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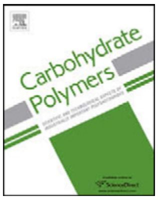

Polysaccharide-covered nanoparticles prepared by nanoprecipitation

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ABSTRACT

Dextran-covered poly(lactic acid) (PLA) nanoparticles were prepared using the nanoprecipitation process. Two alternative procedures were compared. On the one hand, PLA was nanoprecipitated in the presence of dextran-based polymeric stabilizer. On the other hand, a PLA-grafted dextran copolymer was nanoprecipitated without the addition of any external stabilizer. The characteristics of particles (yield, size, colloidal stability in the presence of salt) were correlated to the properties of initial polymers. PLA-grafted dextran led to small particles, with high yield and improved colloidal stability even without any stabilizer in the aqueous phase. Viscometric experiments in organic solvent/water mixtures indicated that PLA-grafted dextran self-organized upon mutual diffusion of water and organic solvent spontaneously, providing nanoparticles with a PLA core and a dextran-enriched surface. These nanoparticles exhibited good colloidal stability in water even at high ionic strengths (4 mol/L).

1. Introduction

Submicron particles find a wide number of applications in various fields like medicine (drug delivery, diagnostic etc.), cosmetics, food etc. Among the great variety of previously developed nanoparticles, polymer-based nanoparticles are a very important class (Léonard et al., 2008; Liu, Jiao, Wang, Zhou, & Zhang, 2008). Poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone), cellulose ethers, pullulan acetate and more recently hydrophobically modified dextrans have been studied as core materials for polymeric nanoparticles (Aumelas, Serrero, Durand, Dellacherie, & Léonard, 2007; Hornig & Heinze, 2008; Hornig, Heinze, Hesse, & Liebert, 2005; Rouzès, Gref, Léonard, De Sousa Delgado, & Dellacherie, 2000; Thioune, Fessi, Devissaguet, & Puisieux, 1997; Zhang et al., 2009). Convenient surface properties are often obtained by setting hydrophilic polymer coils at the particle surface either by covalent attachment, or by physical adsorption, via electrostatic or hydrophobic interactions for example.

Several processes have been extensively described over the past few decades for the preparation of nanoparticles, including emulsion/solvent evaporation, nanoprecipitation, emulsion and miniemulsion polymerization (Pinto Reis, Neufeld, Ribeiro, & Veiga, 2006, Vauthier & Bouchemal, 2009). Nanoprecipitation technique (or solvent displacement method) was first developed by Fessi et al. (Thioune et al., 1997). Briefly, the core material is first dissolved in a fully or partly water-miscible solvent (acetone, THF, ethyl acetate etc.). Then the solution is dropped into an aqueous solution which may contain a surfactant. Rapid desolvation of the core polymer in the presence of water results in nanoprecipitation provided that aggregation phenomena are limited. One main drawback of nanoprecipitation is the large dilution of prepared dispersions, since dilute organic polymer solutions have to be used to avoid aggregate formation during polymer precipitation.

Polysaccharide-covered nanoparticles have been considered for many years since they could combine several key-properties: biocompatibility, colloidal stability (because of the hydrophilicity of polysaccharide chains) and specific interactions with biological systems (Laroui et al., 2007; Lemarchand et al., 2006). The preparation of polysaccharide-covered nanoparticles by nanoprecipitation has been reported following essentially two strategies. The first one consisted in using a hydrophobic core material (like PLA) dissolved in the organic solvent and a polymeric stabilizer dissolved in the aqueous phase (Laroui et al., 2007; Rouzès et al., 2000; Rouzes, Léonard, Durand, & Dellacherie, 2003). The polymeric stabilizer was obtained by hydrophobic modification of a native polysaccharide (dextran, pullulan, chitosan etc.). During the precipitation, polysaccharide-based stabilizers are expected to adsorb onto the formed nanoparticles. An alternative strategy involved the use of graft copolymers with a polysaccharide backbone and various types of side-chains (often polyester-type) (Gref, Rodrigues, & Couvreur, 2002; Guan, Quan, Shuai, Liao, & Mai, 2007; Jeong, Choi, & Song, 2006a; Jeong et al., 2006b; Liu, Xu, Guo, & Han, 2009; Maruyama, Ishihara, Kim, Kim, & Akaike, 1997; Nouvel et al., 2009; Prabu et al., 2008; Rodrigues et al., 2003; Ydens et al., 2005; Yu, Wang, Chen, Deng, & Jing, 2006). With those amphiphilic copolymers, another stabilizer was not required in the aqueous phase. The self-organization of the copolymers allowed by itself the formation of nanoparticles in which polyester chains were essentially in

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Table 1
Reported studies about polyester-grafted polysaccharide nanoparticles. Polymer nature, method for preparing nanoparticles and size range of the final suspensions.

Graft copolymer			Physico-chemical conditions			Nanoparticle suspension		Reference
Backbone	Grafts	Method	Solvent	Non-solvent	Stabilizer	Size range (nm)	Solid content (wt%)	
Chitosan	PCL	Dialysis	THF:Water[a]	Water	–	27–69	0.10	(Yu et al., 2006)
Chitosan	PCL	Nanoprecipitation	DMSO	Water	–	98–215	0.10	(Guan et al., 2007)
Chitosan	PCL, PEG	Simple dispersion	–	1% Acetic acid in water	–	250–350	0.10	(Liu et al., 2009)
Chitosan	PCL	Dialysis	TFE/acetic acid (v/v, 5/1)	Water	–	45–210	Not given	(Cai et al., 2009)
PHCS[b]	PCL	Simple dispersion	–	Water	–	165–549	Not given	(Huang, Li, & Fang, 2009)
Pullulan	PLGA	Dialysis	DMSO	Water	–	77–147	Not given	(Jeong et al., 2006a)
Dextran	PCL	Nanoprecipitation	DMSO	Water	–	100–300	0.5–2.0	(Ydens et al., 2005)
Dextran	PLGA	Dialysis	DMSO	Water	–	137–277	Not given	(Jeong et al., 2006b)
Dextran	PCL	Emulsion/evaporation	CH_2Cl_2	Water	SC[c]	97–114	0.10	(Rodrigues et al., 2003)
Dextran	PCL	Emulsion/evaporation	CH_2Cl_2	Water	PVA[d]	141–191	0.50	(Prabu et al., 2008)
Dextran	PCL	Emulsion/evaporation	CH_2Cl_2[e]	Water	SC[c]	45–140	0.10	(Gref et al., 2002)
Dextran[f]	PLA[f]	Emulsion/evaporation	CH_2Cl_2	Water	PLA-g-Dex[g]	200–270	0.25	(Nouvel et al., 2009)

