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Novel self-assembling nanogels: Stability and lyophilisation studies

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Abstract

The stability of new supramolecular nanoassemblies (nanogels), based on the association of a hydrophobically modified dextran (MD) and a β-cyclodextrin polymer (pβCD), has been studied by two complementary methods: (i) size measurements and (ii) turbidity experiments using a Turbiscan optical analyser. Nanogels of about 120–150 nm were obtained whatever the concentration of the two polymer solutions. At low concentrations, the suspensions presented little mean diameter variations upon storage. However, the concentrated ones tended to destabilize and their mean diameter increased upon time. Size measurements and Turbiscan investigations have demonstrated that destabilization in the MD–pβCD nanogel suspension was only due to particle aggregation and/or fusion, as no sedimentation or creaming occurred. The destabilization of MD–pβCD suspensions led to the formation of a highly viscous phase, as a final state. Moreover, the two methods have shown that aggregation and/or fusion phenomena were more pronounced in the concentrated MD–pβCD suspensions than in the diluted ones. The stability of MD–pβCD suspensions could be improved by their storage at 4 °C. Finally, freeze-drying was found to be a convenient method for the long-time storage of MD–pβCD nanoassemblies.

Keywords: Nanogels; Cyclodextrin; Stability; Turbiscan

1. Introduction

In the past few decades, submicronic polymeric particles have attracted considerable attention as potential drug delivery devices for the controlled release of active molecules and targeting (Brannon-Peppas, 1995; Couvreur et al., 1995; Soppimath et al., 2001; Hans and Lowman, 2002).

However, the preparation methods of these systems are often complex and require the use of potentially toxic surfactants and organic solvents in order to solubilize the commonly used (*co*)polymers such as polyesters (polylactide, poly(lactide-*co*-glycolide), polycaprolactone), polyanhydrides or polyalkylcyanoacrylates, which are insoluble in water (Verrecchia et al., 1995; Bitz and Doelker, 1996; Lin et al., 1999; Birnbaum et al., 2000; Jeon et al., 2000). Therefore, expensive techniques must be employed to remove completely the solvents and surfactants at the end of the preparation process. Nevertheless, solvent and surfactant traces may persist and constitute a drawback for the medical applications of these polymeric systems.

Recently, to overcome these inconveniences, new self-assembling nanogels (NG) were developed by mixing an aqueous solution of a β-cyclodextrin polymer, here designated pβCD with an aqueous solution of a hydrophobically modified polysaccharide, dextran grafted with alkyl moieties, designated MD (Gref et al., 2006). The alkyl moieties in C_{12} were found to form inclusion complexes with cyclodextrins (CDs), leaving also free CDs accessible for the inclusion of active molecules (Fig. 1).

It was demonstrated that stable nanoassemblies could be obtained only in a very narrow range of experimental conditions and that the main parameters governing stability were the dextran substitution yield, the polymers' weight ratio, the polymers' molar masses (M_w) and concentrations (Gref et al., 2006). These submicronic nanoparticles are here named nanogels to highlight the fact that they contain high water amounts. In particular, key parameters were: MD substitution yield $\geq 4\%$; pβCD $M_w > 10^6$ g/mol and weight ratio MD/pβCD = 1.

As the stability of these new nanodevices is a crucial point, the aim of this paper was to set up a methodology to investigate it. Two complementary methods were chosen. On one hand,

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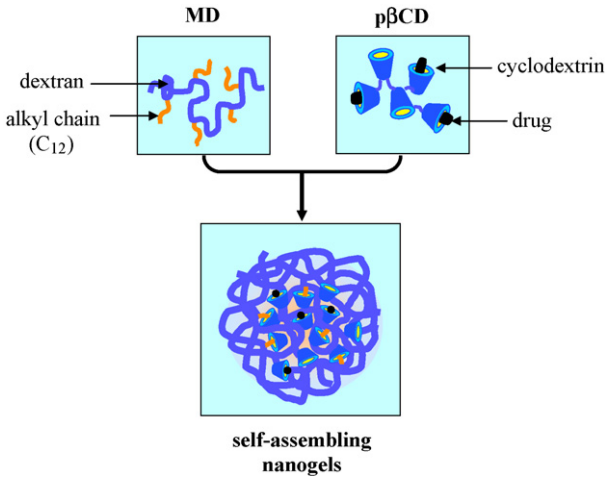

