Characterization of Dextran−Poly(isobutylcyanoacrylate) Copolymers Obtained by Redox Radical and Anionic Emulsion Polymerization

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ABSTRACT: Characteristics of polymer forming poly(isobutylcyanoacrylate) (PIBCA) nanoparticles used as drug delivery systems were investigated. The nanoparticles were prepared by emulsion polymerization of IBCA in the presence of dextran by either anionic or redox radical emulsion polymerization. These methods allowed complete polymerization of IBCA. The nanoparticles were formed by heterogeneous polymers. Characterization of PIBCA and dextran parts after selective hydrolysis of the counterpart highlighted differences in term of composition and structure of polymer. The anionic emulsion polymerization (AEP) led to the simultaneous formation of homopolymer of PIBCA and of copolymers of several short PIBCA chains grafted on a dextran chain. In redox radical emulsion polymerization (RREP), nanoparticles contained only copolymers with bloc structure in which PIBCA chains exhibited a high molecular weight and a broad distribution. These results confirmed hypothesis on formation of diblock copolymers by RREP and of grafted copolymers by AEP.

1. Introduction

In pharmacy, polymer nanoparticles are developed as carriers for drugs to target high drug concentrations in the diseased tissue while reducing the drug concentration in healthy tissues. Indeed, control of the drug distribution in the body by using colloidal carriers prevents the occurrence of unwanted side effects due to the toxicity of the drug molecules. As reported in the literature, promising results were obtained with poly(alkylcyanoacrylate) nanoparticles.[1−3] They are made of a bioerodible and bioeliminable polymer, and many drugs were incorporated in these systems. They are also readily available from emulsion polymerization of the corresponding monomer. Polymerization is performed in an acidic aqueous medium containing dextran which is a neutral polysaccharide and which ensures the colloidal stability of the polymer dispersion. Two types of polymerization were described depending on whether they are based on an anionic or on a redox radical emulsion polymerization.[4] This last method was already used to produce copolymers from poly(ethylene glycol) (PEG) or polysaccharides by a straightforward route of synthesis which only required one step.[5−9] Both types of polymerization lead to the formation of very stable suspension of polymer nanoparticles with a diameter ranging from 80 to 600 nm. However, little is known on the characteristics of the polymer which forms the nanoparticles. Douglas et al.[10] showed that nanoparticles obtained by anionic emulsion polymerization contained a copolymer composed of poly(isobutylcyanoacrylate) (PIBCA) and dextran. More recently, Chauvierre et al.[11] reported that the polymer constituting the nanoparticles prepared by redox radical emulsion polymerization (RREP) was insoluble in most of the organic solvents generally used for polymer characterization. The two types of polymers differed in their emulsifying properties suggesting that their structure might depend on the polymerization mechanism.[11] Structural differences in the polymers forming the nanoparticles can dramatically influence the fate of the nanoparticles once they will be administered in vivo as a drug carrier. Indeed, after incubation with serum, the nanoparticles obtained by the two methods showed opposite capacity to activate the complement systems.[12,13] The pattern of the plasma proteins which adsorbed on the surface of each type of nanoparticles was affected by the method of nanoparticle preparation as well.[12] Thus, the purpose of the present work was to analyze in details the polymers of the two types of nanoparticles in term of both composition and structural characteristics.

Hypothesis. In both methods of preparation of the nanoparticles, the emulsion polymerization of isobutylcyanoacrylate (IBCA) is performed by dispersing the monomer in an acidic solution of dextran.

Anionic emulsion polymerization can be performed according to two distinct procedures. Using a dextran solution in chlorhydric acid at pH 2.5 (AEP-pH 2.5), polymerization may be initiated by the hydroxyl ions of the water leading to the formation of a homopolymer of PIBCA (homo-PIBCA). The polymerization can also be initiated by any hydroxyl groups of the glucose residues of the polysaccharide chains. This may produce copolymers of PIBCA and dextran in which several PIBCA chains can be initiated and grew onto a single chain of dextran as illustrated in Scheme 1A. In the second procedure, the polymerization medium consists of a solution of dextran in nitric acid at pH 1 (AEP-pH 1). In these conditions, it is expected that the anionic polymerization will be preferentially initiated by the hydroxyl groups of the glucose residues of dextran while the initiation by the hydroxyl ions of water is expected to be considerably reduced.

The redox radical emulsion polymerization (RREP) is performed in nitric acid at pH 1 in the presence of dextran and of Cerium IV. Because of the extremely low pH, the glucose units of the dextran chains are partly in the open conformation. When oxidation by Cerium IV occurs, dextran chain is split in two parts, one big fragment and one short fragment as highlighted by Chauvierre et al.[4] in previous work. Simultaneously, a radical is created at a chain end of one of the dextran fragment.[8,9] Thus, it is expected in this case that IBCA polymerization can be

