polymer

Poly(3-hydroxyoctanoate) containing pendant carboxylic groups for the preparation of nanoparticles aimed at drug transport and release

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Abstract

Copolymers of poly(3-hydroxyoctanoate) (PHO) with carboxylic groups in lateral chains have been prepared by chemical modification of unsaturated bacterial polyesters. The oxidation of the pendant alkenes is complete and important loss in molecular weight of polymer was observed. The presence of repeating units containing pendant carboxy groups in the proportion of 25% has enhanced hydrophilicity of these new polymeric structures. Nanoparticles have been prepared from PHO and two functionalized derivatives, characterized by electronic microscopy and compared in view of bioactive molecules transport and release. © 2001 Published by Elsevier Science Ltd.

Keywords: Poly(3-hydroxyoctanoate); Nanoparticles; Oxidation

1. Introduction

A wide variety of microorganisms produce poly(hydroxyalkanoates) (PHAs) as intracellular energy and carbone storage materials [1,2]. More than hundred different repeating units have been identified as constituents of this natural polyester family [3]. Among microorganisms able to produce PHAs, *Pseudomonas oleovorans* has been investigated most extensively [4]. *P. oleovorans* presents the particularity to produce polyesters from a large spectrum of carbon substrates, including *n*-alkanes, alkanoic and alkenoic acids [4–7]. PHAs containing functional terminal groups such as double bonds, triple bonds, epoxy group or halogens, in side chains have also been prepared [8–11]. We have previously reported the production of PHAs with a variable proportion of unsaturated repeating units (0–100%) using sodium octanoate and 10-undecenoic acid mixtures as carbon source [8]. The composition of these materials was the reflection of the nutrient mixtures. The interest to introduce pendant double bonds is evident for further chemical modifications. Unsaturated groups have been transformed into more useful functional groups such as epoxy to crosslink [8] or diol to enhance hydrophilicity of modified biopolyesters [12].

Carboxylic groups are of greatest importance to bind bioactive molecules, hydrolyzable, hydrophilic oligomers or targeting proteins. These groups cannot be directly introduced by bioproduction. Therefore, a chemical route has been carried out from unsatured bacterial polyesters.

In the present work, we have investigated a new chemical modification of poly(3-hydroxyoctanoate-*co*-3-hydroxyundecenoate) ($PHO_{100-x}U_x$) produced by *P. oleovorans* and their ability to form nanoparticles.

2. Experimental part

PHO and PHOUs production was carried out as described in a previous report [8]. Bacterial polyesters were extracted from lyophilized cells with hot chloroform in a Soxhlet extractor, and then purified by precipitation in methanol. The total extraction was controlled by IR analysis of bacteria. As described by Hong et al. [13], IR spectroscopy can be used to carry out rapid qualitative analysis of PHA inside intact bacterial cells. IR analyses were achieved with lyophilized cells before extraction and with bacterial residues after extraction. Cell suspensions were prepared in water (4 mg per water ml) and were directly applied on a KBr optical plate and dried under vacuum. The FTIR spectra showed the strong characteristic band of the ester group at $1742 \, cm^{-1}$ and the CH vibration at $2930 \, cm^{-1}$. We controlled the extraction quality with the significant decrease of both peaks previously mentioned. The polymer

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content per dried cells was determined by gravimetry. The PHA content was of 50% per total dried cells weight (0.5 g of PHA for 1 g of dried cells).

