Novel Polyester–Polysaccharide Nanoparticles

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Purpose. The aim of the present study was to develop a new type of core-shell nanoparticles from a family of novel amphiphilic copolymers, based on dextran (DEX) grafted with poly(ε–caprolactone) (PCL) side chains (PCL-DEX).

Methods. A family of PCL-DEX copolymers was synthesized in which both the molecular weight and the proportion by weight of DEX in the copolymer were varied. The nanoparticles were prepared by a technique derived from emulsion-solvent evaporation, during which emulsion stability was investigated using a Turbiscan. The nanoparticle size distribution, density, zeta potential, morphology, and suitability for freeze-drying were determined.

Results. Because of their strongly amphiphilic properties, the PCL-DEX copolymers were able to stabilize o/w emulsions without the need of additional surfactants. Nanoparticles with a controlled mean diameter ranging from 100 to 250 nm were successfully prepared. A mechanism of formation of these nanoparticles was proposed. Zeta potential measurements confirmed the presence of a DEX coating.

Conclusion. A new generation of polysaccharide-decorated nanoparticles has been successfully prepared from a family of PCL-DEX amphiphilic copolymers. They may have potential applications in drug encapsulation and targeting.

KEY WORDS: nanoparticle; dextran; poly(epsilon-caprolactone); amphiphilic copolymer.

INTRODUCTION

Biodegradable colloidal nanoparticles have received considerable attention as drug delivery systems by several routes of administration (intravenous, oral, pulmonary, nasal, and ocular). They protect the entrapped drug against degradation and to control its site-specific delivery. However, the main drawback of conventional nanoparticles is their nonspecific interaction with cells and plasma proteins, leading to drug accumulation in nontarget organs. Surface-modified nanoparticles have been developed to control their interactions with biologic milieu and therefore their biodistribution. Among them, poly(ethylene glycol) (PEG)-coated ("Stealth") nanoparticles can successfully avoid the mononuclear phagocyte system sequestration and therefore may circulate in blood for much longer periods of time than uncoated ones (1–3). However, one drawback of these PEG-coated nanoparticles is the absence of reactive groups at their surface, which limits ligand coupling (4). Therefore, polysaccharic coatings are attractive alternatives to PEG ones because they possess many recognition functions, allowing specific mucoadhesion or receptor recognition, as well as providing neutral coatings with low surface energy, preventing non specific protein adsorption (5). For example, Österberg *et al.* (5) demonstrated that, similarly to PEG, dextran (DEX) coatings could prevent protein adsorption onto polystyrene surfaces. More recently (6), DEX-coated poly(lactic acid) (PLA) nanoparticles were prepared by using as emulsion stabilizer amphiphilic DEX grafted with phenoxy groups. The DEX corona successfully reduced protein adsorption.

However, the most convenient and direct method for producing DEX-coated nanoparticles would be to use preformed amphiphilic copolymers made of DEX grafted with biodegradable polymers. Only few examples of such materials have been described to date (7,8). They are mostly prepared by polymerizing monomers such as lactide or ε-caprolactone onto a DEX backbone. However, it is difficult to obtain copolymers with controlled structures by this method (8). Moreover, traces of catalysts remaining in the final product are potentially toxic (9).

Very recently, Gref *et al.* (10) synthesized new comb-like materials composed of a polysaccharidic backbone (DEX) onto which preformed poly(ε–caprolactone) (PCL) chains were grafted by means of ester bridges. The synthesis route proposed did not involve the use of any catalyst. Amphiphilic copolymers with various hydrophilic-lipophilic balances (HLB) were successfully obtained.

Nanoparticles can be prepared from preformed (co)polymers by methods such as emulsification-solvent evaporation, nanoprecipitation or salting-out, all of which require the dissolution of the (co)polymers in an organic solvent. To our knowledge, no method has been yet developed to prepare nanoparticles using (co)polymers that are soluble neither in water and nor in organic solvents. In this study, we describe an original "interfacial migration–solvent evaporation" method that leads to nanoparticle formation from a newly synthesized family of insoluble PCL-DEX copolymers. We investigated the structure of these nanoparticles, as well as their mechanism of formation.

MATERIALS AND METHODS

Materials

The detailed synthesis and characterization of the family of PCL-DEX copolymers is described elsewhere (10). It was conducted by grafting preformed PCL chains onto the DEX backbone. Briefly, low-molecular-weight (MW) (2000–3000 g/mol) PCL polymers with low polydispersity (<1.2) bearing one carboxyl end group were obtained by polymerizing freshly distilled caprolactone at 230°C in the presence of capric acid. The carboxyl function was activated with carbonyldiimidazole, allowing coupling to DEX (Fluka, MW 5000 or 40000 g/mol, according to gel permeation chromatography data), to form grafted PCL copolymers. These copolymers were characterized by gel permeation chromatography, ^{1}H-NMR and IR spectroscopy. The copolymers used in this study are named PCL-DEX$_{xkDa}$ y% where x is the DEX MW (5 or 40 kDa) and y is the DEX wt content in the copolymers (5 to 33 wt%). The solvents used for nanoparticle preparation were of analytical grade. All other chemicals were commercially

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available reagent grade. Water was purified by reverse osmosis (MilliQ®, Millipore, Billerica, MA, USA).

Density and Solubility of the Copolymers

The density of the block copolymers samples was determined at room temperature by gas pycnometry (ACCUPYC 1330®, Micromeritics, Londonderry, NH, USA) using a supply of high-purity helium gas (helium N55). The volume measurement was performed with a 12.1408 cm^3 standard chamber, which was previously purged 60 times using helium N55.

The solubility of the copolymers at room temperature was tested in organic solvents, in which the solubility of the starting materials (PCL and DEX) was already described (11,12). Five to 30 mg of copolymer were placed in 1 ml of organic solvent under magnetic stirring during 12 h. The solubility was estimated by visual inspection.