[a] According to the authors, the graft copolymer was not dissolved but suspended in the mixture THF:Water (83:17 vol%).
[b] N-phtaloylchitosan.
[c] Sodium cholate.
[d] Poly(vinyl alcohol).
[e] The polymer was not dissolved but mixed with water and dichloromethane.
[f] In that work, nanoparticles were made from a mixture of PLA-grafted-dextran and PLA.
[g] PLA-grafted-dextran water-soluble copolymers ($\text{Dex}_{29K}\text{-g-}_{18}\text{PLA}_{0.1K}$ and $\text{Dex}_{29K}\text{-g-}_{14}\text{PLA}_{0.2K}$).

the core while polysaccharide backbone was located in the outer part thus forming a hydrophilic coverage. If we particularly consider polyester-grafted-polysaccharide based nanoparticles, a few studies were reported about their preparation (Table 1). Nanoprecipitation itself was considered in only two papers whereas emulsion/solvent evaporation and dialysis were the main reported procedures for nanoparticle preparation. The solid content of the obtained suspensions was generally lower than 1 wt%. An external stabilizer dissolved in the aqueous phase was only used for emulsion/solvent evaporation technique. Finally, in most examples, dimethylsulfoxide and methylene chloride were used for dissolving the graft copolymer. In the previous studies, controlling polymer chemical structure as well as identifying its consequences on final nanoparticle size distribution were the main considered topics. Generally, chitosan and dextran were used for graft copolymer backbone and poly(ε-caprolactone) was by far the most frequent polyester in the grafts (Table 2). While the molar mass of the native polysaccharide can vary largely among the reported studies, the molar mass of side chains is often between 1000 and 5000 g/mol. The weight fraction of polyester grafts in the copolymer is often higher than 50% with a polyelectrolyte like chitosan while it may be lower with neutral polysaccharides like dextran or pullulan, which can be easily explained by solubility requirements associated to nanoparticle preparation. All the reported works showed that the average particle diameter increased with the length of polyester side chains as well as with the amount of grafted side chains. Nevertheless, no detailed investigation has been reported about the effect of physico-chemical parameters on the nanoprecipitation of polyester-g-polysaccharide copolymers either on nanoparticle formation or on the colloidal stability of the final suspension.

Table 2
Macromolecular characteristics of polyester-grafted polysaccharide used for preparing nanoparticles.

Polysaccharide backbone		Grafts		y[a]	$F_w{}^{\text{graft}}$[b] (wt%)	Reference
Nature	M_n (g/mol)	Nature	M_n (g/mol)			
Chitosan	21,000[c]	PCL	4000	4.5–15.2	52.5–78.8	(Cai et al., 2009)
Chitosan	5400	PCL	2400	3.4–52.9	40–73.8	(Yu et al., 2006)
Chitosan	16,000[c]	PCL	1200–11,000	28–49	73.7–93.3	(Guan et al., 2007)
Chitosan	600,000[c]	PEG, PCL	5000 for PEG 3000 for PCL	3 and 0.3 for PEG 4 and 2.1 for PCL	35.1–6.3 for PEG 27.3–26.5 for PCL	(Liu et al., 2009)
PHCS[d]	1,400,000	PCL	1250, 2000, 4000	–	40–54	(Huang et al., 2009)
Pullulan	62,400	PLGA	5800	0.52–1.69	15.7–37.7	(Jeong et al., 2006a)
Dextran	9000	PEG, PCL	750 for PEG 8000 for PCL	10 or 20 for PEG 2 or 3 for PCL	18–31 for PEG 49–59 for PCL	(Qiu et al., 2009)
Dextran	6600–21,300	PCL	200–2900	48–76	52–89	(Ydens et al., 2005)
Dextran	5000[e]	PCL	2100	9.7–22.7	56–74.6	(Rodrigues et al., 2003)
Dextran	10,400[e]	PCL	–	–	9–94[f]	(Prabu et al., 2008)
Dextran	77,000	PLGA	5100	0.36–1.30	10–29	(Jeong et al., 2006b)
Dextran	13,000–33,000	PLA	2300–2500	27–36	81–84	(Nouvel et al., 2009)

[a] y is the average number of grafts for 100 repeat units of the polysaccharide backbone.
[b] $F_w{}^{\text{graft}}$ is the weight fraction of polyester grafts in the copolymer.
[c] Viscometric average molar mass.
[d] N-phtaloylchitosan.
[e] The exact definition of the average molar mass is not given in the paper.
[f] It is assumed that these values of weight fractions but it is not written in the paper.

In this work, we carried out a systematic comparison of the formation of dextran-covered nanoparticles following two alternative strategies: on the one hand, nanoprecipitation of PLA in the presence of a dextran-based stabilizer and on the other hand, nanoprecipitation of a PLA-grafted dextran copolymer. Three important properties were considered: particle size distribution and colloidal stability of the final suspensions and amount of aggregates formed during nanoprecipitation.