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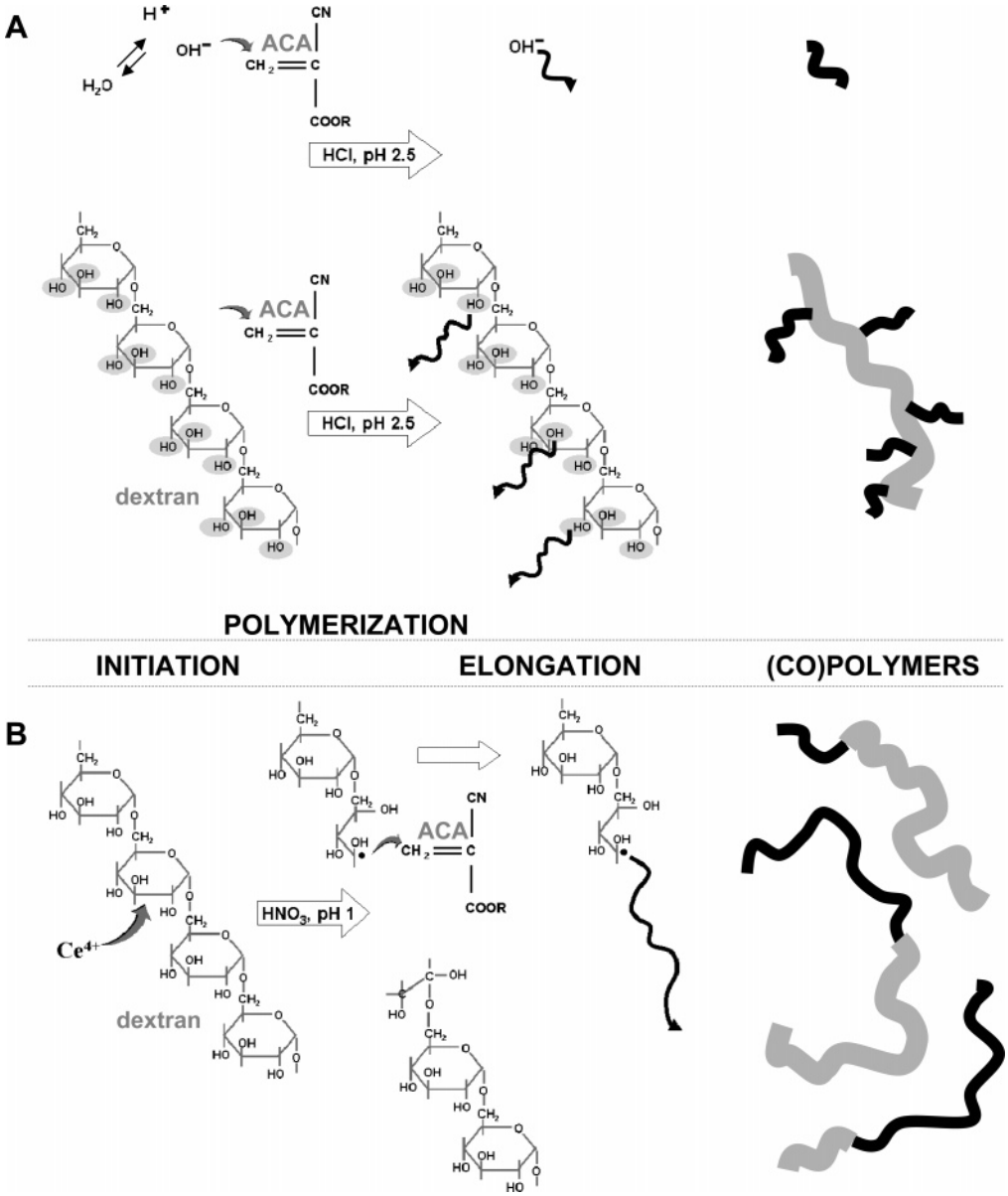
Scheme 1. Mechanisms of Emulsion Polymerization of IBCA in the Presence of Dextran[a]



[a] Key: A, anionic emulsion polymerization; B, redox radical emulsion polymerization.

initiated at the chain end of this particular fragment. The resulting polymer should be composed of a PIBCA chain attached to one extremity of a dextran chain forming a linear block copolymer (Scheme 1B).

2. Materials and Methods

Materials. Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Loctite (Dublin, Ireland). Dextran (66 900 g/mol), dimethyl sulfoxide-d_6 (DMSO-d_6), deuterium oxide (D_2O), and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Cerium (IV) ammonium nitrate, nitric acid, and trisodium citrate dihydrate were purchased from Fluka (Saint Quentin Fallavier, France). DMSO was purchased from Acros Organics (Geel, Belgique) and hydrochloric acid (HCl) from VWR (Fontenay-sous-Bois, France). All chemicals were reagent grade and used as purchased.

Polymerization Procedures. Anionic Emulsion Polymerization at pH 2.5 (AEP-pH 2.5) was performed according to the procedure described by Couvreur et al.[14] Dextran (0.05 g) was dissolved in 10 mL of hydrochloric acid (3×10^{-3} M) at room temperature. Then, 100 μL of IBCA were added dropwise under vigorous magnetic stirring. After 3 h, the pH was adjusted to 7.0 with 1 M sodium hydroxide solution.

Anionic Emulsion Polymerization at pH 1 (AEP-pH 1). Dextran (0.1375 g) was dissolved in 8 mL of 0.2 M nitric acid (pH 1) at 40 °C and under magnetic stirring. After total dissolution, 0.5 mL of IBCA was added under vigorous stirring. The reaction was left to continue under magnetic stirring for 24 h. After cooling to room temperature, the pH was adjusted to 7.0 with 1 M sodium hydroxide solution.

Redox radical emulsion polymerization (RREP) was carried out according to the method described by Chauvierre et al.[4] Briefly, dextran (0.1375 g) was dissolved in 8 mL of nitric acid (0.2 M, pH 1) at 40 °C under gentle magnetic stirring and argon bubbling. After 10 min, 2 mL of a solution of cerium (IV) ammonium nitrate (8×10^{-2} M) in nitric acid (0.2 M) and 0.5 mL of IBCA were successively added under vigorous stirring. Argon bubbling was maintained during 10 min. The reaction was left to continue under gentle magnetic stirring for 50 min. After cooling to room temperature, 1.25 mL of an aqueous solution of 1.02 M trisodium citrate dihydrate was added to the polymerization medium. The pH was then adjusted to 7.0 with 1 M sodium hydroxide solution.

Purification of Samples. After polymerization and adjustment of pH to 7, all suspensions were purified by dialysis using a Spectra/Por membrane with a molecular weight cut off of 100 000 g/mol (Biovalley, Marne la Vallée, France) two times during 1 h 30 min and once overnight against 1 L of distilled water. It was checked that after dialysis no remaining free dextran could be found in the suspension.[15] The purified suspensions were stored at 4 °C until use or freeze-dried. For freeze-drying the suspensions were frozen at −18 °C and freeze-dried over 48 h (Christ Alpha 1-4 freeze-dryer, bioblock Scientific, Illkrich, France) without using cryo-protecting agent.

Composition of Nanoparticles. Dextran and PIBCA Contents. Nanoparticle composition in dextran and PIBCA was determined by elemental analysis and by ^{1}H NMR spectroscopy in DMSO-d_6.

Evaluation of the THF-Soluble Fraction. The THF-soluble fraction was recovered by dissolving 50 mg of the lyophilized nanoparticles in 5 mL of THF for 3 days at room temperature. The limpid solution was recovered and then filtered through a 0.2 μm diameter membrane filter (Millex FG, Millipore, Molsheim, France). The molecular weight was evaluated by size exclusion chromatography in THF (SEC-THF) or by Maldi-TOF. THF-insoluble fractions was recovered by filtration over a sintered glass filter no. 3 and dried residues were analyzed by ^{1}H NMR. The weighted ratio between the soluble part and the insoluble part was deduced from the amount of polymer introduced in THF and the amount of the insoluble fractions of polymer recovered.

Analysis of the Polymer Forming the Nanoparticles. Characterization of Polymer Solutions by Static Light Scattering. The light scattered at 90° by solutions of polymers dissolved in DMSO was measured at room temperature using an experimental device developed in the laboratory (He/Ne LASER 60 mW, wavelength 633 nm, photomultiplicator and correlator BI9000AT, Brookhaven instrument, Les Angles, France). Sample solutions were prepared in carefully washed glassware to remove all trace of dust and with filtered solvents. The lyophilized nanoparticles obtained from the RREP polymerization were dissolved in filtered DMSO at concentration ranging from 0.0128 to 1.77 mg/mL. The polymer was let to dissolve for 24 h and the solutions were filtered over an hydrophilic PTFE membrane (Millex LG pore size 0.20 μm, Millipore, Molsheim, France).