Nanoparticle Preparation

Five milligrams of PCL-DEX copolymer was added to a vial with 1 mL of organic solvent (methylene chloride or ethyl acetate) and 5 mL of pure water or surfactant solution (sodium cholate 0.1%, Lutrol F68® 1% or PVA 1% [w/v]). The block copolymer was insoluble in this mixture. However, it was progressively dispersed, leading the o/w emulsion formation under magnetic stirring (3 min, 500 rpm, room temperature). The size of the emulsion droplets was then considerably reduced by sonication (Microprobe, 60 s, pulses of 1 s each, Vibra Cell™ VC750, Sonics and Materials, Newtown, CT, USA). Finally, the organic solvent was evaporated (Rotavapor®, 20 to 30 min at constant temperature of 25°C, 10–30 mbar), leading to PCL-DEX precipitation in the form of nanoparticles. When surfactant solutions were used as the dispersion media, the nanoparticle suspensions were dialyzed three times against MilliQ® water (dialysis membrane Spectra Por 4, cut-off 12–14000 g/mol, Spectrum) to eliminate free surfactant.

Nanoparticles were also prepared following the above procedure as with water saturated with ethyl acetate in place of the mixture of 1 mL of ethyl acetate and 5 mL of water. The formation of nanoparticles using PCL-DEX copolymers was compared with that using a physical mixture of the two polymers, PCL and DEX, at the same composition as the PCL-DEX copolymers.

Emulsion Stability and Droplet Size

The physical stability of the o/w emulsions prepared with PCL-DEX copolymers was analyzed using the Turbiscan MA 1000 (Formulaction, Ramonville, France). Five milliliters of emulsion samples prepared as previously described, just before the solvent evaporation step, were placed in a cylindrical glass cell. The detection head was composed of a pulsed near-infrared light source (λ = 850 nm) and synchronous transmission (T) and back scattering (BS) detectors. The T detector received the light, which was transmitted through the sample (at 180° from the incident beam), while the BS detector received the light scattered backwards from the sample (at 45° from the incident beam). The detection head scanned the entire height of the sample (about 50 mm), acquiring T and BS at 40-μm intervals, every minute for at least 3 h. The principle of the measurement is based on the variation of the droplet volume fraction (migration) or diameter (coalescence), affecting the BS and T signals.

Emulsions with a volume ratio between the organic and the aqueous phases of 1/10, were placed in a closed glass cell to avoid solvent evaporation. The cells were placed in a nanosizer (Coulter® N4MD, Coulter Electronics, Inc., Hialeath, FL, USA) and the droplet size was measured over a 2-h period at 20°C by quasi-elastic light scattering (QELS).

Emulsion Droplet-Nanoparticle Transition

Size Measurement

To study the transition between emulsion droplets and nanoparticles, the operating conditions were standardized: the solvent was slowly evaporated by magnetic stirring at room temperature. The size of the objects was determined at 10-min intervals at 20°C by QELS.

Residual Solvent Analysis

The amount of ethyl acetate remaining in the preparation during the solvent evaporation step was analyzed by head space gas chromatography. The study was performed with a gas chromatograph GC (HP 6890) equipped with a head space (Agilent 7694 E). During solvent evaporation, emulsion samples of 100 μL were withdrawn. They were immediately diluted in water at 1/100 or were directly analyzed for their ethyl acetate content. A 60-m × 0.53-mm Megabore column with 0.5-μm film thickness (SPB™-5 SUPEL CO, Sigma, France) was used for chromatographic separation. The carrier gas was helium at a constant flow rate of 10 mL/min. The head space conditions were as follows: vial equilibration time, 30 min; vial temperature, 100°C; loop temperature, 120°C; injection temperature 150°C. The column oven temperature was 35°C. Ethanol was used as an internal standard.

Determination of the Coalescence Factor

The coalescence of the emulsion droplets during the evaporation step was quantified by the coalescence factor F_c defined as follows:

$$Fc = \frac{\rho \times v}{m}\left(\frac{d_{NP}}{d_d}\right)^3 \quad (1)$$

where ρ is the density of the PCL-DEX nanoparticles, m the copolymer weight, v the volume of ethyl acetate, d_{NP} the mean diameter of the nanoparticles after solvent evaporation, and d_d the mean diameter of the emulsion droplets.

Physicochemical Characterization of the Nanoparticles

Nanoparticle Size and Surface Charge

The size of the nanoparticles was determined at 20°C by QELS. Their surface charge was investigated through zeta potential measurements after a 1/100 dilution in KCl 1 μM (Zetasizer 4, with a multi-8 correlator 7032, Malvern Instruments). The zeta potential values were calculated using the Smoluchowski equation.

Nanoparticle Density

The density of the nanoparticle suspensions in water was measured at 20°C using a high precision digital densimeter (Analyser Beer 2, Anton-Paar, Austria). The densimeter was standardized using air (0.001238 g/ml) and water (0.998289 g/mL) at 20°C. The density of the nanoparticles was calculated by successive approximation from the density and concentration of nanoparticles suspended in water and the density of water, by considering that there was no interaction between nanoparticles and water.

Freeze-Drying

The nanoparticles dispersed in water, with or without 1–5% of glucose as cryoprotectant were frozen at –20°C for 2 h and freeze-dried (Lyophiliser Christ® Alpha 1-4, Bioblock Scientific, Illkirch, France) under 0.015 mbar vacuum at 30°C for 24 h. The size of the freeze-dried nanoparticles redispersed in water was compared with the initial diameter measured just before freeze-drying.