2. Experimental

2.1. Materials

Dextran T40 ($\overline{M_n}$ 33,800 g/mol, $\overline{M_w}$ 43,000 g/mol, as characterized from multi-angle laser light scattering experiments coupled with a size-exclusion chromatography system, performed in 0.1 M $NaNO_3$) was purchased from Pharmacia Biotech. Polylactide ($\overline{M_n}$ 47,700 g/mol, $\overline{M_w}$ 49,000 g/mol as characterized by SEC MALLS experiments performed in THF using a dn/dC of 0.054 mL/g) was purchased from Sigma–Aldrich.

2.2. Synthesis of polymers

The synthesis and the characterization of the PLA-grafted dextran copolymer (Fig. 1) were performed as previously described (Nouvel et al., 2004). Briefly a three-step synthetic pathway was followed. Native dextran was first hydrophobized by a controlled sylilation reaction resulting in a low number of residual hydroxyl groups available for further ring opening polymerization (ROP) of D,L-lactide. Then, sylilated dextran was used as a multifunctional initiator of ROP of D,L-lactide in the presence of a low amount of tin activator ($SnOct_2$). Finally, deprotection of dextran backbone was carried out by acid hydrolysis of sylilated ether groups. In what follows, Dex_{21K}-g-$_{45}PLA_{3.1K}$ will be used to name the Dex-g-PLA polymer. 21 and 3.1 (in kg/mol) are respectively $\overline{M_n}$ of dextran backbone (determined after deprotection of silylated dextran under mild conditions) and of PLA grafts (determined by ^1H NMR in DMSOd6). 45 is the average number of PLA grafts per 100 glucose units. Dextran molar mass distribution was determined by size exclusion chromatography in aqueous eluent (0.1 M $NaNO_3$ 6.15×10^{-3} M NaN_3) monitored dually by differential refractometry and multi angle laser light scattering (Nouvel, Dubois, Dellacherie, & Six, 2003).

An amphiphilic polymer obtained by covalent attachment of aromatic hydrocarbon groups (phenoxy) onto dextran macromolecules through ether links (Fig. 2) was prepared as previously described (Rouzès, Durand, Léonard, & Dellacherie, 2002). Briefly, 10 g of dextran were reacted with 7 mL of 1,2epoxy-3-phenoxypropane in 100 mL 1 M NaOH under vigorous stirring during 24 h. Then the reaction medium was poured into 1 L ethanol and the resulting precipitate was recovered by filtration and several washings. Finally the solid was dissolved in water and dialyzed against water during 24 h changing water every 3 h. After freedrying, modified dextran was recovered and characterized by ^1H NMR in DMSOd6. Using peaks areas, the phenoxy content, of the polymer was calculated to be 22 grafted phenoxy groups per 100 glucose units of dextran chains. The polymer will be named $DexP_{22}$.

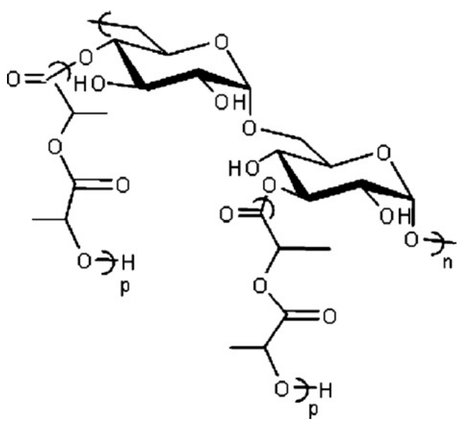

Fig. 1. Schematic chemical structure of Dex_{21K}-g-$_{45}PLA_{3.1K}$. For that polymer $p = 21$.

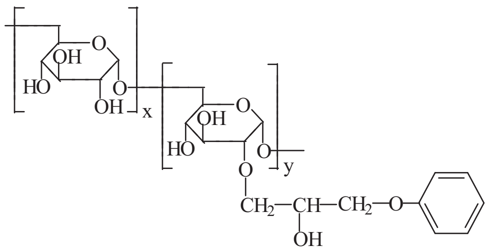

Fig. 2. Schematic chemical structure of $DexP_{22}$. For that polymer $x/y = 78/22$.

2.3. Viscosity measurements

Viscometric measurements of PLA and of Dex_{21K}-g-$_{45}PLA_{3.1K}$ in THF and acetone were carried out using an Ostwald-type capillary viscometer (0.46 mm diameter). The temperature was regulated at 25 °C by a circulating bath. Prior to measurements, the polymer solutions were stirred over 24 h then filtered through 0.22 μm filters. Within the range of polymer concentration explored, the ratio of flow times was assumed to be equal to that of the dynamic viscosities. Eight successive flow time measurements were carried out. The intrinsic viscosity ($[\eta]$ in L/g) and the Huggins' coefficient (k_H) were determined using the following linear equation:

$$\eta_{red} = [\eta] + k_H[\eta]^2 C \qquad (1)$$

In Eq. (1), C is the polymer concentration (in g/L) and η_{red} is the reduced viscosity (in L/g) which is defined by $\eta_{red} = \eta_{sp}/C = \eta - \eta_s/\eta_s C$ where η and η_s are the viscosity of the polymer solution and that of the pure solvent (Pa s), respectively.

2.4. Surface tension measurements

Surface (water/air) tension measurements were determined at 25 °C with a platinum ring, using a K20 EasyDyne tensiometer (Krüss). All solutions were equilibrated for a sufficient time (20 min–1 h) to reach constant reading.