Fig. 1. Schematic representation of the formation of self-assembling MD–pβCD nanogels. NG were spontaneously obtained by mixing two polymeric aqueous solutions: a hydrophobically modified dextran by alkyl chains grafting (MD) and a β-cyclodextrin polymer (pβCD).

the mean diameter of the NG suspensions was followed during storage. On the other hand, the transmission and backscattering variations of the same suspensions were studied over time using a Turbiscan optical analyser, which has already been successfully used to investigate the stability and homogeneity of liquid mixtures on the basis of turbidity measurements (Mengual et al., 1999; Lemarchand et al., 2003a).

2. Materials and methods

2.1. Materials

β-Cyclodextrin polymer (pβCD) was prepared by crosslinking β-cyclodextrin (β-CD) with epichlorohydrin (EP) under strongly alkaline conditions (Renard et al., 1997). 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 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 at 50 °C overnight, neutralized with 6N 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). The average molar mass of pβCD polymer, determined by size exclusion chromatography, was 7×10^5 g/mol.

Dextran bearing hydrophobic lauryl side chains (MD) $M_w = 40\ 000$ g/mol was synthesized as previously described (Arranz and Sanchez-Chaves, 1988; Amiel et al., 2001). Briefly, 0.43 mL of lauryl chloride and 0.031 mL of pyridine were reacted for 3 h at 80 °C with 4 g of dextran solubilized in 100 mL of dimethyl formamide containing 1 g of lithium chloride. 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 yield of MD was 4% of glucose units, according to the ^{1}H NMR spectra.

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

2.2. Methods

2.2.1. Preparation of nanogel suspensions

Nanogels were obtained by mixing at room temperature equal volumes of a 10 g/L aqueous solution of pβ-CD and of a 10 g/L aqueous solution of MD under magnetic stirring. The initial suspension (10 g/L) was immediately diluted with milliQ water to obtain NG suspensions with concentrations comprised between 1 and 5 g/L.

2.2.2. Size determination and stability studies

The mean diameter and the size distribution of the nanogels were determined over 24 h at predetermined 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 one minute at 20 °C and at an angle of 90°. Both unimodal and size distribution processor (SDP) analysis were performed. The measurements were made in triplicate.

For short-term stability studies, the NG suspensions were stored at room temperature without stirring. Their mean diameter and size distribution were determined periodically. The variation of the mean diameter increase (Δd) was calculated as the difference between the diameter at a given time and the diameter measured just after the formation of the nanoassemblies.

For long-term stability studies, NG samples were stored at + 4 °C for 1 month.

2.2.3. Stability of nanogel suspensions using the Turbiscan MA

The stability of NG suspensions (1, 2.5, 5 and 10 g/L) was investigated using a Turbiscan MA 2000 (Formulaction, L'Union, France) (Fig. 2). The Turbiscan MA head is composed of a pulsed near infrared light source ($\lambda = 850$ nm) and two synchronous detectors: a transmission detector which receives the light going across the sample (at 0° from the incident beam) and a back scattering detector which receives the light scattered backward by the sample (at 135° from the incident beam). Samples of 5 mL of NG suspensions, prepared as previously described, were introduced in a cylindrical glass cell. The detection head scanned the entire length of the sample acquiring transmission (T) and backscattering (BS) data every 40 μm and every 30 min over 24 h. The variation of the transmission signal (ΔT) was calculated as the difference between T measured just after the formation of the nanoassemblies and T at a given time.