Recovery of the Dextran Part. The fraction of dextran included in the polymer forming the nanoparticles was recovered from lyophilized nanoparticles after degradation of the PIBCA part by basic hydrolysis. A sample of lyophilized nanoparticles (50 mg) was dispersed in 5 mL of 1 N sodium hydroxide at room temperature and let to hydrolyze (5 h 30 min) under magnetic stirring. The resulted limpid solution was dialyzed three times against 1 L of water (Spectra/Por membrane, MWCO 500 g/mol, Biovalley, Marne la Vallée, France). After dialysis, the high molecular weight compounds retained in the dialysis bag were recovered by lyophilization. To control that dextran was not affected by the hydrolysis method, the same procedure was applied to samples of dextran and of PIBCA. Size Exclusion Chromatography in aqueous medium (SEC-Aq) was used to determine the molecular weight of the remaining compound and composition was determined by ^{1}H NMR in D_2O.

Analysis of the PIBCA Part. The fraction of PIBCA included in the polymer forming the nanoparticles was recovered from lyophilized nanoparticles after degradation of the dextran part by acid hydrolysis. Lyophilized nanoparticles (70 mg) were suspended in 10 mL of hydrochloric acid 35% at 70 °C and incubated under magnetic stirring for 2 h. The remaining insoluble part was carefully recovered by filtration over a scintered glass filter no. 3 and extensively washed with water to remove any water-soluble compounds. The insoluble residue was then dried in an oven (Memmert, Serlabo, Bonneuil sur Marne) at 45 °C for 24 h. The composition of this fraction was analyzed by ^{1}H NMR in DMSO-d_6, and the molecular weight was evaluated by size exclusion chromatography in THF (SEC-THF).

Method of Analysis. Elemental Analysis. The composition in dextran and PIBCA of the different samples was determined by elemental analysis from their content in carbon, nitrogen, oxygen and hydrogen (Service Central d'Analyses du CNRS, Vernaison, France) as described in detail elsewhere.[4,12]

^{1}H NMR Spectroscopy. For the NMR analysis, ^{1}H NMR spectra of polymer solutions were recorded using a spectrometer 200 MHz, ARX 200 (Bruker, Wissembourg, France). The composition was deduced from the spectra by calculating the ratio between the integration of dextran peaks (4.4−5 ppm) and of the CH peak of PIBCA (1.8−2.2 ppm).[16] The solutions were prepared at a concentration of 25 mg/mL in DMSO-d_6 or D_2O. DMSO-d_6 was used to dissolve the lyophilized nanoparticles and the PIBCA fraction recovered after acid hydrolysis of dextran. D_2O served as solvent for the analysis of the dextran recovered from the basic hydrolysis of PIBCA in the nanoparticle samples.

Dosage of Dextran. The amount of dextran in the nanoparticles prepared by RREP was determined by the Anthrone coloric method.[17] First, 5 mg of lyophilized nanoparticles were dispersed in 10 mL of 0.1 M sodium hydroxide solution and homogenized during 10 min using ultrasounds. Then 100 μL of each solution was placed in a glass tube and let to cool in an ice bath. After 10 min, 1 mL of an anthrone (0.75% w/w) solution in sulfuric acid (84% in weight) was added in each tube. The tubes were placed in an oil bath at 110 °C for exactly 10 min. Then, the tubes were cooled in an ice bath during 30 min before reading the optical density at 616 nm (UV/vis spectrophotometer lambda II, Perkin-Elmer, Courtaboeuf, France). The concentration of dextran in each tube was determined using a calibration curve prepared with a series of dextran samples of known concentrations (0.02−0.18 g/L) which were treated by the same procedure. The percentage of dextran in the nanoparticles was calculated from this concentration.

Dosage of PIBCA. Amount of PIBCA was evaluated from the dosage of isobutanol released from the basic hydrolysis of the polymer by gas chromatography.[18] The column used was a capillary column Permabond-FFAP-DF0.1 (Lacheray-Nagel, Düren, Germany), vector gas was Helium at a flow rate of 1 mL/min at 45 °C and injection port and detector were kept at 250 °C. The oven was isothermal for 3 min at 45 °C, then raised from 45 to 150 °C at a rate of 10 °C/min, and kept constant at 150 °C for a further 4 min. Solution of *n*-pentanol in water (1.547 g/L) was used as internal standard.

To prepare the samples for the gas chromatography, 10 mg of lyophilized nanoparticles were hydrolyzed in 5 mL of NaOH (2 mg/mL) at room temperature under magnetic stirring during 24 h. The internal standard (250 μL), was added to 2 mL of sample, and 1 μL of this solution was injected into the gas chromatography system using the technique of hot needle.

Calibration was made by injection of solutions with known concentrations in isobutanol (0.0766−0.544 g/L) and constant concentration in *n*-pentanol (0.155 g/L). Calibration curve was obtained by plotting the ratios between the peak areas of isobutanol and pentanol obtained from calibration samples as a function of the ratios between concentrations of isobutanol and pentanol. A linear calibration curve was obtained with a R^2 of 0.9968.

Determination of Dextran Molecular Weight by SEC-Aq. Size exclusion chromatography in the aqueous phase (SEC-Aq) was used to determine the molecular weight of dextran. The SEC-Aq apparatus consisted of a TSK-G4000PW column (Toyo Soda, Tokyo, Japan), an Hitachi pump (model L-6000), an injection valve (Rheodyne) and two sample detectors: Hitachi multichannel detector (model L-3000) operating between 200 and 360 nm, and differential refractive index detector module (Waters R401). Data were recorded on line on a PC computer. The mobile phase consisted of a Hepes buffer (10 mM, pH 7.35, NaCl 145 mM) and was used at a flow rate of 1 mL/min. All samples were prepared at a concentration of 1 mg/mL in Hepes buffer, and the injected volume was 50 μL.