2.5. Preparation of particles by nanoprecipitation

Nanoprecipitations were performed at ambient temperature by a classic technique (Aumelas et al., 2007; Thioune et al., 1997). The polymer (PLA or Dex_{21K}-g-$_{45}PLA_{3.1K}$) vas dissolved in 5 mL of acetone or THF (5, 10 or 20 mg/mL) for 24 h, then added dropwise to 10 mL of water or of a $DexP_{22}$ solution in water (5 mg/mL) under magnetic stirring. Complete addition was achieved in 3 min. The suspension was maintained under magnetic stirring for 10 min. Acetone or THF were evaporated 2 h at 37 °C. The crude suspensions were then centrifuged 15 min at 3000 rpm to remove large aggregates. The supernatant containing nanoparticles was then freeze dried and weighted for the determination of the yield of the nanoprecipitation step. The yield of nanoparticle formation was calculated as the ratio of recovered polymer to the initial amount of polymer used for the nanoprecipitation. When particles are prepared using $DexP_{22}$ as stabilizer, $DexP_{22}$ remains in the supernatant and thus in the freeze-dried solid. The exact content of PLA or of

Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ in the freeze dried samples was determined by ^{1}H NMR in DMSOd6. The adequacy of this determination method was checked by the ^{1}H NMR analysis of mixtures of PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ containing well defined amounts of DexP$_{22}$.

2.6. Nanoparticle characterization

The size distribution of nanosphere in the supernatant after aggregate removing was determined in 10^{-3} M NaCl, by dynamic light scattering using a HPPS from Malvern and light scattering using a Master Sizer from Malvern in order to make sure that no large particles are obtained. In the first case, the reported diameter, D_z, was the so-called z-average from cumulant analysis, i.e. an intensity–average diameter. In the second case, the reported diameters $D(0.1)$, $D(0.5)$ and $D(0.9)$ are diameters at 10%, 50% and 90% of the volume distribution.

The colloid stability of PLA dispersions toward added electrolyte was assessed by turbidimetry. Typically, 100 μL of dispersions were added to 3 mL of NaCl (from 1×10^{-4} to 4 M). The samples were allowed to stand for 1 h and their absorbance was measured over the range 450–700 nm, at 50-nm intervals. The slope of the straight line log(optical density) versus log(wavelength) was taken as an indication of particle size.

3. Results and discussion

In the present work, nanoparticles were prepared using two different core polymers: one PLA commercial sample and one PLA-grafted dextran copolymer (Fig. 1), Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ synthesized using a previously reported procedure (Nouvel et al., 2004). Both polymers were soluble in tetrahydrofuran (THF) and acetone. These two solvents have been used for surfactant-free nanoprecipitation of PLA (Legrand et al., 2007) and were selected for this work. One polymeric stabilizer was eventually added into the aqueous phase before nanoprecipitation. This amphiphilic polymer was obtained by covalent attachment of phenoxy rings onto a native dextran (Fig. 2). Details about the modification procedure are to be found elsewhere (Rouzès et al., 2002). The degree of substitution of dextran was defined as the molar ratio of attached phenoxy rings to glucose repeat units. It was found to be equal to 22% by ^{1}H NMR and the polymer will be named DexP$_{22}$.

3.1. Polymer behavior in solution

The solution behavior of PLA and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ in acetone and THF was first characterized by capillary viscometry experiments. Intrinsic viscosity and Huggins' constant were determined in both solvents at 25 °C for the two polymers (Table 3). Reduced viscosity of polymer solutions varied linearly with polymer concentration up to at least 30 g/L.

For PLA, both solvents appear to be good solvents. No Mark–Houwink relation is available for PLA in acetone. Nevertheless, an estimation can be obtained using the results of Legrand et al. (2007) and the determined average molar masses of the PLA sample ($\overline{M_n} = 47,700$ g/mol, $\overline{M_w} = 49,000$ g/mol). The resulting value is 47.2 mL/g, which can be considered as satisfactorily close to the experimental value (42.4 mL/g) since the results of Legrand et al. were obtained with broad PLA samples (polydispersity indices higher than 3.4).

Table 3
Viscometric characteristics of PLA and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ at 25 °C in acetone and THF. Intrinsic viscosity ($[\eta]$) and Huggins' coefficient (k_H).

Polymer	Solvent	$[\eta]$ (mL/g)	$k_H \pm 0.1$
PLA	Acetone	42.4	0.5
	THF	48.2	0.5
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$	Acetone	22.8	1.1
	THF	26.1	1.2

Table 4
Reduced viscosity η_{red} (mL/g) of PLA and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ solutions in acetone or THF at 25 °C, with different amounts of water. The polymer concentration is 15 g/L.

Water (wt%)	Reduced viscosity (mL/g)			
	PLA		Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$	
	Acetone	THF	Acetone	THF
0	52.9	57.0	32.0	40.0
2.5	51.7	56.1	44.0	56.3
5.0	49.1	50.6	46.0	52.3
10.0	42.0	44.1	27.4	39.0

For results in THF, several Mark–Houwink equations are available and give values between 49 and 56 mL/g at temperatures between 30 and 35 °C (Dorgan et al., 2005; Garlotta, 2001). The intrinsic viscosity of PLA is slightly higher in THF than in acetone, indicating that PLA coils are more extended in THF. The critical overlap concentration can be estimated as the reverse of the intrinsic viscosity, which gives 24 and 21 g/L for acetone and THF solutions, respectively. Thus, we can consider that solutions of the PLA sample with concentrations lower than 20 g/L are in the dilute domain.

For Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$, both acetone and THF are bad solvents. This fact is reflected by the value of Huggins' coefficients (>1) and the rather low intrinsic viscosities in both solvents. Indeed, the number-average molar mass of the graft copolymer is about 200,000 g/mol, which is more than four times the number-average molar mass of PLA sample, while its intrinsic viscosity is about half that of PLA. Thus, the graft copolymer is dissolved in acetone or THF under the form of compact coils in which the dextran backbone is collapsed and the PLA grafts protrude into the surrounding solvent. The intrinsic viscosity is slightly higher in THF than in acetone, which could be attributed to the contribution of PLA grafts (see above). The upper limit of the dilute domain is difficult to estimate when Huggins' coefficient is higher than unity because intermolecular associations may take place even below the overlap concentration that may be derived from the value of the intrinsic viscosity (as for PLA, see above). Previously, we showed that for hydrophobically modified dextrans dissolved in water, the upper limit of the dilute domain could be estimated using a group of empirical equations including $[\eta]$ and k_H (Durand, 2007). Applying this equation to solutions of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ provides the following values of the upper limit of dilute domain in acetone and THF: 43 and 34 g/L, respectively. These values are close to the reverse of intrinsic viscosities. The authors are aware that this is only a rough estimation based on empirical equations established for dextran derivatives in aqueous medium. Nevertheless, it shows that solutions of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ in acetone or THF with concentrations lower than 20 g/L, can be considered as dilute.

Further investigations were carried out to clarify the effect of water addition into organic solutions of PLA and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ on polymer conformation (Table 4). The reduced viscosity of dilute organic solutions was measured at a fixed polymer concentration (15 g/L) with increasing amounts of water (up to 10 wt%).

As a first comment, we must notice that, for each polymer, the variations observed are similar with both solvents, either qualitatively or quantitatively. With PLA solutions, a continuous decrease of reduced viscosity is observed when the amount of added water is increased up to 10 wt%. This is an indication of a continuous decrease of solvent quality, leading to a contraction of polymer coils. Turning to Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ graft copolymer solutions, the variation of reduced viscosity upon water addition appears totally different. For intermediate water amounts (2.5 and 5 wt%), the reduced viscosity is significantly higher than that obtained

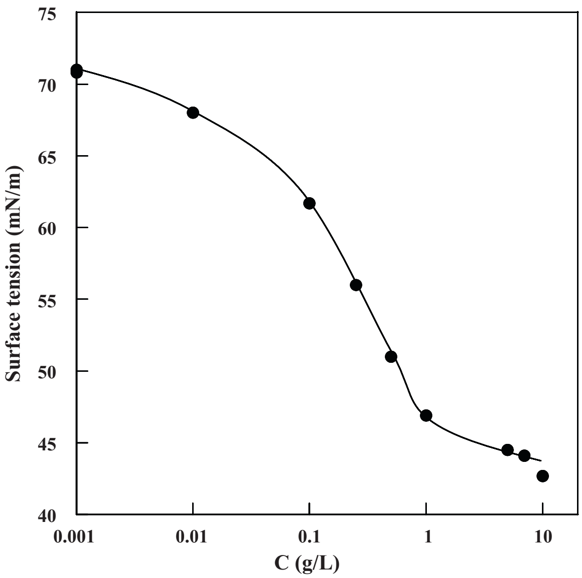

Fig. 3. Surface tension of $DexP_{22}$ aqueous solutions as a function of concentration (line is guide for the eye).

for extreme compositions (0 and 10 wt%). Since dilute solutions are considered, variations in reduced viscosity can directly result from modifications in polymer conformation. Thus, starting from purely organic solutions, polymer coils first swell in the presence of limited amounts of water and finally collapse. This behavior can be interpreted by the expansion of polysaccharide backbone which is triggered out by water molecules provided that the solvation of PLA side chains is preserved. When the amount of water exceeds a critical value, the collapse of PLA side chains becomes predominant. Thus the addition of limited amounts of water into organic solutions of Dex_{21K}-g-$_{45}PLA_{3.1K}$ induces a re-arrangement of macromolecules. Because of the energy of hydrogen bonds, this re-arrangement occurs at water amounts as low as 2.5 wt%. Similar maxima of intrinsic viscosity in selective solvent mixtures have been reported for random copolymers. In particular, for partly hydrolyzed poly(vinyl acetate), a maximum of intrinsic viscosity was found in mixtures of water and dioxane. This maximum was displaced toward higher water contents when the degree of hydrolysis was increased (Bai, Qian, Sun, & An, 2006).

As a conclusion, during nanoprecipitation of PLA, the mutual diffusion of water and organic solvent should induce a transient contraction of polymer coils preceding precipitation itself and particle formation. On the contrary, nanoprecipitation of Dex_{21K}-g-$_{45}PLA_{3.1K}$ first induces a re-arrangement of macromolecules with dextran backbone swelling before a contraction driven by the aggregation of PLA grafts leading to the formation of nanoparticles which can be expected to have a dextran-enriched surface.

3.2. Adsorption of polymeric stabilizer at the surface of polymer particles

For the preparation of nanoparticles by nanoprecipitation, a polymeric stabilizer ($DexP_{22}$) was sometimes dissolved in the aqueous phase. It was necessary to characterize the adsorption of that polymer at the surface of hydrophobic solid particles.

As a first characterization, the surface tension of $DexP_{22}$ aqueous solutions was measured over a wide range of polymer concentration (10^{-3}–10 g/L, Fig. 3). As previously reported for similar dextran derivatives, the minimum surface tension was reached at 1 g/L (Rouzès et al., 2002). The adsorption isotherm of $DexP_{15}$ onto preformed PLA nanoparticles (160 nm diameter) was reported previously (Rouzès et al., 2000). With $DexP_{15}$, the maximum surface coverage was found equal to 6.6 mg/m^2 and reached for $DexP_{15}$ concentrations above 1 g/L. These data will be taken as orders of magnitude for $DexP_{22}$ in what follows. When present in the aqueous phase, the concentration of $DexP_{22}$ was always equal to 5 g/L. A simple mass-balance shows that, for fully saturated 100 nm diameter PLA nanoparticles, with the highest concentration of PLA in the organic solvent (20 g/L), the concentration of excess $DexP_{22}$ in the aqueous phase should be still of the order of 1 g/L. Since 100 nm is generally close to the lower limit of all particle size distributions obtained in that work (see below), we can consider that, for all nanoprecipitation experiments with PLA, the initial amount of $DexP_{22}$ is never a limiting parameter for obtaining nanoparticles with a maximum surface coverage. For nanoparticles prepared with Dex_{21K}-g-$_{45}PLA_{3.1K}$ the same assumption will be made. Indeed, the surface hydrophobicity of such particles should be expected to be always lower than that corresponding to PLA particles. Thus the surface coverage by $DexP_{22}$ should not exceed that found for PLA nanoparticles. As a conclusion, the initial amount of $DexP_{22}$ will not be considered as a limiting parameter with regard to particle surface coverage.