2.2.4. Cryotechniques for transmission electron microscopy

Cryotechniques procedures involved three steps: (i) high pressure freezing (HPF) which comprises sandwich preparation and freezing of the specimen; (ii) freeze fracture (FF) which includes fracturing, replication and cleaning of the replicas; (iii)

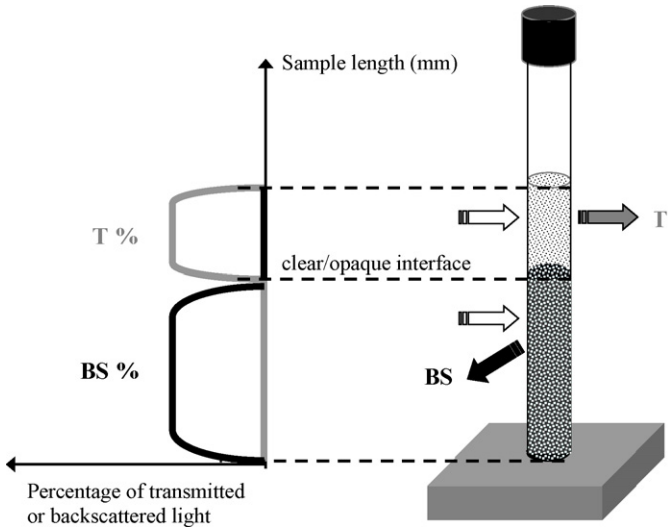

Fig. 2. Principle of Turbiscan. The detection head is composed of a pulsed near infrared light source (λ = 850 nm) and two synchronous detectors: a transmission (*T*) detector which receives the light going across the sample and a backscattering (BS) detector which receives the light scattered backward by the sample. The head scans the entire length of the sample acquiring *T* and BS data.

transmission electron microscopy (TEM) investigation of the replicas.

For the preparation of the sandwich, a small amount of NG 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 amount of 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 under the temperature of vitrification in liquid nitrogen in order to avoid any modification.

For fracturing, the sandwiches were mounted on a cold table which was inserted inside the vacuum chamber of a Bal-Tec Model BAF 400T 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 to 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 to deposit 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 specimens were removed. The replicas of the surface were then floated off the specimen by submerging in successive baths of water, dimethylsulfoxid, and acetone and finally collected onto naked 400 mesh grids which were subsequently mounted in a transmission electron microscopy (TEM) for inspection. TEM observations were performed on a LEO 912 Omega high resolution microscope working at 120 kV.

2.2.5. Freeze-drying

A 2 mL of each NG 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 evaluated for size.

3. Results and discussion

3.1. Short-term stability studies

The stability of MD–pβCD nanogel suspensions was investigated over 24 h using both mean diameter measurements and Turbiscan analysis.

Firstly, the variations of the mean diameter of the MD–pβCD suspensions were studied as a function of the total polymer concentration, ie 1, 2.5, 5 and 10 g/L (Fig. 3). Whatever the concentration, nanoassemblies smaller than 150 nm were initially formed. It was observed that the most diluted NG suspensions were the most stable. Indeed, for the 1 g/L suspensions, the mean diameter d remained below 200 nm over 24 h: *d* was equal to 125 ± 9 nm at *t* = 0 and to168 ± 5 nm at *t* = 24 h.

In the case of higher polymer concentrations, the diameter increased fast in the first eight hours and tended to reach a plateau at *t* = 20 h (Fig. 3). For example, in the case of the 5 g/L MD–pβCD suspension, the nanogels presented an initial diameter of 154 ± 7 nm. After 8 h, the diameter increased of about 40 nm whereas the mean diameter variation (Δ*d*) was lower than 10 nm between 16 and 24 h.