Nanoparticle Morphology

The morphology of the nanoparticles was analyzed by scanning electronic microscopy (SEM; LEO 9530, France) with a Gemini Column. The nanoparticles were freeze-dried without any cryoprotectant. They were mounted on supports and coated with a Pt/Pd layer (Cressington, 208 HR) under an argon atmosphere.

PCL-DEX nanoparticles were also observed by transmission electron microscopy after freeze-fracture. A small drop of an aqueous suspension was deposited on a copper planchet and rapidly cooled by a jet of liquid nitrogen under high pressure (HPM 010, Balzers Union). Fracturing, etching and shadowing, using Pt and C, were performed in a Balzers BAF 400. After washing, the replicas were observed in a MET LEO 912 microscope.

RESULTS

Solubility of the Copolymers

The solubility of PCL-DEX copolymers was determined in water and in different organic solvents. As shown in Table I, all copolymers were insoluble in most of the organic solvents commonly used for the preparation of nanoparticles (acetone, THF, ethyl acetate, methylene chloride, chloroform), in contrast with the PCL homopolymer, which was soluble in most of them. With the exception of PCL-DEX_{40kDa}, all PCL-DEX copolymers were only soluble in DMAC and DMSO, which are solvents for both their constituents, DEX and PCL.

Emulsion Study

The stability of the emulsions was studied using a Turbiscan. PCL-DEX copolymers were unable to stabilize the methylene chloride-in-water emulsions. A typical transmission and backscattering profile, suggesting instability, is shown in Fig. 1a. In the first 30 min, the backscattering signal rapidly decreased from the top to the bottom of the cell (10 mm to 50 mm), and increased in the region from 7.5 to 10 mm (Fig. 1b). After 7 min, the backscattering signal decreased in the bottom region (6.5 to 7.5 mm; Fig. 1b). After 50 min, the transmission signal increased progressively in the region from 7 to 7.5 mm (Fig. 1a). Just after 1 min, the transmission signal increased progressively in the region from 45 to 49 mm (Fig. 1a).

When ethyl acetate was used as the organic phase, Turbiscan studies showed the appearance of transmission and backscattering peaks at the bottom of the cell, which increased with time (Fig. 1c). At the end of monitoring, a backscattering signal was observed at the top of the cell (43.5 to 49 mm).

In parallel with these studies, the size of the droplets was monitored by QELS. Whichever PCL-DEX copolymer was used for the stabilization of ethyl acetate-in-water emulsions, the size of the oil droplets remained constant for two hours (Fig. 2a). The presence of the surfactant sodium cholate reduced the mean droplet diameter (Fig. 2a). The size of the methylene chloride-in-water emulsions prepared from PCL-DEX copolymers could not be measured because of the opacity of the emulsion.

Emulsion Droplet-Nanoparticle Transition

Using the emulsions described above, we investigated the size of the objects (nanodroplets followed by nanoparticles) during the evaporation of ethyl acetate. As shown in Fig. 2b, while the solvent evaporated, the mean diameter of the droplets decreased quickly during the first 10 min and remained constant thereafter, whatever the composition of

Table I. Solubility of the PCL Homopolymer and of PCL-DEX Copolymers in Water and in Different Organic Solvents

Solvent	DEX	PCL	PCL-DEX_{5kDa} 5, 20, or 33%	PCL-DEX_{40kDa} 10, 25, or 33%
Water	++	–	–	–
Acetone	–	+	–	–
Ethyl acetate	–	+	–	–
THF	–	++	–	–
Methylene chloride	–	++	–	–
Chloroform	–	++	–	–
DMSO (room temperature)	+/–	++	–	–
DMSO (60°C)	+	++	+	+
DMAC	+/–	++	+	–

Note: ++ very soluble; + soluble; +/– slightly soluble; – insoluble.