3.3. Preparation of polymeric nanoparticles by nanoprecipitation

In what follows, PLA and Dex_{21K}-g-$_{45}PLA_{3.1K}$ were used for preparing nanoparticles. For all nanoprecipitation experiments, the polymer solution in organic solvent (PLA or Dex_{21K}-g-$_{45}PLA_{3.1K}$ dissolved in acetone or THF) was added to the aqueous phase (containing the polymeric stabilizer, $DexP_{22}$, or not). When $DexP_{22}$ was present in the aqueous phase, its concentration was always 5 g/L. To summarize, four different systems were considered (PLA and Dex_{21K}-g-$_{45}PLA_{3.1K}$ with or without $DexP_{22}$ in the aqueous phase).

3.3.1. Experimental procedure

Two ways of adding the organic solution to the aqueous phase were compared: either dropwise addition or direct injection in the aqueous phase (5 mL of organic solution added into 10 mL of aqueous phase for all experiments). When comparing the diameters of the obtained particles as well as the yield of nanoparticle formation, no clear difference could be evidenced.

Similarly, three different procedures were compared for evaporating the organic solvent after nanoprecipitating PLA at different concentrations (5, 10, 15 and 20 g/L) in the presence of $DexP_{22}$ (5 g/L): at room temperature and normal atmosphere during 24 h, at 37 °C during 2 h and under vacuum during 45 min. No significant difference could be detected for the final particle diameters.

Finally, the rate of centrifugation of the final suspensions was varied between 3000 and 5500 rpm for the 4 different systems at various polymer concentrations in THF (5, 10 and 20 g/L). Except in the case of PLA without polymeric stabilizer in water, for the three other systems, the average particle diameter was systematically decreased when passing from 3000 to 5500 rpm. These results show that for the three systems, centrifugation extracted aggregates and a certain fraction of primary particles which depended directly on the rate of centrifugation. On the contrary, for stabilizer-free PLA particles, the average particle diameter was increased after centrifugation at 10 g/L and at 20 g/L a macroscopic aggregation took place. This fact was a direct consequence of the absence of stabilizer covering the surface of PLA particles. Consequently, centrifugation induced particle aggregation which was even increased when the solid content of the suspensions was higher. No particle aggregation could be detected with the three other systems. These results suggested that, in these three systems, particle aggregation was prevented either by the presence of $DexP_{22}$ or by the self-organization of Dex_{21K}-g-$_{45}PLA_{3.1K}$ during the formation of nanoparticles. In addition, for these three systems, particle aggre-

Table 5
Yield and mean diameter of nanoparticles prepared after dissolving PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ in THF or acetone (5 g/L) after aggregate separation by centrifugation. PDI = polydispersity index.

System	Solvent	D_z (nm)	PDI	$D(0.1)$ (nm)	$D(0.5)$ (nm)	$D(0.9)$ (nm)	Span[a]	Yield (wt%)
PLA	Acetone	188	0.09	148	160	177	0.2	83
	THF	359	0.18	234	301	399	0.5	45
PLA/DexP$_{22}$	Acetone	224	0.06	125	192	275	0.8	84
	THF	224	0.04	95	163	254	1.0	83
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$	Acetone	141	0.10	125	151	180	0.4	84
	THF	178	0.05	94	148	209	0.8	81
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/DexP$_{22}$	Acetone	145	0.11	83	118	163	0.7	55
	THF	187	0.08	83	179	360	1.5	94

[a] The span is calculated by $(D(0.9) - D(0.1))/D(0.5)$ and used as an indication of the broadness of particle size distribution.

gates were easily eliminated using a centrifugation rate of 3000 rpm and it was not necessary to apply higher rates.

3.3.2. Effect of solvent and polymeric stabilizer on nanoparticle size and yield

The nature of the organic solvent is known to have a significant influence of the characteristics of nanoparticles formed by nanoprecipitation. The two solvents employed in that study strongly differ by their dielectric constants and dipole moment. When comparing the particle size distributions obtained using both solvents (Table 5), it appears that for all 4 systems, particle diameters are lower and particle size distributions are narrower when the polymer is initially dissolved in acetone as compared to THF. A similar trend has been evidenced in the case of nanoprecipitation of PLA without surfactant. Our results indicate the same tendency with a PLA-grafted dextran copolymer. Interestingly both polymers (PLA and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$) exhibited a higher intrinsic viscosity in THF than in acetone (Table 3). This fact agrees with the conclusions of Legrand et al. who showed that intrinsic viscosity was a relevant parameter for the control of nanoparticle diameter (Legrand et al., 2007).

As for the yield of nanoparticle formation, it is generally similar for both solvents except for PLA alone.

The presence of DexP$_{22}$ has a strong effect on the nanoprecipitation of PLA, particularly of the size of the formed nanoparticles (which is significantly reduced when the polymeric stabilizer is present). This strong effect underlines the necessity of having a polymeric stabilizer for preventing particle aggregation in the aqueous phase because of the hydrophobic surface of PLA nanoparticles. On the contrary, with Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ the particle size distribution is very similar with or without DexP$_{22}$. This result shows that when Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ is used, the aggregation of formed nanoparticles is limited even when no stabilizer is added in the aqueous phase. This can be explained by the spontaneous formation of a hydrophilic layer at the surface of the nanoparticles because of the organization of graft copolymer chains upon mutual diffusion of water and the organic solvent (as indicated by viscometric measurements, cf. Section 1). The accumulation of hydrophilic sequences at the surface of the particles has two direct consequences: a good colloidal stability of these nanoparticles and a limited adsorption of DexP$_{22}$ which should be present as loosely attached chains because of the lack of superficial hydrophobic zones. These two aspects will be re-examined later through the colloidal stability of the suspensions as a function of ionic strength (cf. Section 4).