2.1. Carboxylation of PHOU containing 10% of unsaturated groups

0.22 g PHOU was dissolved in a mixture of 10 ml of CH_2Cl_2, 1.8 ml of acetic acid and 0.119 g of 18-crown-6-ether (3 equiv. per unsaturated units in PHOU). The mixture was vigorously stirred and cooled at 0 °C with an ice bath. Then, 0.95 g of potassium permanganate ($KMnO_4$) (4 equiv. per unsaturated units in PHOU) was added. The reaction medium is instantaneously violet (as indicated by the presence of aqueous $KMnO_4$) then became brown within several minutes as manganese oxide was formed. The suspension was stirred overnight. Ten milliliters of water were added and sodium bisulfite was added to reduce any oxidant excess until complete colorless of the suspension. The organic layer was washed twice with water, and then dried on $MgSO_4$. The organic layer solution was concentrated under vacuum and the carboxylated polymer was precipitated twice in methanol as a nonsolvent from the chloroform solution. The oxidation was quantitative, according to ^{1}H NMR, based on the disappearance of both peaks at 4.9 and 5.7 ppm assigned to the unsaturated groups.

#### 2.1.1. Purification of PHOU contained 25% of unsaturated groups

Purification by dialysis: after oxidation, the organic layer was evaporated under vacuum and brought into an acetone/water (90/10 v/v) mixture, then dialyzed against the same solvents composition in a regenerated cellulose membrane tubing (6-8000 Da).

Purification by centrifugation: after oxidation, the organic layer was evaporated under vacuum and water was added to the crude product. $PHO_{75}D_{25}-COOH$ is not hydrosoluble. The solution was slowly basified with a concentrated sodium hydroxide aqueous solution until pH = 11. The polymer is soluble. Then, the solution was acidified with a concentrated chlorohydric acid aqueous solution until pH = 5. The weak acidic pH leads to the precipitation. The precipitate was isolated by centrifugation at 5000 rpm for 30 min. The solid, very sticky, was washed with water and centrifugated again. The product obtained was dried under vacuum.

2.2. Polymer characterization

Infrared spectra were recorded on a Perkin 1760 model spectrometer.

Molecular weights were determined by size exclusion chromatography (SEC) using two columns in serial 'mixed C (5 μm)' (Polymer Laboratories) using THF as eluent with a flow rate of 1 ml min^{-1} and connected to an RI detector. Sample concentration of 10 mg ml^{-1} and injection volume 50 μl. A calibration curve was generated with
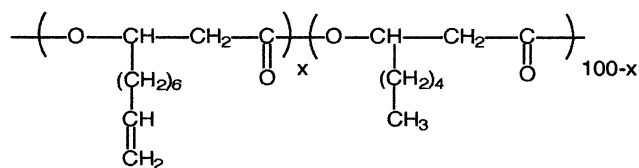
Scheme 1.

polystyrene standards of low polymolecularity purchased from Polysciences (*M* (g mol^{-1}): 2,656,000; 841,700; 320,000; 148,000; 59,500; 28,500; 10,850; 2930 and 580). The correlation coefficient was 0.999.

^{1}H NMR spectra were recorded in $CDCl_3$ using a Bruker AM200.

2.3. Preparation and characterization of nanoparticles

Nanoparticles were prepared by a nanoprecipitation-solvent evaporation method. Seventy-five milligrams of the corresponding polyester were dissolved in 15 ml of acetone. This organic phase was poured slowly into 45 ml of water containing Pluronic F-68 surfactant (1% w/v) with moderate stirring at room temperature. Particles were immediately formed, and the colloïdal suspension was continuously stirred for 4 h at room temperature under air flow until complete evaporation of the organic solvent. The whole dispersed system was centrifuged (900 g for 10 min) to separate microparticules (>1 μm). The nanoparticles dispersed in the supernatant were sedimented by centrifugation (3500 g for 30 min) and dispersed into a glass tube in phosphate-buffered saline (pH = 7.4) containing 1% Pluronic F-68 (w/v) to prevent aggregation.

The resulting nanoparticles were characterized by light scattering, (Zeta Sizer 3 Malvern) allowing their size to be determined and by zeta potential measurement, giving access to surface characteristics.

3. Results and discussion

3.1. Preparation of carboxylated PHOD

Two series of ($PHO_{100-x}U_x$) with two different percentages of double bonds ($PHO_{90}U_{10}$ and $PHO_{75}U_{25}$) have been prepared and characterized by SEC (Scheme 1 (structure of $PHO_{100-x}U_x$) and Table 1).