Calibration of the column was performed using dextran standards (Polymer Laboratories, Marseille, France) with a molecular weight ranging from 182 to 1 660 000 g/mol. The total volume of the column V_t, was evaluated from the peak of glucose (M = 180 g/mol). The effective void volume of the column, V_0, was taken as

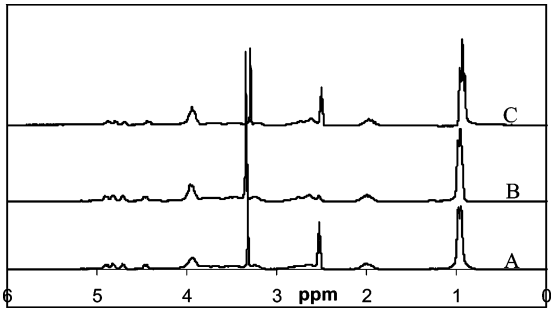


Figure 1. ^{1}H NMR spectra of copolymer made by (A) RREP, (B) AEP-pH 2.5, and (C) AEP-pH 1 in DMSO-d_6. Peaks from dextran: 4.4−5 ppm. Peaks from PIBCA: δ $2CH_3$, 1 ppm; δ CH, 2 ppm; δ CH_2, 2.5−3 ppm; δ O−CH_2, 4 ppm. Peak for DMSO: 2.5 ppm. Peak for H_2O: 3.4 ppm.

the elution volume given by the excluded standard of dextran (M_w 1 660 000 g/mol). The elution volume of the samples, V_e, was determined using EZChrom Elite, (Scientific Software Inc., Asnière sur Seine, France). The column parameter, K_d, was calculated using eq 1 and used to determined the weight-average molecular weight of dextran detected in the samples prepared as described above.

$$K_d = (V_e - V_0)/(V_t - V_0) \quad (1)$$

Determination of PIBCA Molecular Weight by Size Exclusion Chromatography. Molecular weight of products obtained from the acid hydrolysis of the nanoparticles was determined by size exclusion chromatography in THF (SEC-THF). Samples were prepared by dissolving 15 mg of the lyophilized polymer in 5 mL of THF. All samples were filtered before injection in the SEC-THF apparatus using a PTFE membrane (Millex FG, 0.2 μm, Millipore, France).

The SEC-THF apparatus included a guard column (GMH HR, Viscotek, Irigny, France) and two columns GMH HR_M (Viscotek, Irigny, France) maintained at 30 °C (Module TCM, Waters, Saint Quentin en Yvelines, France) during the analysis. Triple detection for SEC with right angle light scattering, viscometer (270 dual detectors, Viscotek Irigny, France) and differential refractometer (Module 410 Waters, Saint Quentin en Yvelines, France) was used to detect the eluted polymers. The mobile phase, THF, was used at a constant flow rate (1 mL/min) using a pump (VE 1121 pump, Viscotek, Irigny, France). The sample volume was 100 μL. The data were analyzed with the OmniSEC software version 3.1 (Viscotek, Irigny, France). The universal calibration of the columns was obtained from the analysis of six poly(styrene) standards with molecular weight ranging from 1270 to 354 000 g/mol and the elution time of toluene was 24.7 min.

Determination of PIBCA Molecular Weight by MALDI−TOF. Analysis of THF soluble part of polymers constituting the nanoparticles was made by MALDI−TOF (ICSN, Gif sur Yvette, France) using a MALDI−TOF mass spectrometer (Voyager-DE STR, Perspective Biosystems, Foster. City, CA), a 2,5-dihydroxybenzoic acid matrix and calibration with PEG 1000 g/mol.

This method allowed the determination of the molecular weight of small molecular weight homo-PIBCA. It was unsuitable for the analysis of products containing dextran which gave no signals and for the analysis of high molecular weight polymers.

3. Results

3.1. Composition of the Nanoparticles. The ^{1}H NMR spectra obtained from DMSO-d_6 solutions of all types of nanoparticles revealed signals of PIBCA (δ $2CH_3$, 1 ppm; δ CH, 2 ppm; δ CH_2, 2.5−3 ppm; δ O−CH_2, 4 ppm) and signals of dextran protons (4.4−5 ppm) (Figure 1). This indicated that the nanoparticles were composed of both dextran and PIBCA. As shown in Table 1, the percentage of PIBCA in the nanoparticles evaluated by the different methods agreed quite well with the content of PIBCA which was expected from the composition in IBCA monomer and dextran of the corresponding polymerization medium. This indicated that the composition of the nanoparticles was defined by the composition of the polymerization medium. The RREP and the AEP-pH 1 nanoparticles showed a closer composition whereas the AEP-pH 2.5 nanoparticles contained a slightly lower percentage of PIBCA.

Table 1. Percentage in Weight of PIBCA in Nanoparticles by Elemental Analysis, ^{1}H NMR, and Dosage of PIBCA and Dextran:

method of polymerization	RREP	AEP-pH 2.5	AEP-pH 1
^{1}H NMR	72 ± 5	67 ± 5	70 ± 5
elemental analysis	81 ± 8	66 ± 6	77 ± 7
PIBCA dosage	77 ± 7	nd	nd
dextran dosage	82 ± 8	nd	nd
expected from polymer composition medium	77	64	77

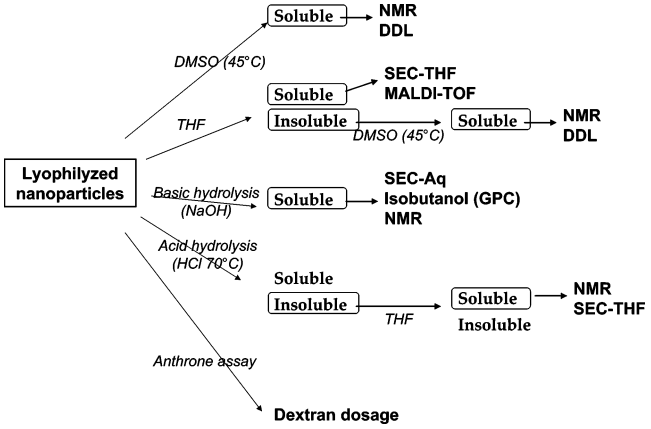
Only part of the nanoparticles could be dissolved in THF. The insoluble weight fraction represented 21% of the total amount of polymer of the RREP nanoparticles, 26% of the nanoparticles prepared by AEP-pH 2.5 and 25% of the nanoparticles prepared by AEP-pH 1. The content in PIBCA in these insoluble fractions was respectively 22%, 5%, and 8.9% as determined from the ^{1}H NMR spectra obtained from DMSO-d_6 solutions. The PIBCA content of the THF insoluble fraction was much lower than the PIBCA content of the whole nanoparticles indicating that the nanoparticles were formed by polymers of heterogeneous composition. These values also suggested that the composition of the polymers varied with the method of synthesis. It can be deduced that more than 94% in weight of the total amount of PIBCA which formed the nanoparticles was included in the fraction of the polymer which was soluble in THF.