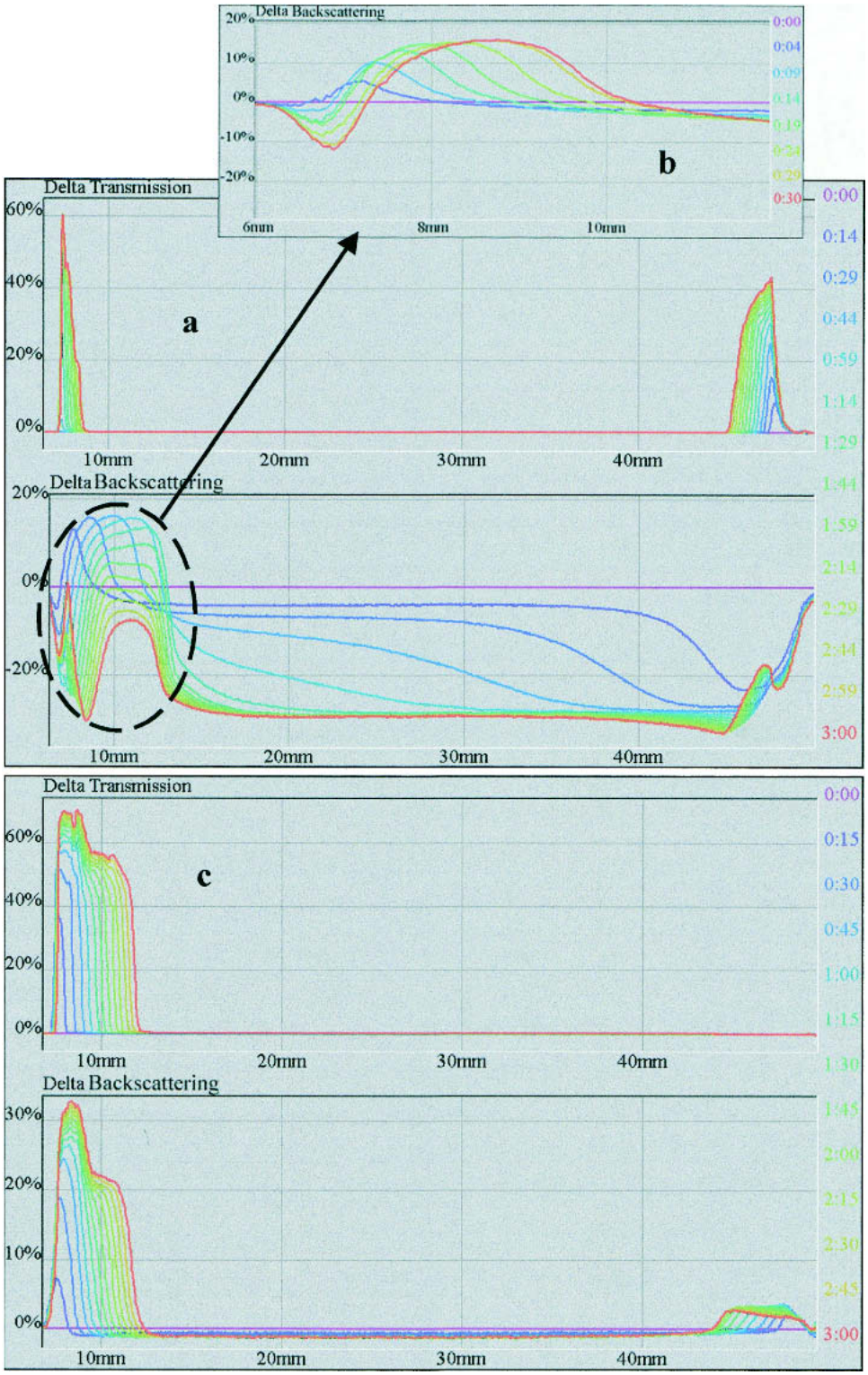

Fig. 1. Delta transmission and delta backscattering data of the PCL-DEX$_{5kDa}$ 33% emulsion prepared with methylene chloride and water from t_0 to t_{180min} (a) and from t_0 to t_{30min} (b) or with ethyl acetate and water from t_0 to t_{180min}(c). To better visualize modifications in the signal with time, the transmission and backscattering profiles at t_0 were subtracted from all subsequent profiles.

the PCL-DEX copolymer and the nature of the dispersion medium.

The factor of coalescence was calculated from Eq. (1) on the basis of the mean diameter of the oil droplets and nanoparticles as measured by QELS. It was between 3.6 and 4.

During the evaporation step, measurement of ethyl acetate showed a decrease of solvent concentration from 69 g/L in the original emulsion to less than 0.02 g/L in the final suspension of nanoparticles after evaporation was complete (Fig. 3).

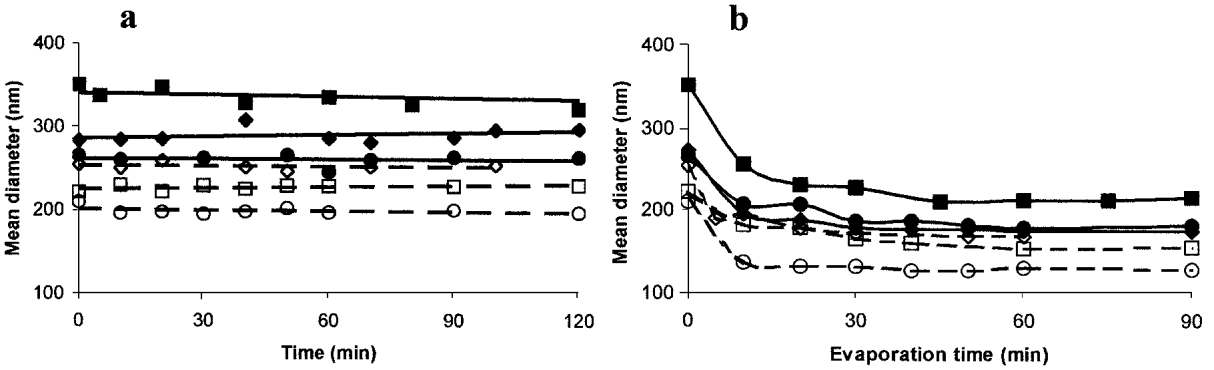

Fig. 2. Evolution of the size of the droplets of ethyl acetate-in-water emulsions (a) and the size of nanoparticles during the evaporation step (b) using different copolymers (PCL-DEX$_{40kDa}$ 33% [rhombus], PCL-DEX$_{40kDa}$ 10% [squares], and PCL-DEX$_{5kDa}$ 5% [circles]) in the presence (empty symbols) or in the absence (full symbols) of sodium cholate as a surfactant.

Nanoparticle Characterization

Nanoparticle Size

All PCL-DEX copolymers formed nanoparticles with a mean diameter comprised between 200 and 400 nm (polydispersity 0.16 to 1.4) and a process yield close to 100%, whatever the nature of the organic solvent (methylene chloride or ethyl acetate) used (Table II). The copolymer PCL-DEX$_{40kDa}$ 33% was an exception, leading to a diameter close to 1 μm, when methylene chloride was used in the nanoparticle preparation. All copolymers were able to form nanoparticles spontaneously in the absence of any additional surfactant, as opposed to the homopolymer PCL, which needed a surfactant (sodium cholate) to form nanoparticles. Similarly, the use of mixtures of DEX and PCL in the same proportions as the PCL-DEX copolymers failed to produce nanoparticles in the absence of surfactant.