Table 1
Physical characteristics of $PHO_{100-x}U_x$

Polymer	M_n (g mol^{-1})	M_w (g mol^{-1})	T_g (°C)	T_m (°C)
$PHO_{90}U_{10}$	46,000	115,000	−38	43
$PHO_{90}U_{10}$	43,000	109,000	–	–
$PHO_{75}U_{25}$	80,000	179,000	−39	41
$PHO_{75}U_{25}$	74,000	160,000	–	–
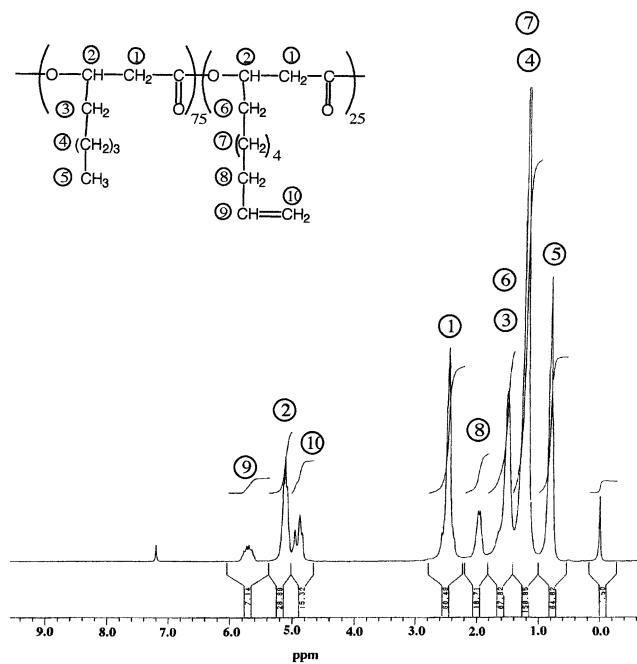
Fig. 1. ^{1}H NMR spectrum of $PHO_{75}U_{25}$.

Fig. 2. ^{1}H NMR spectrum of $PHO_{75}D_{25}$–COOH.

PHOUs production was carried out as described in a previous report [3]. High molecular weights were obtained in both cases. Characteristic temperatures explain thermoplastic elastomer properties. T_g (glass transition temperature) is very low (-40 to -30 °C) and crystalline regions act as physical crosslinks ($T_m \sim 40$ °C). ^{1}H NMR spectrum of $PHO_{75}U_{25}$ is shown in Fig. 1. The proportion of unsaturated repeating units was determined from areas of peaks 9 and 5. The percentage of unsaturated units (25%) corresponds to the proportion of the alkenoic acid in a mixture of substrates. Polymers prepared from a mixture of sodium octanoate and 10-undecenoic acid (80/20) contain 25% of reactive ethylene groups.

Unsaturated groups of PHOU were converted to carboxylic groups by modification of a method using $KMnO_4$ in the presence of potassium bicarbonate ($KHCO_3$) [14].