The ^{1}H NMR spectra of the THF-soluble polymer which dissolved in THF confirmed that PIBCA was the major component of these fractions. Peaks of dextran found for the polymers dissolved in this fraction were modified and shifted to higher values indicating that the polysaccharide parts of the polymers were modified during the polymerization reaction.

3.2. Characterization of the Polymers. The main problem encountered for the characterization of polymers which formed the nanoparticles was due to their solubility behavior. Indeed, nanoparticles could only be completely dissolved in hot DMSO while they were totally insoluble in water and only partly soluble in THF. Thus, attempts were made to characterize DMSO solutions obtained by the dissolution of RREP nanoparticles by light scattering and to evaluate the molecular weight of the polymers which dissolved in THF by SEC-THF and MALDI−TOF (Scheme 2). A further analysis of the polymers was performed after the nanoparticles were selectively hydrolyzed to recover either their dextran or their PIBCA content as illustrated in Scheme 2. The content of all isolated fractions were identified by ^{1}H NMR and the molecular weights were estimated by the appropriate SEC method.

3.2.1. Light Scattering Given by Nanoparticles Dissolved in DMSO. Figure 2 shows the intensity of the light scattered at 90° by solutions of RREP nanoparticles dissolved in hot DMSO. The graph clearly presented two linear parts with an intercept at a concentration of 1.1 mg/mL. Below the intercept of the lines, the intensity of the signal slightly increased with increasing concentrations of dissolved nanoparticles while beyond this point the increase in the signal intensity was more pronounced. This indicated a polymer chain associations which led to larger aggregates.

Scheme 2. Methodology Used for Characterization of Polymers Obtained by RREP, AEP-pH 2.5 and AEP-pH 1



3.2.2. Characterization of the Polymer Soluble in THF. As mentioned above, polymer fractions from nanoparticles which dissolved in THF contained both PIBCA and dextran but PIBCA content was predominant in all preparations. The molecular weights of the polymers were analyzed by SEC-THF and MALDI−TOF. While the SEC-THF analysis allowed to reveal the molecular weight distribution of all the polymer species present in the sample to be analyzed, MALDI−TOF could only be used to analyze homo-PIBCA. Indeed, polymers containing both PIBCA and dextran were not detected by this last method.

The SEC-THF chromatogram of the THF-soluble polymers obtained by RREP showed a single broad peak with elution time ranging from 13.5 to 22.6 min (Figure 3A). The corresponding molecular weights were 61 500 (M_n) and 130 200 (M_w) g/mol with a polymolecular index M_w/M_n of 2.1 (Table 2). As revealed by the SEC-THF chromatogram, this polymer sample did not contain low molecular weight compounds. By MALDI−TOF, no signal could be detected suggesting that the polymer sample did not contain free homo-PIBCA which was the only species in our samples which could be detected by this technique.

THF-soluble polymer from the nanoparticles prepared by AEP-pH 2.5 showed a chromatogram with a major peak appearing at higher elution time (18.82−22.88 min) indicating that the polymer chains were much shorter than those produced by the previous polymerization method (Figure 3B). Molecular weights deduced from the universal calibration curve were much lower (M_n 13 400 g/mol) than what could be expected from a copolymer containing dextran (49 500 g/mol) and PIBCA (Table 2). The big difference observed may be due to the fact that the calibration curve used was drawn for homopolymers in good solvent and that the analyzed polymer corresponded to an amphiphilic hydrophobized dextran. In THF, the dextran part of such a copolymer may be collapsed or the amphiphilic copolymer may adsorb on the column changing the separation mode from size exclusion to absorption. Both of these phenomena result in the determination of an apparent lower molecular weight then expected. On the right of the major peak, a small peak of elution appeared showing retention times ranging from 22.9 and 24.2 min. This peak corresponded to polymer chains with a low molecular weight. The MALDI−TOF analysis of this sample showed a single peak including a series of signals with a space corresponded exactly to the molecular weight of the IBCA monomer (153 g/mol). The molecular weights of the polymers detected by MALDI−TOF are given in Table 2. The molecular weight of all major signals corresponded to the composition given in eq 2, indicating that the chemical species detected by MALDI−TOF corresponded to short chains of homo-PIBCA which were not linked to dextran and resulted from a polymerization of isobutylcyanoacrylate initiated by water.

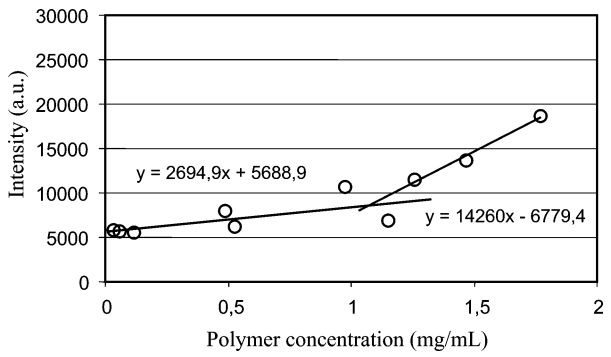


Figure 2. Measurement of 90° light scattering intensity from solutions of copolymers prepared by RREP in DMSO.

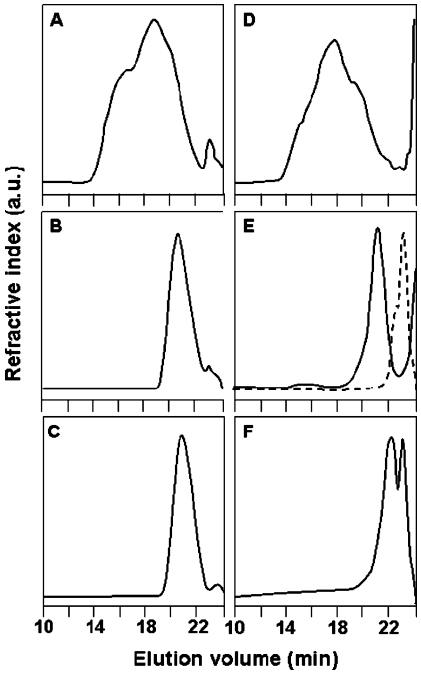


Figure 3. Chromatograms given by SEC-THF analysis (RI signal) of the fraction of polymer forming the nanoparticles soluble in THF (A−C) and of the polymer isolated after 2 h (black line) or 14 h (dashed line) of acid hydrolysis of the nanoparticles (D−F). Samples from the RREP nanoparticles (A, D), AEP-pH 2.5 nanoparticles (B, E), and AEP-pH 1 nanoparticles (C, F).