The influence of various surfactants on the size of PCL-DEX nanoparticles was studied. PVA, a commonly used stabilizer (13) failed to decrease the mean diameter. For example, in the case of PCL-DEX$_{40kDa}$ 33%, the size was similar (260 nm) at a PVA concentration in the aqueous phase of 0.1 wt % (data not shown). However, the addition of surfactants such as sodium cholate or Lutrol F68® were able to decrease the mean diameter of the obtained nanoparticles significantly by a factor of 1.3 to 3.1 (Table II). Nanoparticles with a mean diameter close to 100 nm could be produced from PCL-DEX$_{40kDa}$ 10% or PCL-DEX$_{40kDa}$ 25% in this way. The nanoparticle mean diameter remained constant after storage (4 weeks, 4°C), with a size variation of less than 5% (data not shown).

Moreover, PCL-DEX$_{40kDa}$ 33% nanoparticles could also be prepared by simply mixing this copolymer with water saturated with ethyl acetate. After sonication, objects of 240 nm were obtained, whereas after solvent evaporation, the nanoparticle size was 195 nm.

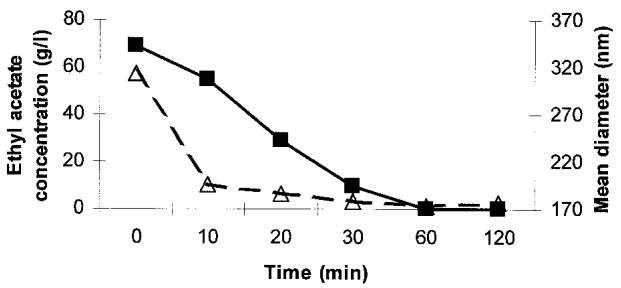

Fig. 3. Evolution of the concentration of ethyl acetate (■) and the mean diameter of the objects (△) prepared with PCL-DEX$_{40kDa}$ 33% as a function of time during the evaporation step.

Nanoparticle Density

As shown in Table III, the density of the nanoparticles increased with the DEX wt% in the PCL-DEX copolymer. The density of the nanoparticles was always slightly lower than that of the polymer used to prepare them.

Nanoparticle Surface Charge

PCL nanoparticles exhibited a negative surface charge, as indicated by their high negative zeta potential (–57.4 mV; Table III). The zeta potential of PCL-DEX nanoparticles was related to the DEX weight content in the copolymer. For DEX wt% as low as 10%, the zeta potential was dramatically increased, reaching –9.4 mV. With PCL-DEX$_{40kDa}$ 33%, zeta potential values were even closer to zero (–4 mV).

Nanoparticle Freeze-Drying

Freeze-drying of PCL nanoparticles led to extensive and irreversible aggregation (Table IV) since after redispersion in water, the diameter increased to more than 1 μm. Addition of glucose (up to 1 wt%) tended to decrease aggregation but the size still remained above 1 μm, the upper detection limit of the Nanosizer.

In contrast, freeze-dried PCL-DEX nanoparticles were much easier to redisperse. Although in the absence of cryoprotectant, the mean diameter of the nanoparticles still increased to above 1 μm, 1% of glucose was enough to maintain the size of nanoparticles when PCL-DEX$_{40kDa}$ 33% was used as starting material. In the case of nanoparticles with a lower DEX content (10% to 20 wt%), the mean diameter increased by about 80 nm in the presence of glucose, but no large aggregates were observed after redispersion in water. For the lowest DEX wt% (PCL-DEX$_{5kDa}$ 5%), lyophilization induced a considerable increase of the nanoparticle size (from 188 to 310 nm) even in the presence of glucose at 5%.

Table II. Mean Diameter of the Nanoparticles Prepared with a Family of PCL-DEX Copolymers as a Function of the Nature of the Organic Solvent Used and of the Surfactant Added to the Aqueous Phase

	Particle diameter ± SD (nm) (PI)			
	Ethyl acetate			Methylene chloride
		Aqueous surfactant		
(Co)Polymer	No	Sodium cholate 0.1%	Lutrol F68® 1%	No
PCL	–[a]	200 ± 70.8 (0.25)[b]	–[a]	151 ± 52 (0.17)[c]
PCL-DEX$_{5kDa}$ 5%	211 ± 85 (0.50)	161 ± 57 (0.33)	122 ± 51 (0.58)	225 ± 63 (0.09)
PCL-DEX$_{5kDa}$ 20%	395 ± 170 (0.73)	146 ± 46 (0.22)	149 ± 54 (0.40)	235 ± 85 (0.32)
PCL-DEX$_{5kDa}$ 33%	211 ± 71 (0.20)	136 ± 43 (0.18)	155 ± 54 (0.25)	297 ± 114 (0.38)
PCL-DEX$_{40kDa}$ 10%	284 ± 118 (0.60)	188 ± 55 (0.16)	107 ± 46 (0.62)	261 ± 86 (0.24)
PCL-DEX$_{40kDa}$ 25%	326 ± 139 (0.70)	154 ± 49 (0.20)	101 ± 34 (0.28)	269 ± 104 (0.39)
PCL-DEX$_{40kDa}$ 33%	269 ± 109 (0.50)	145 ± 60 (0.35)	153 ± 58 (0.36)	1080 ± 485 (1.40)

Note: The sizes given are the means and standard deviation of populations that were reported by the instrument ($n = 3$) from 3 independent preparations.
[a] Did not form nanoparticles.
[b] Formed nanoparticles only if ethyl acetate was heated.
[c] Formed nanoparticles only in the presence of a surfactant (sodium cholate 0.1% w/v).
PI, Polydispersity index; SD, standard deviation.

Nanoparticle Morphology

Freeze-fracture electron microscopy was performed on emulsion droplets before solvent evaporation (Fig. 4a) and on nanoparticles after solvent evaporation (Fig. 4b). The general appearance of emulsion droplets and nanoparticles was quite similar, showing individualized spherical objects, although some of them were aggregated or grouped in larger raspberry-like particles. SEM also revealed spherical particles which were covered by a film when a copolymer with a high DEX content (25 wt %) was used (Fig. 4c).

