 (1999) 65–67.
[12] J.F. Gohy, S.K. Varshney, R. Jerome, Morphology of water-soluble interpolyelectrolyte complexes formed by poly(2-vinylpyridinium)-*block*-poly(ethylene oxide) diblocks and poly(4-styrenesulfonate), Macromolecules 34 (2001) 2745–2747.
[13] S. Daoud-Mahammed, P. Couvreur, C. Amiel, M. Besnard, M. Appel, R. Gref, Original tamoxifen-loaded gels: an in situ self-assembling system for cancer treatment, Drug Deliv. Sci. Technol. 14 (2004) 51–55.
[14] A. Hashidzume, F. Ito, I. Tomatsu, A. Harada, Macromolecular recognition by polymer-carrying cyclodextrin: interaction of a polymer bearing cyclodextrin moieties with poly(acrylamide)s bearing aromatic side chains, Macromol. Rapid Commun. 26 (2005) 1151–1154.
[15] R. Gref, C. Amiel, B. Sébille, P. Couvreur, Aqueous dispersion of nanometric or micrometric particles for encapsulating chemical compounds, PCT 2004/006897 A1, 22 January 2004.
[16] E. Renard, A. Deratani, G. Volet, B. Sébille, Characterization of water-soluble high molecular weight β-cyclodextrin-epichlorhydrin polymers, Eur. Polym. J. 33 (1997) 49–57.
[17] A. Harada, H. Adachi, Y. Kawaguchi, M. Kamachi, Recognition of alkyl groups on a polymer chain by cyclodextrins, Macromolecules 30 (1997) 5181–5182.
[18] R.I. Gelb, L.M. Schwartz, Complexation of carboxylic acids and anions by alpha and beta cyclodextrins, J. Incl. Phenom. 7 (1989) 465–476.
[19] G. Crini, C. Cosentino, S. Bertini, A. Naggi, G. Torri, C. Vecchi, L. Janus, M. Morcellet, Solid state NMR spectroscopy study of molecular motion in cyclomaltoheptaose (beta-cyclodextrin) crosslinked with epichlorohydrin, Carbohydr. Res. 308 (1998) 37–45.
[20] S.C. Yu, A. Bochot, M. Chéron, M. Seiller, J.L. Grossiord, G. LeBas, D. Duchêne, Design and evaluation of an original multiple emulsion containing natural cyclodextrins as the emulsifier, STP Pharma Sci. 9 (1999) 273–277.
[21] K.A. Udachin, L.D. Wilson, J.A. Ripmeester, Solid polyrotaxanes of polyethylene glycol and cyclodextrins: the single crystal X-ray structure of PEG-β-cyclodextrin, J. Am. Chem. Soc. 122 (2000) 12375–12376.
[22] I. Brigger, P. Chaminade, V. Marsaud, M. Appel, M. Besnard, R. Gurny, M. Renoir, P. Couvreur, Tamoxifen encapsulation within polyethylene glycol-coated nanospheres. A new antiestrogen formulation, Int. J. Pharm. 214 (2001) 37–42.
[23] R.D. Armstrong, T.J. Ward, N. Pattabiraman, C. Benz, Separation of tamoxifen geometric isomers and metabolites by bonded-phase beta-cyclodextrin chromatography, J. Chromatogr. 414 (1987) 192–196.
[24] A. Gerloczy, M. Vikmon, J. Szejtli, E. Redenti, P. Ventura, Mutual solubility enhancement by binary and multicomponent complexation of clomiphene and tamoxifen, Proc. Int. Symp. Cyclodextrins, 9th, 1999, pp. 277–280.
[25] Y. de Kozak, K. Andrieux, H. Villaroya, C. Klein, B. Thillaye-Goldenberg, M.C. Naoud, E. Garcia, P. Couvreur, Intraocular injection of tamoxifen-loaded nanoparticles: a new treatment of experimental autoimmune uveoretinitis, Eur. J. Immunol. 34 (2004) 3702–3712.
[26] A. Monza da Silveira, G. Ponchel, F. Puisieux, D. Duchêne, Combined poly(isobutylcyanoacrylate) and cyclodextrins nanoparticles for enhancing the encapsulation of lipophilic drugs, Pharm. Res. 15 (1998) 1051–1055.