Table 2. Molecular Weight Obtained for THF-Soluble Part of Polymer and Fraction Recovered from Acid Hydrolysis by SEC-THF or MALDI−TOF[a]

		M_n (g/mol)	M_w (g/mol)	I_p
THF soluble	RREP	61 500	130 200	2.1
	AEP-pH 2.5	1157[a]	1313[a]	1.1
		13 400	28 500	2.12
	AEP-pH 1	1174[a]	1293[a]	1.1
		16 800	25 900	1.5
after hydrolysis	RREP 2 h	46 100	139 900	3.3
	AEP-pH 2.5 2 h	18 600	28 400	1.5
	14 h	1285	1740	1.3
	AEP-pH 1 2 h	3770	7231	1.9

[a] MALDI-TOF.

$$M \text{ measured} = M \text{ polymer} + M_{Na} = nM_{IBCA} + M_{H_2O} + M_{Na} \quad (2)$$

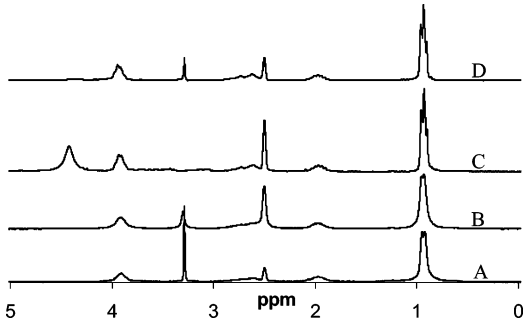


Figure 4. ^{1}H NMR spectra in DMSO-d_6 of polymer after acid hydrolysis: (A) homo-PIBCA (control); (B) polymer made by RREP; (C) polymer obtained by AEP-pH 2.5; (D) polymer made by AEP-pH 1.

With n being the number of IBCA residues in the chain, M_{IBCA} = 153 g/mol, M_{Na} = 23 g/mol and M_{H_2O} = 18 g/mol.

Thus, it can be calculated that the number of IBCA residues of these PIBCA chains varied from 3 to 15. The maximum of the peak corresponded to a chain of 7 IBCA residues. These short PIBCA chains can be attributed to the small elution peak appearing at the highest retention times on the SEC-THF chromatogram.

The THF-extracted polymer constituting the nanoparticles prepared by the AEP-pH 1 method showed a SEC-THF elution profile (Figure 3C) very similar to the profile given by the polymers recovered from the AEP-pH 2.5 nanoparticles. In addition, the major peak ranged roughly within the same retention time interval (19.2−23.2 min), indicating no drastic influence of the pH of the polymerization medium on the polymer molecular weight. MALDI−TOF analysis also revealed the presence of PIBCA short chains (Table 2). At the maximum of the peak, the PIBCA chains extracted from the AEP-pH 1 nanoparticles were composed of 8 IBCA residues.

3.2.3. Characterization of the PIBCA Part Recovered after Acid Hydrolysis. The chemical structure of the residue obtained after acid hydrolysis which removed the dextran part of the polymer, was determined by ^{1}H NMR (Figure 4). Figure 4A gives a reference ^{1}H NMR spectrum obtained by the analysis of homo-PIBCA.

Acid hydrolysis of nanoparticles prepared by the RREP method provided a residue totally soluble in THF. Only signals corresponded to PIBCA appeared on ^{1}H NMR spectra (Figure 4B). The SEC-THF analysis showed a broad peak with elution times ranging from 13.4 to 22.7 min corresponding to number and weight-average molecular weights M_n 46 100 and M_w 139 900 g/mol, respectively (Figure 3D, Table 2). The molecular weight distribution of these polymers was broader with a slight shift of the distribution toward lower molecular weight compared to the nonhydrolyzed polymer fraction which was soluble in THF (Figure 3, parts A and D, and Table 2).

Polymers recovered after acid hydrolysis of the AEP-pH 2.5 nanoparticles were partly soluble in THF and in DMSO. ^{1}H NMR spectra showed signals given by PIBCA and a peak at 4.4 ppm which may be attributed to some remaining of degraded dextran (Figure 4C). After 2 h of hydrolysis the chromatogram profile presented one peak with the retention time close to nonhydrolyzed THF-soluble polymers (Figure 3E). There was still an insoluble fraction of polymer in the sample. After 14 h of hydrolysis, the SEC-THF chromatogram of the part of polymer which solubilized in THF showed a dissymmetrical peak with a shoulder at 21.6 min and a maximum at 23.1 (Figure 3E). The molecular weight of the corresponding polymers was much lower than that of the polymer found in the nonhydrolyzed THF-soluble fraction (Table 2).

Polymers recovered after 2 h of acid hydrolysis of the AEP-pH 1 nanoparticles were totally soluble in THF. ^{1}H NMR analysis showed a signal attributed to PIBCA and a little additional signal at 4.4 ppm which may be due to remaining degraded dextran (Figure 4D). SEC-THF chromatogram showed a double peak with maxima at 22.1 and 23.1 min (Figure 3F). This sample contained polymers of low molecular weight (Table 2).

3.2.4. Characterization of the Dextran Fraction Recovered from Basic Hydrolysis. To analyze the dextran part of the polymer forming the nanoparticles, the PIBCA was removed by basic hydrolysis. The action of sodium hydroxide on PIBCA is of 2-folds. It may induce an extremely rapid depolymerization−repolymerization reaction producing short chains of PIBCA (Scheme 3A).[19] NaOH may also hydrolyze the ester bonds of the isobutylcyanoacrylate residues producing water-soluble molecules including isobutanol and short fragments of poly(cyanoacrylic acid) (Scheme 3B).[3,18] After hydrolysis, the water-soluble compounds of molecular weight higher than 500 g/mol and corresponding to the molecular weight cutoff of the dialysis membrane were analyzed both by ^{1}H NMR and SEC-Aq. Dextran used for the preparation of the nanoparticles was the reference for the ^{1}H NMR spectra and for the SEC-Aq analysis. After basic hydrolysis, the K_d of dextran was not significantly different from the K_d measured for the untreated dextran indicating that the method of basic hydrolysis did not altered the dextran molecule.

The ^{1}H NMR spectra obtained from the polymer recovered from basic hydrolysis of RREP nanoparticles showed dextran peaks and a signal at 2.4 ppm which can be attributed to CH_2−CN groups of PIBCA backbones (Figure 5B). No signal of isobutyl ester function was detected (Figure 5A**)**. Chromatograms obtained by SEC-Aq exhibited 2 peaks attributed to dextran (K_d: 0.529) and to the hydrolyzed and depolymerized PIBCA (K_d: 0.779) (Table 3). Dextran extracted from polymer made by RREP had very similar molecular weight than the reference dextran treated in the same conditions (Table 3).