DISCUSSION

Whatever their composition, PCL-DEX copolymers appeared to be amphiphilic materials. Indeed, they were insoluble in water and in most of the organic solvents commonly used for the preparation of nanoparticles (Table I). In this study, an original interfacial migration–solvent evaporation technique was proposed that allowed the preparation of nanoparticles with this type of polysaccharide-based copolymers. The hypothetical mechanism of nanoparticle formation is shown schematically in Fig. 5. The copolymers were first allowed to migrate to the o/w interface to form a stabilizing layer around the solvent droplets. The droplet size was then reduced by sonication and the organic solvent was removed by evaporation, leading to the formation of a fine aqueous suspension of nanoparticles. The size of the resulting PCL-DEX nanoparticles was between 200 and 400 nm in the absence of surfactants and could be reduced to about 100 nm by using Lutrol F68® (Table II). The interfacial migration–solvent evaporation process could only be applied to DEX-PCL copolymers. Indeed, PCL or mixtures of PCL and DEX failed to produce nanoparticles in the absence of additional surfactants (Table II).

Moreover, interestingly, PCL-DEX copolymers swelled and self-organized when sonicated in the presence of a single phase composed of water (solvent for DEX) saturated with ethyl acetate (solvent for PCL). After solvent evaporation, nanoparticles were recovered.

Because of their amphiphilic properties, all PCL-DEX copolymers were able to form nanoparticles spontaneously. However, the presence of an additional surfactant could be supposed to decrease the interfacial tension of o/w emulsions, thereby reducing the final size of the nanoparticles (Table II). The high hydrophilicity of the surfactants used (sodium cholate or Lutrol F68®) compensated for the considerable hydrophobicity of the copolymers, with HLB ranging between 1 and 7. This HLB was calculated as the ratio of the MW of the hydrophilic part divided by the total MW of the copolymer and multiplied per 20 (14). However, due to its polymeric nature, Lutrol F68® might remain entrapped within the nanoparticle matrix during the emulsification process by intermingling with the polymer forming the core (13).

The advantage of sodium cholate is its high tensioactive power, decreasing two or three times the size of nanoparticles

Table III. Density and Zeta Potential of (co)Polymers and Nanoparticles Prepared with PCL and a Family of PCL-DEX Copolymers

	Density of the (co)polymer	Density of nanoparticles	Zeta potential (mV)
PCL	1.151	1.0640	–57.4 ± 0.8
PCL-DEX$_{5kDa}$ 5%	1.151	1.0469	–46.1 ± 0.3
PCL-DEX$_{5kDa}$ 20%	ND[a]	1.0944	–21.8 ± 0.3
PCL-DEX$_{5kDa}$ 33%	ND[a]	1.1375	–17.2 ± 0.6
PCL-DEX$_{40kDa}$ 10%	1.185	1.0597	–9.4 ± 0.6
PCL-DEX$_{40kDa}$ 33%	1.261	1.1481	–4.5 ± 0.3

Note: Densities of block (co)polymers (n = 10) and nanoparticles ($n = 3$) were determined on the whole sample and the zeta potential values ($n = 5$) are the means of two to four independent preparations.
[a] Not determined

Table IV. Influence of Freeze-Drying in the Presence of Glucose as Cryoprotectant on the Size of the Nanoparticles Prepared with PCL and a Family of PCL-DEX Copolymers

	Size before freeze drying (nm) (PI)	Size after freeze drying and redispersion in water (nm) (PI)			
		Without glucose	Glucose 1%	Glucose 2.5%	Glucose 5%
PCL	151 ± 52 (0.17)	>1 μm	>1 μm	>1 μm	>1 μm
PCL-DEX$_{5kDa}$ 5%	161 ± 57 (0.33)	>1 μm	748 ± 310 (0.59)	664 ± 301 (1)	416 ± 173 (0.54)
PCL-DEX$_{5kDa}$ 20%	146 ± 46 (0.22)	>1 μm	224 ± 89 (0.45)	204 ± 79 (0.37)	224 ± 90 (0.47)
PCL-DEX$_{5kDa}$ 33%	136 ± 43 (0.18)	>1 μm	136 ± 43 (0.24)	132 ± 42 (0.21)	132 ± 45 (0.26)
PCL-DEX$_{40kDa}$ 10%	188 ± 55 (0.16)	>1 μm	285 ± 99 (0.26)	288 ± 90 (0.21)	191 ± 52 (0.10)
PCL-DEX$_{40kDa}$ 33%	145 ± 60 (0.35)	>1 μm	154 ± 51 (0.22)	158 ± 35 (0.10)	157 ± 49 (0.16)

Note: Sodium cholate (0.1% w/v) was used as surfactant. The sizes given are the means and standard deviations of populations that were reported by the instrument ($n = 3$).
PI, polydispersity index.

at very low concentration (0.1%) (Table II). Furthermore, it has previously been shown that this surfactant can be efficiently eliminated by washing (6,15).

To investigate the mechanism of nanoparticle formation, we measured the evolution of the droplets' mean diameter with time (Fig. 2) and used the Turbiscan to follow the emulsion stability directly over prolonged periods (Fig. 1). This methodology reveals irreversible (coalescence or aggregation) or reversible (creaming or sedimentation) destabilization much earlier than the operator's naked eye (16).

Methylene chloride is one of the most commonly used organic solvents for the preparation of polyester (PLA, PLGA, PCL) nanoparticles by emulsion-solvent evaporation because of its low water solubility (2% (w/v) at 25°C), easy emulsification, good solvent properties, and low boiling point (17). Surprisingly, according to the Turbiscan studies, the use of methylene chloride for the preparation of PCL-DEX nanoparticles led to the formation of very unstable emulsions (Fig. 1a and b). Indeed, as shown by the variation of the initial backscattering signal, the organic phase droplets first migrated from the top to the bottom of the cell, probably because of their higher density. This sedimentation was followed by droplet coalescence promoted by their increased concentration in the middle and bottom of the cell, until, finally, phase separation was observed. This irreversible destabilization corresponded to the appearance of a transmission peak at the bottom of the cell (Fig. 1a).