3.3.3. Effect of the ionic strength of the aqueous phase

A simple way to enhance hydrophobic effect is to increase the ionic strength. We thus compared the results obtained by carrying out nanoprecipitation from THF into pure water or a 0.15 M NaCl solution with 3 systems (Table 6). The case of PLA alone was not considered here since it has been showed previously that bare PLA nanoparticles are not stable in 0.15 M NaCl solution (Rouzès et al., 2000). For the three studied systems, the yield of nanoparticle formation was strongly reduced in 0.15 M NaCl as compared to pure water. The presence of DexP$_{22}$ seems thus necessary to obtain convenient particle sizes either with PLA or with Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$. Nevertheless, with the latter, the yield is significantly higher than for PLA. In addition, the suspension exhibits no significant variation of particle size distribution after two days, which confirms its good colloidal stability. To summarize, the presence of NaCl strengthens the hydrophobic effect which in turn induces particle aggregation despite the presence of DexP$_{22}$. Upon solvent diffusion, polymer precipitation may be too sharp to allow a fast enough diffusion of DexP$_{22}$ at the surface of the forming particles. Nevertheless, this effect is less pronounced when Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ is used with DexP$_{22}$ which may be explained by the dextran sequences which retain hydrophilicity even in 0.15 M NaCl and allow the establishment of interactions between the two copolymers. These interactions limit the formation of aggregates. Indeed, when Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ is precipitated alone in 0.15 M NaCl it does not form nanoparticles and thus behaves very similarly to PLA.

3.3.4. Effect of polymer concentration on nanoparticle size and yield

Polymer concentration in THF was systematically varied from 5 to 20 g/L for the four different systems. For each one, the particle size distribution as well as the yield of nanoparticle formation was determined at three different concentrations. Except for PLA alone, particle size distribution was displaced toward higher values when polymer concentration was increased (Table 7). A broadening of particle size distribution accompanied this displacement toward higher sizes. Nevertheless, this effect was relatively limited which is consistent with previous work about nanoprecipitation of PCL-grafted dextrans (Ydens et al., 2005). With the four systems, the yield of nanoparticle formation was strongly reduced upon increasing polymer concentration. The probability of aggregate formation upon solvent diffusion is greatly enhanced when polymer concentration is increased. In addition, the variation of the viscosity of organic solvent with polymer concentration has been also suggested as one of the relevant parameters (Galindo-Rodriguez,

Table 6
Mean particle diameter and yield of nanoparticle formation after dissolving the polymer (PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$) in THF (5 g/L) and nanoprecipitating either in pure water or in 0.15 M NaCl.

System	Non-solvent	D_z (nm)	PDI	Yield (wt%)
PLA/DexP$_{22}$	Water	224	0.04	83
	0.15 M NaCl	189	0.10	9
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$	Water	178	0.05	81
	0.15 M NaCl	n.d.	n.d.	8
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/DexP$_{22}$	Water	187	0.08	94
	0.15 M NaCl	241	0.10	23

Table 7
Yield and mean diameter of nanoparticles prepared in THF (5, 10 and 20 mg PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/mL after aggregate separation by centrifugation. PDI = polydispersity index.

Sample	Polymer concentration (g/L)	D_z (nm)	PDI	D(0.1) (nm)	D(0.5) (nm)	D(0.9) (nm)	Span[a]	Yield (wt%)
PLA	5	359	0.18	234	301	399	0.6	45
	10	514	0.22	267	456	652	0.8	8
	20	472	0.09	261	341	542	0.8	1
PLA/DexP$_{22}$	5	224	0.04	95	163	254	1.0	83
	10	246	0.05	141	214	318	0.8	44
	20	242	0.11	109	231	581	2.0	13
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$	5	178	0.05	94	148	209	0.8	81
	10	197	0.05	114	179	262	0.8	61
	20	225	0.13	99	198	425	1.7	12
Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/DexP$_{22}$	5	187	0.08	95	148	251	1.1	94
	10	215	0.06	99	167	254	0.9	70
	20	215	0.13	83	179	360	1.6	23

[a] The span is calculated by (D(0.9) – D(0.1))/D(0.5) and used as an indication of the broadness of particle size distribution.

Allémann, Fessi, & Doelker, 2004). Nevertheless, the variation of the yield of nanoparticle formation (as well as that of the average particle size) with polymer concentration was found to vary significantly with the nature of the organic solvent in which the polymer is dissolved (Galindo-Rodriguez et al., 2004; Legrand et al., 2007; Thioune et al., 1997). The decrease of the yield was much more pronounced for PLA alone while it was more limited for the three other systems in which a component acted as a stabilizer (either DexP$_{22}$ or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ itself or both). The three systems led to similar values of yield of nanoparticle formation and these values did not exceed 23% at the highest polymer concentrations (20 g/L) even in the presence of DexP$_{22}$. In addition, the use of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ provides similar results to PLA in the presence of DexP$_{22}$, a fact that has been already noted (see Section 3.2).

3.4. Colloidal stability of nanoparticles in the presence of salt

The colloidal stability of PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ nanospheres was then examined in pure water as a function of storage time and at various NaCl concentrations (ranging from 10^{-4} to 4 mol/L).

For PLA or Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ nanospheres prepared in the presence of DexP$_{22}$, the average particle diameters measured either immediately after preparation or after a 7 day storage at room temperature were not significantly different, whatever the initial concentration of the polymer in THF (data not shown). This result demonstrates that the surface coverage of nanoparticles is always enough for preventing any aggregation upon storage at room temperature.