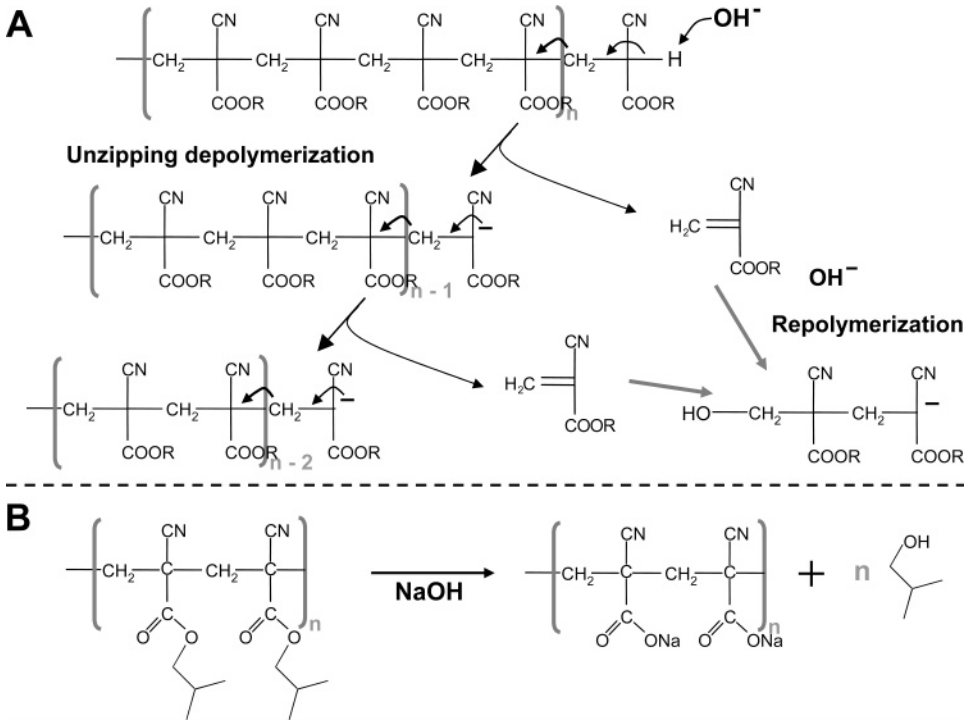
In the case of polymer obtained from the hydrolysis of AEP-pH 2.5 nanoparticles, the ^{1}H NMR spectra showed the peaks attributed to dextran (Figure 5C). The SEC-Aq chromatogram showed one peak with a K_d of 0.478 corresponding to the elution of dextran of apparently higher molecular weight than the dextran actually used for the preparation of the nanoparticles (Table 3).

The basic hydrolysis of the AEP-pH 1 nanoparticles led to the recovery of a compound which showed roughly the same characteristics than the polymer obtained after hydrolysis of AEP-pH 2.5 nanoparticles (Figure 5D, Table 3).

4. Discussion

As indicated by the composition of the nanoparticles, all the monomer introduced in the polymerization medium was incorporated in the nanoparticles suggesting that polymerizations were completed. All types of nanoparticles contained both PIBCA which is hydrophobic and dextran which is hydrophilic. The difficulty encountered to dissolve the nanoparticles indicated that they contained PIBCA-dextran copolymers. The fact that part of the polymer was soluble in THF suggested that the nanoparticles were composed of a heterogeneous population of copolymers with some being more hydrophobic (i.e., soluble in THF) and others being more hydrophilic (i.e., insoluble in THF). The ^{1}H NMR analysis confirmed that the most hydro-

Scheme 3. Hydrolysis of PIBCA by Reaction with NaOH[a]



[a] Key: A, depolymerization−repolymerization; B, hydrolysis of ester function.

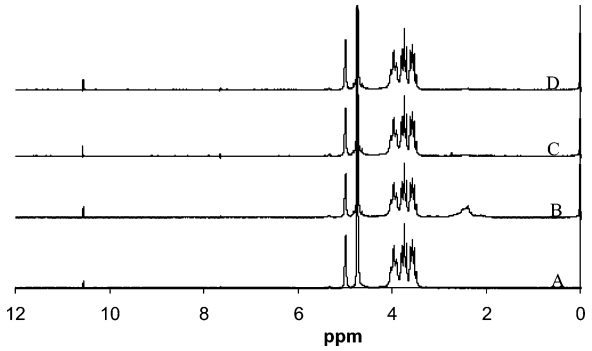


Figure 5. ^{1}H NMR spectra in D_2O of polymer hydrolyzed by NaOH 1 N: (A) dextran (control). Polymer made by (B) RREP, (C) AEP-pH 2.5, and (D) AEP-pH 1.

Table 3. Column Parameter (K_d) and Molecular Weight of Compounds Obtained after Basic Hydrolysis and Measured by SEC-Aq

	K_d	mol wt (g/mol)
dextran	0.518 ± 0.008	49500 ± 2000
dextran NaOH	0.524 ± 0.008	47800 ± 2000
dextran/PIBCA NaOH	0.529 ± 0.008	46300 ± 2000
RREP	0.529 ± 0.008	46300 ± 2000
	0.779 ± 0.008	5600 ± 200
AEP-pH2.5	0.478 ± 0.008	62000 ± 3000
AEP-pH1	0.495 ± 0.008	56000 ± 3000

phobic fraction was enriched in PIBCA whereas dextran was the main component of the most hydrophilic fraction. The THF-soluble/insoluble balance of polymers depended on the method of polymerization. In addition, a small amount of short chains of homo-PIBCA were found in the nanoparticles prepared by either AEP-pH 2.5 or AEP-pH 1. The polymerization which led to the formation of the homopolymer was initiated by the water molecules. In contrast, the nanoparticles prepared by RREP did not contain any detectable amount of homo-PIBCA. This result can be explained by a difference in the kinetic of polymerization which was extremely fast in the RREP conditions.[4] Thus, in the presence of cerium, the polymerization was preferentially initiated on the radical created on the dextran chains. Therefore, the monomer was consumed before it could react with the small amount of hydroxyl ions of the acidified water to form homo-PIBCA by an anionic polymerization initiated by the water. In the absence of cerium, the two competing initiators (i.e., hydroxyl ion of the water and hydroxyl groups of dextran) can both initiate an anionic polymerization which kinetics mainly differ from the reactivity of the initiators. In this case, the consumption of the monomer will be partitioned between chains initiated by each initiator depending on their reactivity and on the pH of the polymerization medium which define the concentration in hydroxyl ions of the water.

Differences between the polymerization methods appeared more clearly considering the analysis of each parts of copolymers. They were obtained after selective hydrolysis of either PIBCA or dextran.