The chemical modification reaction was carried out with potassium permanganate, ($KMnO_4$) as oxidation agent (Scheme 2 (oxidation of PHOU)) and crown-ether as phase transfer and dissociating agent of $KMnO_4$. The reaction was carried out in a biphasic medium constituted
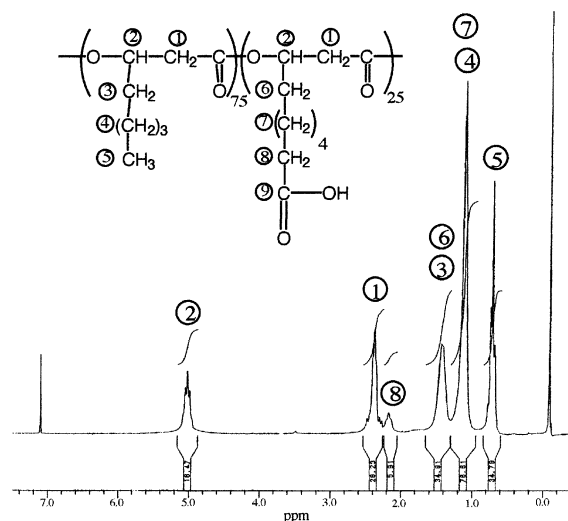
by dichloromethane/acetic acid mixture. Acidic condition was used to reduce hydroxide ions produced during the reaction and to avoid overoxidation [15]. We have studied the influence of $KMnO_4$ and crown-ether amounts on the oxidation (Table 2). In the case of $PHO_{75}U_{25}$ high amounts of $KMnO_4$ and crown-ether caused strong emulsion formation after water and sodium bisulfite addition. The recovery of the oxidized product from the organic layer was very delicate and consequently losses were observed. In an attempt to overcome difficulties in working with a biphasic medium, a homogeneous system was obtained using a mixture of 3 equiv. of crown-ether and 4 equiv. of $KMnO_4$ (based on unsaturated units). An additional important reactional parameter is the polymer concentration. Indeed, polymer concentration must be higher or equal to 20 g l^{-1} to prevent the formation of diol groups.

$PHO_{75}U_{25}$ is totally oxidized in 16 h. On the contrary, after 7 h of reaction, the ^{1}H NMR presented a significant decrease of the signals corresponding to the unsaturated peaks but not a total disappearance. Results show that the reaction is unaffected by amounts of $KMnO_4$ and crown-ether. For example, in the NMR spectrum of $PHO_{75}D_{25}$–COOH (Fig. 2) two characteristic peaks at 4.9 and 5.7 ppm assigned to the unsaturated groups have completely disappeared. The total oxidation into carboxylic groups for all samples was also confirmed by the absence of signals
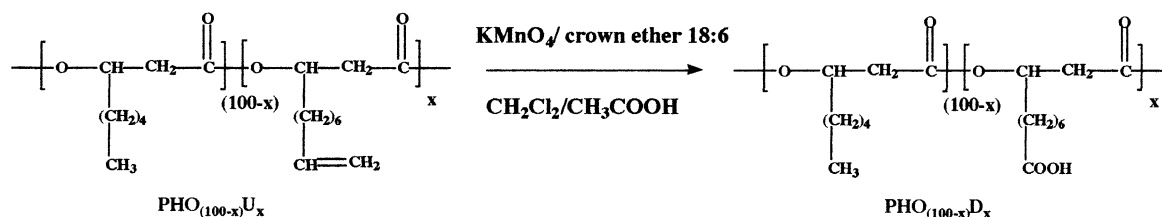
Scheme 2.

Table 2
Results and conditions of the oxidation of PHOU (equiv: Equivalent based on the unsaturated unit content of PHOU)

No.	PHOU precursor	KMnO$_4$ (equiv.)	Crown-ether (equiv.)	Yield (%)	Conversion (%)	Reaction duration (h)	
1	PHO$_{90}$U$_{10}$	6	4	50	100	16	
2	PHO$_{90}$U$_{10}$	4	3	70	100	16	
3	PHO$_{75}$U$_{25}$	8	6	30	100	16	Strong emulsion
4	PHO$_{75}$U$_{25}$	4	6	33	100	16	Strong emulsion
5	PHO$_{75}$U$_{25}$	4	3	70	97	7	
6	PHO$_{75}$U$_{25}$	4	3	70[a]	100	16	

[a] Calculated yield after dialysis in acetone/water mixture.

around 3.3–3.7 ppm due to *CH*–OH and *CH$_2$*–OH as reported by Lee et al., in the preparation of hydroxylated PHAs with KMnO$_4$ as oxidative agent [12]. The peak assigned to the carboxylic groups is not perceptible on the ^{1}H NMR spectrum. But its presence was clearly confirmed by the addition of trimethylsilyldiazomethane inside the NMR tube. Free carboxylic groups have quantitatively been transformed in methyl ester groups. A single peak corresponding to methoxy groups is present at 3.6 ppm (Fig. 3).