The higher was the concentration of the MD–pβCD suspensions, the higher was the size increase of the nanoassemblies over time. For example, at *t* = 12 h, the mean diameter of the nanogels was 154 ± 4, 190 ± 2, 201 ± 18 and 387 ± 15 nm, respectively, for 1, 2.5, 5 and 10 g/L MD–pβCD suspensions and corre-

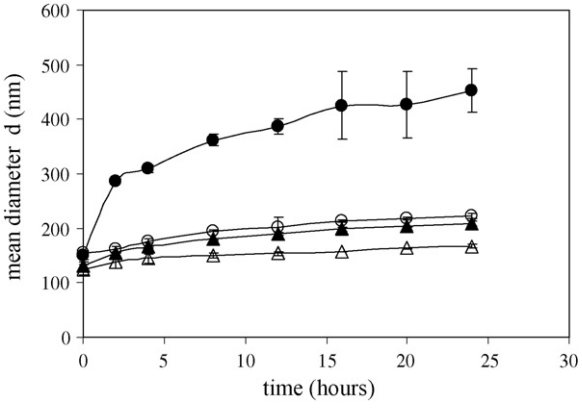

Fig. 3. Mean diameter (*d*) of MD–pβCD nanogel suspensions: (△) 1 g/L; (▲) 2.5 g/L; (◯) 5 g/L; (●) 10 g/L vs. time.

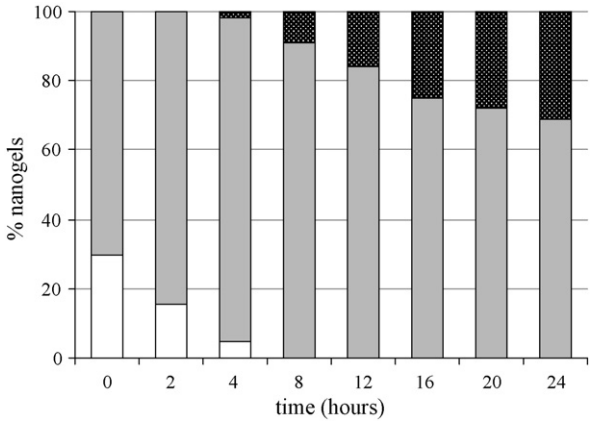

Fig. 4. Size distribution as a function of time in case of a 5 g/L MD–pβCD nanogel suspension showing three distinct populations at 115, 178 and 274 nm. The NG size distribution was analyzed at predetermined time intervals (0–4–8–12–16–20–24 h).

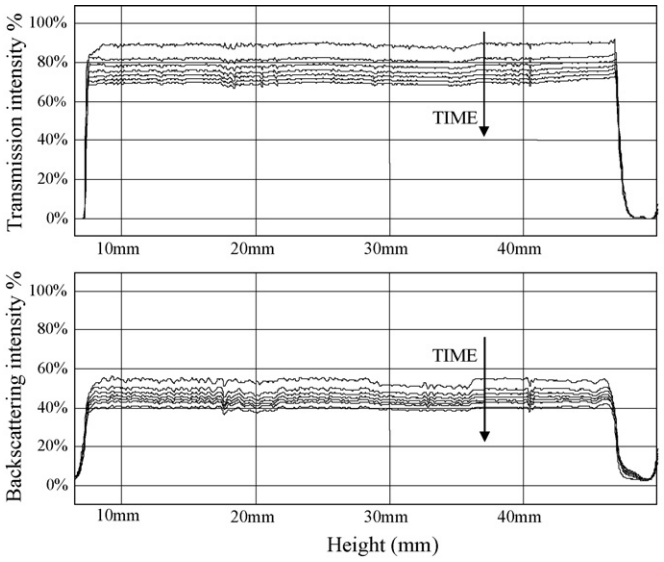

Fig. 5. Transmission (T) and backscattering (BS) data (%) of a 5 g/L MD–pβCD nanogel suspension as a function of tube height (0–50 mm). Data are given for different period of time (arrows from top to bottom, 0–4–8–12–16–20–24 h).

sponded to a Δd of 22 ± 4, 58 ± 8, 48 ± 12 and 240 ± 15 nm, respectively.