This instability of the methylene chloride-in-water emulsion can probably be explained by the inadequate HLB of the copolymers. This HLB varied in our case from 1 to 7, and therefore would be more in favor of the stabilization of w/o emulsions rather than o/w ones (18).

However, even if the PCL-DEX copolymers could not stabilize methylene chloride-in-water emulsions, nanoparticles with controlled size (mean diameter from 225 to 297 nm, except for PCL-DEX$_{40kDa}$ 33%) were obtained (Table II). It appears that the rate of evaporation was faster than to the rate of coalescence. Evaporation led to polymer precipitation, thus avoiding coalescence.

Nevertheless, the disadvantage of methylene chloride is its toxicity (Class 2 in the ICH for residual solvents) and for this reason ethyl acetate was also used in this study, as a more acceptable organic solvent. In the presence of ethyl acetate, all copolymers were able to stabilize the o/w emulsions (Fig. 1c). No coalescence occurred during the 3 h of scanning. Indeed, in the middle of the cell, the backscattering signal remained constant and the only phenomenon observed was droplet migration from the bottom to the top of the sample. This creaming was probably due to the lower density of ethyl acetate compared with the dispersing media.

During the first steps of emulsion formation according to our interfacial migration–solvent evaporation technique, ethyl acetate is drained out of the nanodroplets into the dispersing media (Fig. 5), because of its high water solubility (8–10% w/v). This process led to a dramatic decrease of the dispersed solvent volume, from 1 ml, the initial volume, to 0.1

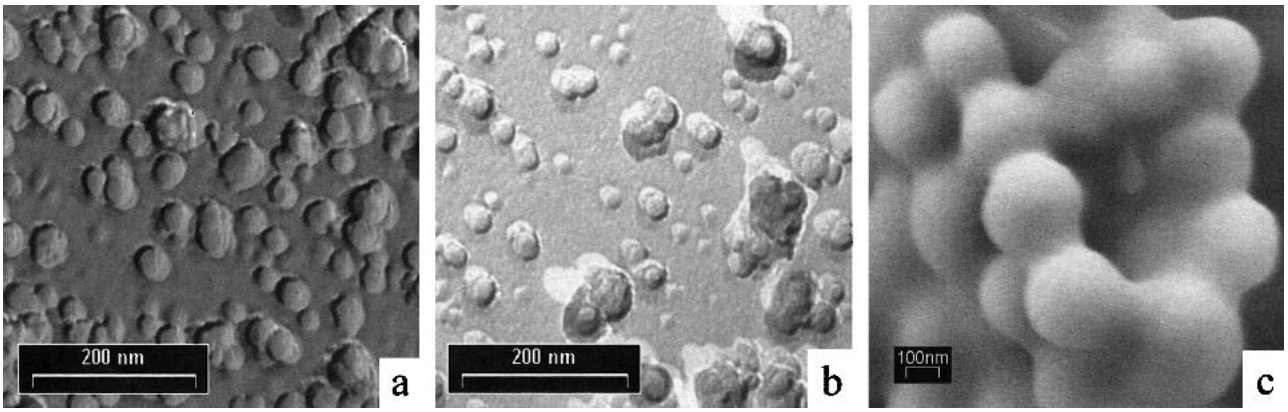

Fig. 4. Morphology of emulsion and nanoparticles: freeze-fracture images of PCL-DEX$_{40kDa}$ 33% emulsion (a) and suspension of nanoparticles (b) and SEM images of nanoparticles made of PCL-DEX$_{40kDa}$ 25% (c).