The purification of PHOD containing 25% of carboxylic groups is impossible by simple precipitation in methanol (as used for the PHOD containing 10% of carboxylic groups) because the oxidized polymer is soluble in polar solvents as methanol. Three different methods (Table 3) have been tested to eliminate crown-ether as well as to purify the PHO$_{75}$D$_{25}$–COOH: precipitation from a chloroform solution of the oxidized PHOD into cyclohexane, dialysis in an acetone/water (90/10 v/v) mixture and the precipitation in weak acidic

Fig. 3. ^{1}H NMR spectrum of PHO$_{75}$D$_{25}$–COOH in the presence of trimethylsilyldiazomethane.

condition. The purification percentage obtained was calculated on the basis of ^{1}H NMR signal assigned to the crown-ether centered at 3.5 ppm, in comparison with spectra before and after purification. The best method is the dialysis in comparison.

3.2. Characteristics of PHOD–COOHs

Molecular weights after oxidation were investigated by comparison with the values for PHOUs and PHOD–COOHs found by SEC (Table 4). Concerning PHO$_{90}$D$_{10}$–COOH, no significant modification of the macromolecular chain was observed. On the contrary, an apparent reduction in molecular weight for PHO$_{75}$D$_{25}$–COOH is observed. This seems to indicate a macromolecular chain degradation during the oxidation step. However, it is worthwhile to note that column calibration standard in SEC analysis were polystyrene standards. So, the difference observed could be explained by changes in hydrodynamic radii between precursor and carboxylated polymers. An additional indication is the unmodified polymolecularity index. The last result in Table 4 shows that a significant molecular weight reduction from $M_n = 77{,}000$–6400 g mol^{-1}. This degradation did not occur during the carboxylation step but during the purification. Under strong basic conditions (pH = 11), the polyester chain with 25% of carboxylic groups was hydrolyzed. This result can be compared with those referred in the citation [14]. Even though the oxidation procedure is different (KMnO$_4$ as oxidative agent under basic condition), there is an important similar parameter: the basic medium. In both cases, significant degradations were observed.

Our results are different from those cited by Lee et al. [14] concerning the conversion of the double bonds in carboxylic groups and the macromolecular chain length of resulting
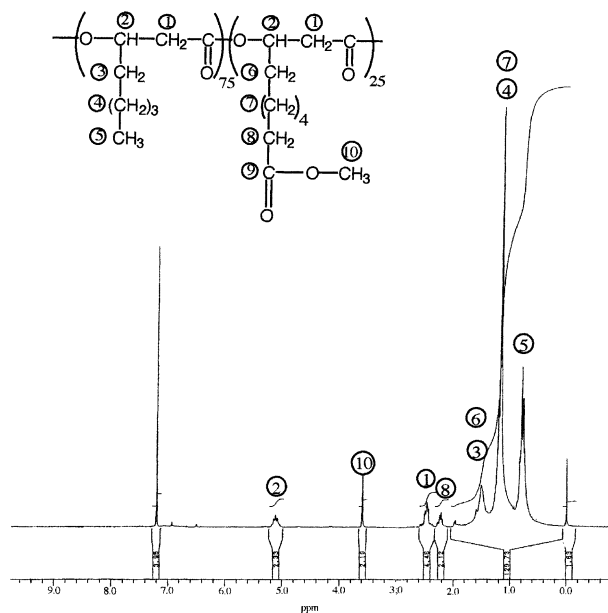
Table 3
Purification methods for PHO$_{75}$D$_{25}$–COOH

Method	Purification (%)	Yield (%)
Precipitation in C$_6$H$_{12}$	50	98
Dialysis in acetone/water	98	70
Precipitation at pH = 4–5	98	40