Then, the mechanism of NG suspension destabilization was investigated more in details (Fig. 4). At $t = 0$, the SDP analysis of the 5 g/L NG suspension showed that they were composed of 30% of NG with a mean diameter of 115 nm and of 70% of NG sizing 178 nm. After 4 h incubation, the proportion of the smallest NG decreased whereas the second one increased and a new population of 274 nm appeared. Then, in the following 4 h of incubation, the percentage of the largest ones (274 nm) continuously increased while the smallest NG completely disappeared. At $t = 24$ h, the suspension was composed of 69% of NG of 178 nm and 31% of NG sizing 274 nm. The disappearance of the population of the smallest nanoassemblies in favour of the largest one was typical of an aggregation or of a fusion mechanism. Similar observations were drawn from the analysis of samples with different polymer concentrations (results not shown). Turbiscan optical analyser was further useful to study if other phenomena such as creaming or sedimentation took place during particle aggregation or fusion.

Fig. 5 presents T and BS intensities as a function of sample height and time, in the case of a 5 g/L MD–pβCD suspension. These profiles were similar for all the other concentrations studied (1, 2.5, 5 and 10 g/L—data not shown). In all cases, both the intensity of the transmitted light and the intensity of the backscattered light decreased as a function of time. However, as the T signal was not nil, the partial reflection of the light crossing the sample by the faces of the measurement cell interfered with the BS signal. For this reason, only the T signals could be taken into account.

The first important observation was that whatever the time when the measurements were made, BS signal was constant, within the error of the experiment, all over the sample length. This means that no creaming or sedimentation occurred during the 24 h period of the scanning. Therefore, it was possible to choose arbitrarily a sample portion (20–25 mm) to study T variation as a function of time. In all cases, the percentage of light going across the sample decreased over the time. For example, in the case of the 5 g/L MD–pβCD suspension, at $t = 0$, T intensity were 90% and reached 69% after 24 h. Thus, the T signal decreased first quickly with a variation of 7.5% in the first 4 h and then the decrease was slower with a variation of 3.5% between 4 and 8 h. The decrease was only of 1.9% between 20 and 24 h.

Fig. 6 compares the variation of the T signal as a function of time for four MD–pβCD nanogel suspensions: 1, 2.5, 5 and 10 g/L. The higher was the sample concentration, the higher was the T variation. For example, after 20 h of scanning, ΔT was 0.98, 9.8, 18.1 and 46.1%, respectively, for 1, 2.5, 5 and 10 g/L MD–pβCD suspensions. The destabilization increased as the concentration of the NG suspension increased.

Thus, Turbiscan investigations have clearly demonstrated that destabilization of the NG suspension was the result of particle aggregation and/or fusion, as no sedimentation nor creaming occurred during the time course of the experiment.

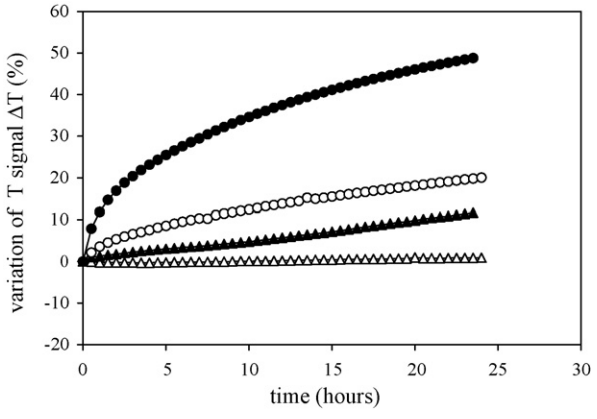

Fig. 6. Variation of transmission signal (ΔT) vs. time for MD–pβCD nanogel suspensions of various concentrations: (△) 1 g/L; (▲) 2.5 g/L; (◯) 5 g/L; (●) 10 g/L. The mean ΔT was measured over 24 h, every 30 min, on a sample portion comprised between 20 and 25 mm.

Moreover, Turbiscan allowed a qualitative comparison of the stability of the four suspensions studied in accordance with the measurements of size distribution. The T variation studies (Fig. 6) perfectly corroborated the light scattering investigations (Fig. 3) performed on the same NG suspensions. Indeed, for the 1 g/L suspension, only low ΔT and Δd variations were observed whereas largest variations were recorded in case of the concentrated 10 g/L sample. The two methods, quasi-elastic light scattering and turbidity analysis, demonstrated that aggregation and/or fusion phenomena were more pronounced in the concentrated MD–pβCD suspensions (5 and 10 g/L) than in the diluted ones (1 and 2.5 g/L). Such observations could be explained by the fact that high concentrations imposed proximity between suspended nanogels which probably promotes contact between nanoassemblies leading to their aggregation and/or fusion.