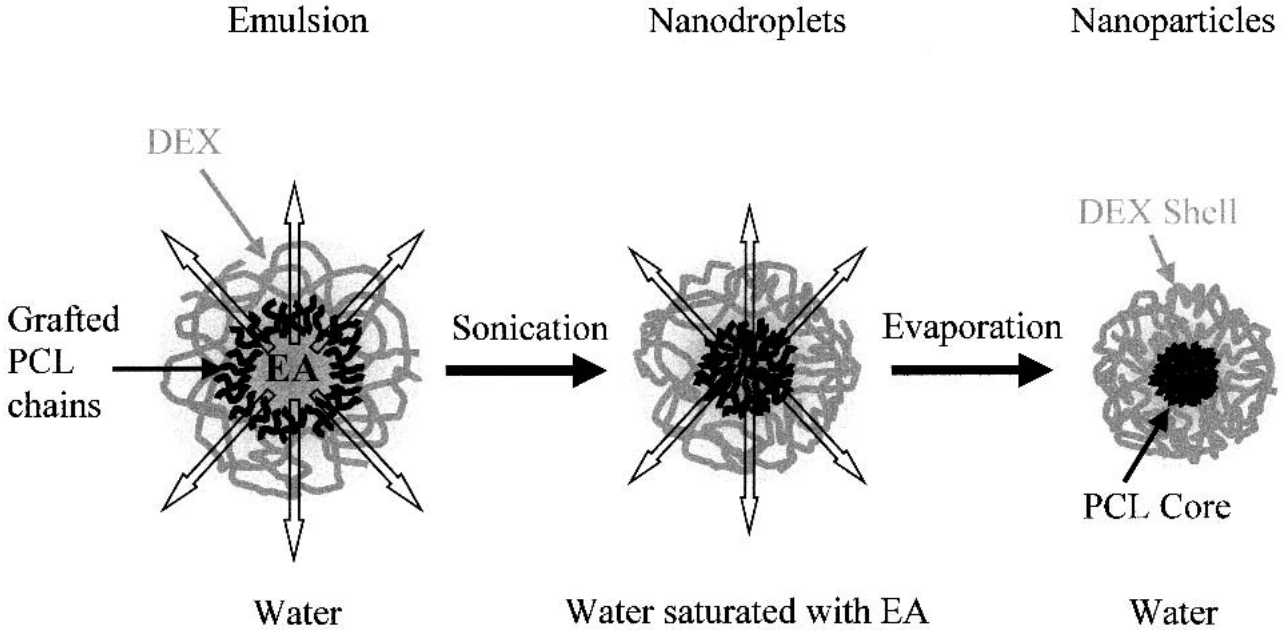

Fig. 5. Schematic representation of the hypothetical mechanism of nanoparticle formation by the interfacial migration–solvent evaporation method using insoluble PCL-DEX copolymers. The size of the emulsion droplets is reduced by sonication. Nanoparticles are formed after solvent evaporation. The dispersion medium is water or water saturated with ethyl acetate. The diffusion of ethyl acetate (EA) out of the nanodroplets is symbolized by empty arrows.

mL. The rapid diffusion of the solvent in the dispersion media has been described as a factor in favor of Ostwald ripening, responsible for coalescence. However, whatever the PCL-DEX copolymer used to stabilize the emulsions, no coalescence of the emulsion droplets was observed, in either Turbiscan or droplet size measurement studies (Fig. 1c and 2). Moreover, when nanoparticles were prepared under conditions in which solvent diffusion could not occur (directly in 0.1 ml ethyl acetate and 10 mL of water saturated with ethyl acetate), similar sizes were obtained. These results tend to prove that solvent diffusion occurred early during stirring and sonication steps.

Solvent evaporation led to polymer precipitation in the form of nanoparticles. During this step, the dispersion media played a role of reservoir of ethyl acetate before its removal by evaporation. Indeed, 10 min after the beginning of solvent evaporation, the decrease in volume of the nanodroplets was lower than the volume of solvent removed by evaporation. After 60 min of solvent evaporation, no more ethyl acetate could be detected in the nanoparticle suspensions (Fig. 3).

Figure 4a and b show typical images of PCL-DEX emulsions immediately after sonication and of the resulting nanoparticles after solvent evaporation respectively. These freeze-fracture studies revealed the dense inner structure of the nanoparticles. Indeed, in cases where particles were sectioned, no porous cores were detected (data not shown). The low porosity of the nanoparticles is supported by the fact that the density differences generally observed between the block copolymer samples and the nanoparticles were less than 10% (Table III).

The nanoparticles (Fig. 4b) and the nanodroplets (Fig. 4a) appear as groups of small-sized objects of less than 100 nm, packed together. The size of the aggregates could reach 200-300 nm, in accordance with the sizes determined by light scattering (Table II). Aggregation also explains the relative high polydispersity of the samples (Table II). If we take into account the mean diameters of the small-sized objects, the low factor of coalescence calculated (3.6) indicates that no or very limited coalescence occurred during solvent evaporation. This is in accordance with the Turbiscan and emulsion stability studies (Fig. 2a).

Because freeze-drying is a convenient technique for nanoparticle storage, we looked at its possible adverse effects on the redispersibility of the PCL-DEX nanoparticles. After freeze-drying, these nanoparticles were redispersed in water and their mean diameter was compared to the initial one measured just before freeze-drying (Table IV). When the copolymers had a high DEX content, the nanoparticles' redispersibility in water was improved. Indeed, even in the presence of the lowest concentration of glucose (1%), the size of the nanoparticles was preserved. Thus, DEX as a polysaccharide, can be assumed to play an additional cryoprotectant role during freeze-drying.

The morphology of the freeze-dried PCL-DEX nanoparticles was also investigated by SEM (Fig. 4c). When the DEX content in the copolymer was high, the nanoparticles seemed to be covered by a film. Possibly, this aspect is the result of the presence of a DEX coating layer at the nanoparticles' surface.

The presence of DEX at the nanoparticles' surface was also investigated by zeta potential measurements (Table III). PCL nanoparticles had a strongly negative zeta potential (–57 mV). Similar values have already been found with other polyesters (6) and have been attributed to the presence of carboxyl end groups located near the surface. When the DEX content increased in the copolymers, the zeta potential of the nanoparticles tended toward zero, probably because the neutral DEX coating shielded the strong negative surface charge of the PCL core. Therefore, these studies suggested that DEX preferentially migrates to the nanoparticle surface during the preparation process, as illustrated in Fig. 5. Taking into ac-

count the comb-like structure of the PCL-DEX copolymers, DEX should adopt a "side-on" configuration. Österberg *et al.* (5) demonstrated that this type of DEX configuration at the surface of polystyrene films was as efficient as a PEG "end-on" (or "brush") configuration at preventing protein adsorption. These authors also showed that DEX in an end-on configuration was less efficient at limiting this adsorption. We can therefore predict that the new DEX-coated nanoparticles developed in this study will have potential applications as long-circulating drug carriers for intravenous administration, because they will undergo limited plasma protein adsorption.

CONCLUSION

Nanoparticles were successfully prepared in the absence of surfactants from a family of novel amphiphilic comb-like copolymers, according to an interfacial migration–solvent evaporation technique. These materials were found to be able to self-organize and precipitate in the presence of mixtures of water and ethyl acetate. Furthermore, nanoparticles could be prepared from o/w emulsions by using ethyl acetate or methylene chloride as the organic solvent. Ethyl acetate-in-water emulsions were stable and produced the best nanoparticles. These studies of emulsion stability yielded much interesting information such as an understanding of the ability of the copolymers to migrate to the solvent-water interface, a means of following the procedure of nanoparticle preparation and of determining the factor of coalescence, a key parameter responsible for emulsion instability.

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