Table 4
Physical characteristics of PHO$_{100-x}$D$_x$–COOH

No. (oxidation condition from Table 2)	Polymer	M_n (g mol^{-1})	M_w (g mol^{-1})	I_p
2	PHO$_{90}$U$_{10}$	43,000	109,000	2.5
	PHO$_{90}$D$_{10}$–COOH	41,000	100,000	2.4
	PHO$_{90}$D$_{10}$–COOH	48,000	103,000	2.1
5	PHO$_{75}$U$_{25}$	80,000	179,000	2.2
	PHO$_{75}$D$_{25}$–COOH	54,000	135,000	2.5
5	PHO$_{75}$U$_{25}$	74,000	160,000	2.2
	PHO$_{75}$D$_{25}$–COOH	49,000	105,000	2.1
5	PHO$_{75}$U$_{25}$	77,000	180,000	2.3
	PHO$_{75}$D$_{25}$–COOH[a]	6400	35,000	5.5

[a] Purified by precipitation in water under weak basic condition after solubilization at pH = 11.

polymer (from $M_n = 77,000$–6400 g mol^{-1}). The difference can be explained by a higher proportion of unsaturated pendant groups (between 40 and 90%) and experimental conditions (acidic medium).

The introduction of polar groups such as carboxylic acid groups has modified polymer physical properties. As reported in Table 5, initial PHO$_{100-x}$U$_x$ are soluble in organic solvents such as dichloromethane, chloroform, tetrahydrofuran, but are insoluble in polar solvents such as methanol, acetone/water 85/15 (v/v). After oxidation, PHO$_{90}$D$_{10}$–COOH has the same feature as the precursor polymer and remains insoluble in polar solvents.

In the case of PHO$_{75}$D$_{25}$–COOH, the presence of 25% polar groups modifies the hydrophobicity/hydrophilicity balance. This polymer is soluble in methanol. In an acetone/water mixture 85/15 (v/v), PHO$_{75}$D$_{25}$–COOH is perfectly soluble while PHO$_{90}$D$_{10}$–COOH gives a bluish clear solution. This blue color is typical of the presence of aggregates in solution resulting in hydrophobic macromolecular chain associations. The same situation was also observed for PHO$_{75}$D$_{25}$–COOH when the water amount in the solvent is higher than 17%. Furthermore, solubility of PHO$_{100-x}$D$_x$–COOH in polar mixture acetone/water depends on pH. When pH is acid (<5) PHO$_{100-x}$D$_x$–COOH precipitates. The protonated carboxylic groups do not enhance enough hydrophilicity to solubilize these
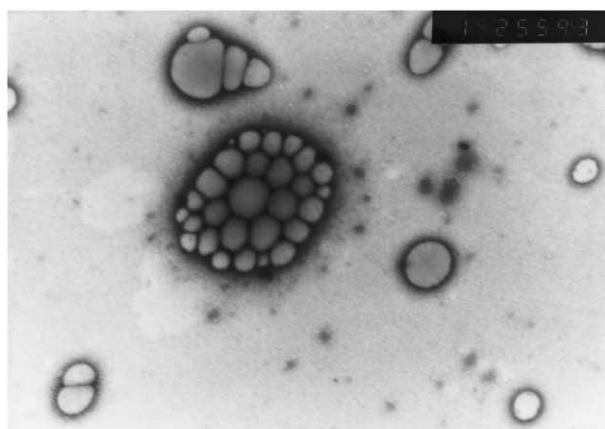
polymers. When pH weakly increases, ionization of acid groups is insufficient to solubilize polymer. At pH = 11, the product is soluble but at the same time, the strong basic conditions caused the hydrolysis of the macromolecular chain. Furthermore, the short length of the chain could be also explained by the solubility of PHO$_{75}$D$_{25}$–COOH at this pH. This fast hydrolytic degradation under strong basic conditions may be associated with the presence of polar groups in the side chain of the PHOD. It is a promising first result because biodegradation or simple hydrolysis of PHAs with long chain-length as PHO or PHOU are largely disturbed by the hydrophobicity of long pendant chains [16]. Experiments on the degradation of films led with PHO$_{75}$D$_{25}$–COOH are currently in progress.