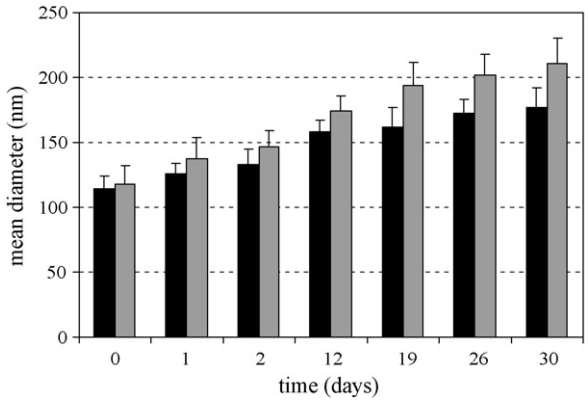

Fig. 7. Mean diameter (d) of MD–pβCD nanogel suspensions: 1 g/L (black) and 2.5 g/L (grey) over one month storage at 4 °C.

3.2. Long-term stability studies

It is well established that the thermal agitation is reduced by decreasing the temperature. Thus, at low temperature, the probability of contact between nanogels in suspension is lower than at room temperature. On the other hand, it was found that the complex formation constants of β-CD with various alkanoic carboxylic acids were higher at low temperature (Gelb and Schwartz, 1989). Therefore, in order to improve the stability of the NG suspensions, it was chosen to store them at 4 °C. Fig. 7 illustrates the variation in the mean diameter of 1 and 2.5 g/L MD–pβCD nanogels over 1 month. As expected, for both concentrations, NG exhibited a slow diameter growth at 4 °C than at room temperature. Indeed, for example, after 24 h, the 1 and 2.5 g/L NG suspensions stored at room temperature showed a diameter increase Δd of 43 and 76 nm, respectively, whereas Δd was only 11 and 20 nm for the same NG suspensions stored at 4 °C. After 30 days storage, the mean diameter was 180 ± 10 and 205 ± 8 nm for the 1 and 2.5 g/L nanogels, respectively. Conversely, the 5 and 10 g/L NG suspensions were very instable, even at 4 °C: a highly viscous phase settled in the bottom of the vial after 3 and 2 days storage, respectively. Thus, the storage of the diluted MD–pβCD nanogel suspensions at 4 °C could be successfully employed to improve their stability.

Other systems such as solid lipid nanoparticles (SLN^{TM}) exhibit similar behaviour (Heurtault et al., 2003). Westesen and Siekmann observed that phospholipids stabilized tripalmitate suspensions showed a fast and considerable particle growth followed by gel formation which was attributed to the crystallization of the lipid phase (Westesen and Siekmann, 1997). Moreover, the particle growth was enhanced by the introduction of energy, like temperature and light, in the SLN^{TM} systems (Freitas and Muller, 1998).

3.3. Microscopic observations

Fig. 8 shows the evolution of a 10 g/L MD–pβCD nanogel suspension. The replicas obtained by FF techniques after HPF and observed by TEM were of excellent quality and presented the same homogeneous features. The morphological characterization confirmed the information concerning the evolution of the NG size. Two hours after their preparation, MD–pβCD suspensions (10 g/L) were composed of spherical individualized nanoassemblies (Fig. 8A) whereas after one day storage at +4 °C (Fig. 8B), bigger structures appeared indicating NG fusion. Meanwhile, groups of nanoassemblies were also observed, indicating that agglomeration took place too (Fig. 8B).