The acid hydrolysis of the nanoparticles prepared by RREP provided PIBCA as indicated by the ^{1}H NMR spectrum. The molecular weight of PIBCA was high, with a very broad distribution. This corresponded to a general characteristic of polymers obtained under uncontrolled radical polymerization reactions and is in agreement with the preparation conditions used in the present study. Indeed, the emulsion polymerization occurred in less than 10 min. No particular efforts were made to control the progress of this radical polymerization unless it occurred faster than a competing anionic polymerization which could be only slightly delayed in our experimental conditions.[20] Formation of very high molecular weight PIBCA chains (M_w 139 900 g/mol) can be explained by a termination of the radical polymerization by a recombination mechanism. However, according to Topp et al.,[5] once this kind of redox radical polymerization is initiated, micelles of copolymer are formed and reaction continues according to a quasi living polymerization mechanism implying that radicals can survive in the micelle core. This mechanism, which is in contrast with the hypothesis based on termination of polymer chains by recombination,

agrees with the fact that radical recombination may be difficult in the case of IBCA polymerization. Indeed, the radical bearing carbon atom of the PIBCA growing chain is substituted by both a nitrile and an isobutylester group creating a steric hindrance which may prevent radical recombination. The basic hydrolysis of the nanoparticles gave a mixture of dextran and of a low molecular weight water-soluble degradation product of PIBCA as shown by the presence of two distinct peaks of elution in the SEC-Aq chromatograms. According to the SEC-Aq analysis, the molecular weight of dextran was not significantly reduced by the treatment with cerium during the initiation stage of the polymerization. This was in agreement with previously reported data.[4] The SEC-Aq analysis did not show the presence of a small molecular weight dextran chain which can be expected to be found in the nanoparticles because of the mechanism of the polymerization initiation (Scheme 1). This suggested that only the longer fragment of dextran, which resulted from the splitting of the dextran chain by cerium, could initiate the polymerization and was therefore included in the nanoparticles. The small fragment of dextran which remained soluble in the polymerization medium could be eliminated by dialysis. From the molecular weight of the dextran (46 300 g/mol) and the molecular weight determined for the PIBCA found in the nanoparticles (139 900 g/mol) and taking into account the mean compositions in dextran and PIBCA found in the nanoparticles, it can be calculated that the number of PIBCA chains per dextran molecule is equal to 1.2. This simulation, provided a ratio very close to 1 but did not take into account the broad distribution of the PIBCA molecular weight. However, since the dominating termination mode for acrylates during radical polymerization is disproportionation, such value could be expected and hence, mainly diblock copolymers should be formed. Thus, our result strongly supports the hypothesis that the copolymer synthesized by the RREP method corresponded to a diblock copolymer of PIBCA and dextran.

Compounds resulted from the selective hydrolysis of the nanoparticles obtained by the anionic emulsion polymerization performed either at pH 2.5 (AEP-pH 2.5) or at pH 1 (AEP-pH 1) led to a slightly modified PIBCA after acid hydrolysis. The new signal showed on the ^{1}H NMR spectra can be attributed to the presence of a degradation product of dextran remaining at the chain end of PIBCA where the initiation of the polymerization took place. The molecular weight of the PIBCA chains were low and the size distribution was better defined than in the case of the RREP. They were lower than those of the polymer which dissolved in THF directly from the nanoparticles before degradation of dextran by acid hydrolysis. This means that the hydrophobic polymer of high molecular weight detected in the THF-soluble fraction of the nanoparticles consisted of dextran which was made hydrophobic by linkage of low molecular weight PIBCA chains. The PIBCA chains which were formed at pH 1 showed a higher molecular weight than the PIBCA chains which were formed at pH 2.5. This is also in agreement with the anionic polymerization mechanism because at pH 2.5, the acid proton of the hydroxyl groups of dextran can be more labile than at pH 1. So there can be more initiation groups for the anionic polymerization of IBCA on dextran at pH 2.5 compared to pH 1 leading to the formation of lower molecular weight PIBCA chains and in turn to a higher degree of substitution of the dextran chain. Because of this effect, a more acid polymerization medium should lead to longer PIBCA chains in agreement with what was observed. ^{1}H NMR spectra of the dextran part did not reveal any chemical modification of dextran after hydrolysis of the PIBCA part. However, the dextran molecules showed an apparent higher molecular weight as determined by the SEC-Aq analysis. The increase of the molecular weight could be explained by the introduction of charges on the dextran molecules but this disagreed with the results of the ^{1}H NMR analysis. Nevertheless, considering the hydrolytic reaction used to degrade PIBCA, a few cyanoacrylic acid residues may still remain attached to dextran where PIBCA chains were anchored. These residues may bring enough charges to dextran, which behaved like a molecule larger than the original one when analyzed by SEC-Aq (Hepes buffer, pH 7.35). To explain the fact that such a modification of dextran did not appear on the ^{1}H NMR spectra, it can be proposed that only a low number of hydroxyl groups of glucose residues per dextran chains were actually modified and that no specific proton were created. From the molecular weights of PIBCA chains and dextran and from the mean composition of nanoparticles, it can be calculated that copolymers formed by polymerization AEP pH 2.5 was composed of 47 PIBCA chains per dextran. In the AEP pH 1 polymerization conditions the copolymers were composed of 21 PIBCA chains per dextran. This simulation slightly overestimated the number of PIBCA chains per dextran because it did not take into account the homo-PIBCA detected by MALDI−TOF. However, it supported the hypothesis that several rather defined low molecular weight PIBCA chains were grafted onto a single dextran molecule during the preparation of the nanoparticles by both AEP pH 2.5 and AEP pH 1 methods. It also supported the hypothesis that pH can control the degree of grafting of the dextran chain.

5. Conclusion

This study showed that the emulsion polymerization of IBCA performed in acidic aqueous solutions containing a polysaccharide led to the formation of nanoparticles which were composed of heterogeneous polymers. The structure of copolymers including hydrophobic and hydrophilic moieties could be investigated by the selective degradation of one part and characterization of the remaining counterpart. The anionic emulsion polymerizations led to the simultaneous formation of homo-PIBCA and of copolymers of several short PIBCA chains grafted onto dextran. In contrast, diblock copolymers were found in nanoparticles obtained by RREP in which PIBCA chains were characterized by a high molecular weight and a broad distribution. Results obtained in this study agreed with hypothesis suggested about the corresponding mechanisms of polymerizations. They confirmed that the structure of polymers forming nanoparticles prepared by the two types of polymerization (i.e., anionic or redox radical) were different. This can explain the different behaviors which were observed when the nanoparticles were interacting with serum proteins.[12,13] In the view of in vivo applications, further developments of the nanoparticles obtained by RREP will require a better control of the polymerization reaction to reduce the molecular weight and the polydispersity of the PIBCA chains.

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