The stability of nanoparticles in the presence of salt is also an important point to consider in view of potential uses in biological fluids. Furthermore, differences in critical flocculation salt concentration provide a simple means to detect modification in the surface characteristics of nanoparticles according to their conditions of preparation. Above 10^{-3} M NaCl, suspensions of uncoated PLA nanospheres were no more stable, due to the screening of residual surface charges by electrolyte (Fig. 4). This flocculation originated from Van der Waals attractions which occurred upon increasing ionic strength.

For PLA/DexP$_{22}$ system, flocculation was not observed below 0.1 M NaCl. The presence of dextran loops and tails onto the surface of nanoparticles led to steric repulsions, due to osmotic and elastic compression potentials. However, in a previous paper, we had showed that, with PLA nanospheres coated with DexP$_{15}$, no flocculation could be observed at NaCl concentrations up to 4 M if the amount of adsorbed DexP$_{15}$ was high enough (Rouzès et al., 2000). Differences between PLA/DexP$_{22}$ and PLA/DexP$_{15}$ nanoparticles should be explained either by an insufficient amount of polymer adsorbed onto the surface of the particles or by an excess of phenoxy groups at the PLA/DexP$_{22}$ nanoparticles surface, leading to attractive hydrophobic interactions between nanoparticles at high enough ionic strengths. The latter seems the most probable if we refer to previous work which evidenced the existence of non-adsorbed phenoxy groups for degrees of substitution higher than 10% (Fournier et al., 1998). Nevertheless, further studies would be required to investigate that point. Finally, the occurrence of flocculation for salt concentrations exceeding 0.1 M is consistent with the low yield of nanoparticle formation resulting from nanoprecipitation in 0.15 M NaCl (see Section 3.3).

For Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ nanoparticles prepared without addition of polymeric stabilizer, no flocculation was observed up to 4 M NaCl. This result confirmed that the self-organization of the graft copolymer in the presence of water led to core–shell nanoparticles with a superficial layer enriched in polysaccharide chains. The thickness of that external hydrophilic layer was enough to prevent particle aggregation and ensure colloidal stability up to very high salt concentrations. When nanoprecipitation was carried out in 0.15 M NaCl instead of pure water (see Section 3.3) the yield of nanoparticle formation was greatly lowered. This result may appear in contradiction with the fact that a suspension of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ nanoparticles remained stable up to 4 M NaCl. Nevertheless, it seems in line with the proposed interpretation for the results of nanoprecipitation in 0.15 M NaCl. Indeed, the decrease of the yield of nanoparticle formation with Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$

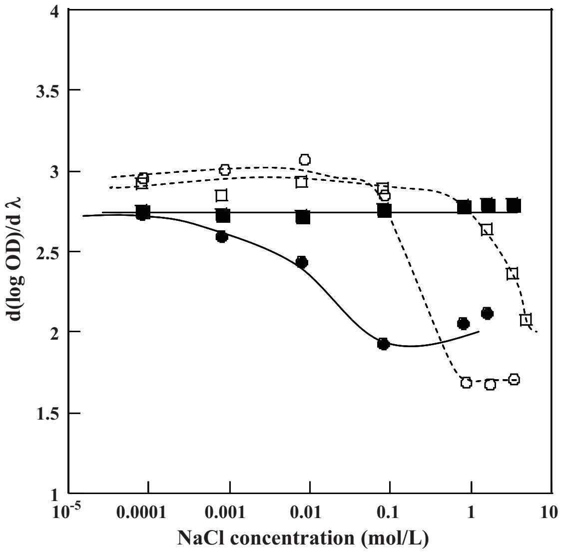

Fig. 4. Dependence of the stability coefficient n on NaCl concentration for bare PLA (●), PLA/DexP$_{22}$ (○), Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ (■), and Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/DexP$_{22}$ (□). Lines are guides for the eye.

when salt is present in the aqueous phase should be related to the kinetics of nanoprecipitation rather than the colloidal stability of the obtained suspension.

Finally, the colloidal stability of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ suspension prepared in the presence of DexP$_{22}$ appeared to be worse than that of the suspension prepared without DexP$_{22}$. Indeed, flocculation was detected for salt concentrations exceeding 1 mol/L. This flocculation may be attributed to the presence of loosely adsorbed DexP$_{22}$ macromolecules at the surface of nanoparticles. Increasing the ionic strength of the aqueous phase increases the hydrophobic effect and leads to an aggregation driven by these dangling DexP$_{22}$ chains. The flocculation of nanoparticles obtained with Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$/DexP$_{22}$ system occurs at much higher ionic strengths than the one occurring with the PLA/DexP$_{22}$ system. This can be explained by both the hydrophilic character of the surface of Dex$_{21K}$-g-$_{45}$PLA$_{3.1K}$ nanoparticles and the low amount of DexP$_{22}$ adsorbed on their surface.

4. Conclusion

Nanoprecipitation was used for preparing suspensions of submicronic dextran-covered PLA nanoparticles following two strategies. The first involved the use of hydrophobically modified dextrans as polymeric stabilizers during the nanoprecipitation of PLA. Alternatively, PLA-graft-dextran copolymers were used without the addition of stabilizer in the aqueous phase. It was shown that this second strategy led to the formation of suspensions of polymeric nanoparticles with convenient yields and size distributions. In addition these suspensions exhibited colloidal stability up to high ionic strengths (4 mol/L). The formation of dextran-covered nanoparticles was investigated in details by examining the variation of polymer conformation in water/THF mixtures and by considering the effects of various physico-chemical parameters (polymer concentration, ionic strength, solvent nature).

Applications of nanoparticles obtained with PLA-g-dextran copolymer for encapsulating fragile active molecules are currently investigated.

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