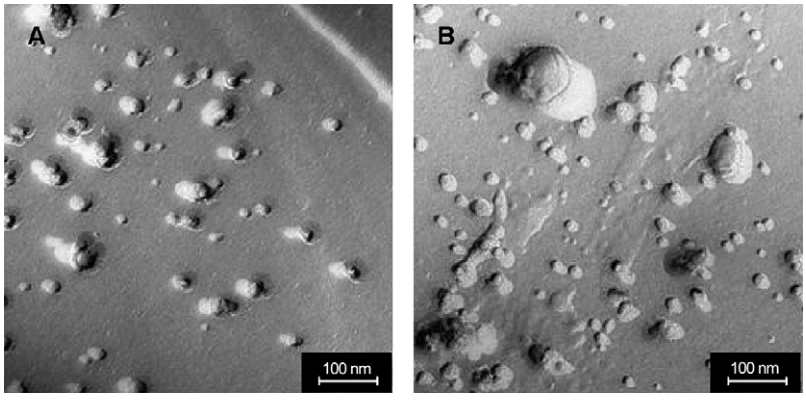

Fig. 8. Photomicrographs of a MD–pβCD nanogel suspension (10 g/L) taken by transmission electron microscopy after freeze-fracture: (A) 2 h after preparation and (B) after 1 day storage.

Table 1
Mean diameter and polydispersity index of MD–pβCD nanogels before and after freeze-drying, as a function of the polymer concentration

Polymer concentration (g/L)	NG before freeze-drying		NG after freeze-drying	
	Mean diameter (nm)	Polydispersity index	Mean diameter (nm)	Polydispersity index
0.5	120 ± 4	<0.2	115 ± 5	<0.2
1	136 ± 10	<0.2	123 ± 8	<0.2
1.5	176 ± 6	<0.2	149 ± 10	<0.2
2	186 ± 9	<0.2	145 ± 8	<0.2
2.5	195 ± 12	<0.2	Gel	–

Numbers are the average of three independent determinations.

3.4. Freeze drying

Because freeze-drying is one of the most convenient methods for long-time storage of colloidal systems, this method was tested on MD–pβCD nanoassemblies. The mean diameter and polydispersity index obtained for MD–pβCD nanoassemblies with concentrations comprised between 0.5 and 2.5 g/L, before and after freeze-drying are shown in Table 1. All the NG preparations were successfully lyophilized, except the 2.5 g/L suspension, and the dried cakes could be easily re-dispersed after addition of milliQ water. The Tyndall effect was preserved and the size after lyophilization was even slightly smaller than the initial one. For example, the mean diameter of 1 g/L NG was 136 ± 10 nm and 123 ± 8 nm before and after freeze-drying, respectively. Advantageously, the nanoassemblies could be freeze-dried without adding any cryoprotective agent, possibly due to the presence of dextran at their surface. Indeed, this polymer has been already successfully employed as cryoprotectant for biologic cells and enzymes (Strumia and Strumia, 1964; Ashwood-Smith and Warby, 1972; Pellerin-Mendes et al., 1997). More recently, Lemarchand et al. reported that core (poly-ε-caprolactone)-shell (dextran) nanoparticles could be freeze-dried in the absence of cryoprotectants, due to the presence of dextran at their surface (Lemarchand et al., 2003b).

At a concentration of 2.5 g/L, the re-hydration of the dried nanoassemblies' cake led to the formation of a highly viscous phase, suggesting that there is a concentration threshold (between 2 and 2.5 g/L) above which the NG cannot be lyophilized. One possible explanation for this limit is that during the freeze-drying process, the suspension underwent a drastic transition. From an aqueous diluted state, the sample reached a dried and highly concentrated state. The NG became in intimate contact with each other, and this favoured potential bridging by the formation of inclusion complexes between the alkyl moieties of one nanoassembly and the CDs of another one and conversely. This phenomenon was enhanced as the concentration of the suspensions was increased.

4. Conclusion

This study has shown that MD–pβCD nanoassemblies in suspension have a tendency to perform aggregation and/or fusion depending on polymer concentration and temperature. Freeze-drying was found to be a convenient method for the long-time storage of MD–pβCD nanogel suspensions.

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