3.3. Preparation and characterization of nanoparticles

Nanoparticles were prepared by a nanoprecipitation-solvent evaporation method. This method very often used to prepare colloïdal carriers of a matricial type (nanospheres) or of a vesical type (nanocapsules) was described by Fessi et al. [17]. Briefly, polyesters were dissolved in acetone. This organic phase was poured slowly into water containing Pluronic F-68 surfactant with moderate stirring at room temperature. Particles were immediately formed,

Table 5
Solubility of PHOD–COOH ((+) soluble, (±) partialy soluble, (−) insoluble (insoluble sticky feature)

Solvent	PHO$_{90}$U$_{10}$	PHO$_{90}$D$_{10}$–COOH	PHO$_{75}$U$_{25}$	PHO$_{75}$D$_{25}$–COOH
Chloroform	+	+	+	+
Acetone	−	−	−	+
Water	−	−	−	−
Acetone/water (v/v)				
90/10	−	±	−	+
85/15	−	±	−	+
83/17	−	−	−	±
80/20	−	−	−	−
Methanol	−	−	−	+

PHO nanoparticles (magnification 5000 x)
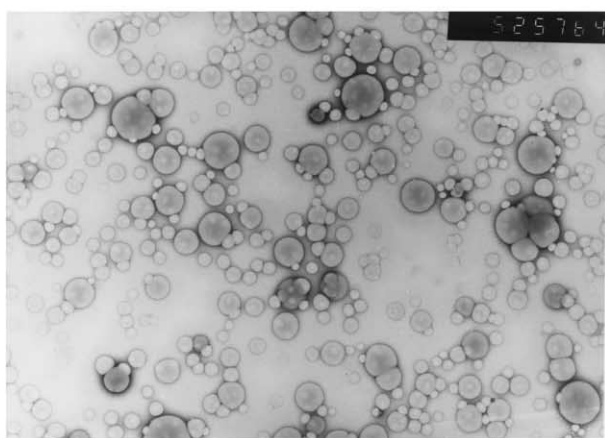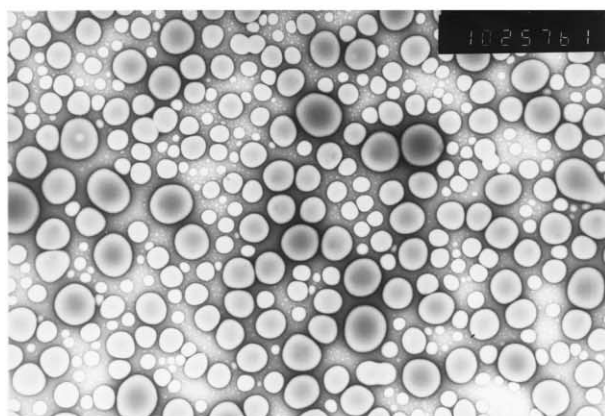

PHO$_{90}$D$_{10}$-COOH nanoparticles (magnification 5000 x)

PHOU$_{75}$D$_{25}$-COOH nanoparticles (magnification 10000 x)

Fig. 4. TEM photographs of nanoparticles prepared by nanoprecipitation method of the corresponding polyester.

and the colloïdal suspension was continuously stirred for 4 h at room temperature under air flow until complete evaporation of the organic solvent. The whole dispersed system was centrifuged to separate microparticles (>1 μm). The nanoparticles dispersed in the supernatant were sedimented by centrifugation and dispersed into a glass tube in phosphate-buffered saline (pH = 7.4) in the presence of 1% Pluronic F-68 (w/v) to prevent aggregation. It is noteworthy that in

Table 6
Characteristics of nanoparticles prepared by nanoprecipitation method of the corresponding polyester

Nanoparticles	Size (nm)	
	Mean diameter	Dispersity (90%)
PHO	271 ± 48	249–289
PHO$_{90}$D$_{10}$–COOH	213 ± 17	177–283
PHO$_{75}$D$_{25}$–COOH	206 ± 26	205–240

the absence of surfactant, nanoparticles would sediment and form a cake difficult to redisperse.

Taking into consideration the introduction of carboxylic groups for PHO$_{100-x}$D$_x$–COOH and then the solubility of these polymers in an acetone/water mixture, nanoparticles from PHO$_{100-x}$D$_x$–COOH precipitates when water phase is acid (<5).

The resulting nanoparticles were characterized by light scattering allowing their size to be determined (Table 6) and morphological examination of nanoparticles was performed using a transmission electron microscope (TEM) (Fig. 4).

The mean diameter of nanoparticles measured by a dynamic light scattering method was around 200–300 nm and the standard deviation is rather low (monodispersity) as shown in Table 6 and the values agree with the observation by transmission electron microscopy (TEM) (Fig. 4).

TEM revealed that spherical nanoparticles were produced from polymers but some have collapsed depending on the type of polymer. PHOs resultant nanoparticles collapsed dramatically compared to that of nanoparticles prepared from PHO$_{90}$D$_{10}$–COOH. On the contrary, there was no evidence of collapsed nanoparticles with PHO$_{75}$D$_{25}$–COOH. The difference might be due to the nanoparticles surface hydrophobicity as assessed by the hydrophobicity/hydrophilicity balance of the polyesters.

In preliminary experiment, PHOs nanoparticle preparations were performed with different Pluronic F68 concentrations. No surface modification (size and zeta potential) was detectable indicating that hydrophobic PHOs nanoparticles were not coated with the poloxamer (Table 7) as previously described for polystyrene nanoparticles [18] and then not prevent clumping mentioned earlier.

Table 7
Effect on the size and the charge of PHOs nanoparticles performed with different surfactant concentrations

Pluronic F68% (w/v)	Size (nm)	Zeta potential (mV)
0.0	271 ± 48	−30.0
0.1	272 ± 48	−32.8
0.3	283 ± 34	−33.8
0.6	267 ± 19	−34.7
1.0	246 ± 21	−30.6

4. Conclusions

Targeted drug delivery has gained recognition in modern therapeutics and numerous attempts have been made to explore the potentials of carriers in the development of drug transport and release [19].

PHOs and $PHO_{90}D_{10}$–COOH nanoparticles aggregates and so could not represent an appropriate drug delivery system. For this type of application, the preparation of nanoparticles suspension with suitable properties is necessary. On the contrary, an increasing proportion of hydrophilic groups allow the suspension of $PHO_{75}D_{25}$–COOH nanoparticles in the presence of the biocompatible surfactant Pluronic F-68. Pluronic F-68 (Poloxamer 188) is a poly(oxyethylene)–poly(oxypropylene) surfactant that has been used as a stabilizer of fluorocarbon emulsion for in vivo oxygen delivery during, for example, percutaneous transluminal coronary angioplasty [20].

The therapeutic effectiveness of drug delivery systems is often limited by the inability to sustain therapeutic drug levels at the target site. Site-specific drug delivery is yet largely empirical and the methods available are presently imperfect [21].

For this purpose, surface properties of $PHO_{75}D_{25}$–COOH nanoparticles will be interesting in order to obtain a long term delivery of drug combined with a targeting efficiency. The presence of repeating units containing pendant carboxy groups in the proportion of 25% of the polymeric structure of $PHO_{75}D_{25}$–COOH could be considered to synthesize nanoparticle-drug conjugates, including for example anti-cancer agents, anti-infectious agents and analgesics. Moreover, specific adhesion systems could be prepared by covalent coupling of ligands to nanoparticles such as monoclonal antibodies.

$PHO_{75}D_{25}$–COOH nanoparticles are a promising site-specific drug delivery system to provide optimum therapeutic activity at the active sites, and so reduce systemic exposure and toxicity.

Acknowledgements

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