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Near Infrared with Principal Component Analysis as a Novel Analytical Approach for Nanoparticle Technology

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Purpose. To progress in the characterization of a poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) (poly(PEGCA-co-HDCA) copolymer and the nanoparticles formed from this copolymer.

Methods. Poly(PEGCA-co-HDCA) at a MePEG/hexadecyl ratio of 1:4 was investigated by ^{1}H-NMR and near infrared spectroscopy. The nanoparticle suspensions, obtained by the methods of nanoprecipitation or emulsion—solvent evaporation, as well as the crude nanoparticles and their dispersion medium—were analyzed by MePEG measurement, ^{1}H-NMR, and near infrared spectroscopy.

Results. The ^{1}H-NMR results showed that the (poly(PEGCA-co-HDCA) copolymer obtained bore lateral hydrophilic MePEG chains and lateral hydrophobic hexadecyl chains in a final ratio of 1:4. However, this ratio, although reproducible from batch to batch, represented only a mean value for different molecular species. Indeed, our results demonstrated the formation of more hydrophobic poly(alkylcyanoacrylate) oligomers (with a higher content of hexadecyl chains) and other more hydrophilic oligomers (with a higher MePEG content). Only the more hydrophobic oligomers were able to form solid pegylated nanoparticles. As far as these nanoparticles were concerned, determination of their MePEG content allowed the calculation of a distance of 1.2 nm and 1.05 nm between 2 grafted MePEG chains at the nanoparticle surface, when obtained by nanoprecipitation and emulsion–solvent evaporation, respectively. Moreover, when the same copolymer batch was used, different nanoparticles were obtained according to the preparation method, as seen by near infrared spectroscopy.

Conclusions. The nanoparticles obtained by nanoprecipitation or emulsion–solvent evaporation of poly(PEGCA-co-HDCA) 1:4 copolymer displayed a different supramolecular organization, as evidenced by the near infrared spectroscopy results. Moreover, these nanoparticles showed surface characteristics compatible with a long circulating carrier.

KEY WORDS: amphiphilic copolymer; nanoparticles; near infrared spectroscopy; poly(ethylene glycol); poly(alkylcyanoacrylate); principal component analysis.

INTRODUCTION

The near infrared (NIR) energy spectral range, as defined by IUPAC, extends from 12800–4000 cm^{-1}, between the middle infrared (mIR) and the visible region of the spectrum (1). The absorption bands observed in NIR spectra are mainly due to overtones of hydrogen-stretching vibrations or to combinations of stretching and bending modes of vibration (2). These bands are therefore broader than in the mIR spectra and are considerably more complex. Thus, unlike mIR where spectral variations can be assigned to functional groups, NIR cannot be used as a single method for structural analysis (3). Sophisticated methods of data treatment such as Principal Component Analysis (PCA) are necessary to extract the information contained in NIR spectra (3). However, since the absorption in the NIR region is much weaker than in the mIR region, NIR spectra can be obtained from direct measurement on samples without dilution. This also makes the diffuse reflection of solid samples amenable to direct analysis (4,5). Finally, as the reflectance of solid samples varies with the concentration, the absorptivity, and the scattering coefficient, according to the Kubelka–Munk theory, the NIR spectrum of a solid material depends on both its chemical composition and its physical properties, such as particle size and surface characteristics (3,5). For this last reason, NIR spectroscopy, followed by PCA, would be an interesting and useful technique for nanoparticle analysis. Such analysis has not yet been undertaken.

In this study, NIR spectroscopy has been applied to both the characterization of the poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) (poly(PEGCA-co-HDCA) copolymer and the nanoparticles prepared from this material (6,7). Poly(PEGCA-co-HDCA) nanoparticles are colloidal drug carriers which have shown efficient protein-rejecting properties *in vitro* (8), enhanced blood-circulation time, and reduced liver accumulation *in vivo* compared with nonpegylated poly(hexadecylcyanoacrylate) (PHDCA) nanoparticles (9). Although these nanoparticles are efficiently sterically stabilized, the physico-chemical properties of the poly(PEGCA-co-HDCA) copolymer of which they are constituted have not been completely characterized. In particular, although it is well accepted that the copolymer has a cyanoacrylic backbone with hydrophilic MePEG and hydrophobic hexadecyl side chains, it is still unclear whether the material is composed of one molecular species or whether it contains a mixture of different molecular entities. In this case, because of the amphiphilic nature of the copolymer, it is not clear whether all the different macromolecular species forming the copolymer sample participate in nanoparticle formation. Therefore, the reproducibility of MePEG-coated nanoparticles—an important requirement for efficient tumor targeting—can be questioned.

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ABBREVIATIONS: DCC, dicyclohexylcarcodiimide; DMA, dimethylamine; DMAP, 4-(dimethylamino)pyridine; MePEG, methoxypoly(ethylene glycol); mIR, middle infrared; MPS, mononuclear phagocyte system; MSC, multiplicative scatter correction; NIR, near infrared; OD, optical density; PC, principal components; PCA, principal component analysis; PEG, poly(ethylene glycol); PHDCA, poly(hexadecylcyanoacrylate); PLA, poly(lactic) acid; Poly(PEGCA-co-HDCA), poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate); PPEGCA, poly(MePEGcyanoacrylate)

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Using NIR spectroscopy as well as ^{1}H-NMR and MePEG measurement, we have answered these questions and characterized in detail the poly(PEGCA-co-HDCA) copolymer and the nanoparticles formed from it.

MATERIALS AND METHODS

Materials

Materials used for the preparation of the hexadecyl cyanoacetate and MePEG cyanoacetate monomers, the PHDCA and poly(MePEGcyanoacrylate) (PPEGCA) polymers, and the poly(PEGCA-co-HDCA) copolymer are described elsewhere (6), except for the catalyst of the copolymerization where dimethylamine (DMA) was replaced by pyrrolidine (Fluka, Buchs, CH). Solvents and reagents used in the different synthesis were pure.

Preparation of nanoparticles by nanoprecipitation (10) or emulsion–solvent evaporation (11) was performed with acetone and methylene chloride of analytical grade.

Synthesis and Purification of the Monomers, the Poly(PEGCA-co-HDCA) Copolymer and the PHDCA and PPEGCA Polymers

Preparation of the Monomers

The cyanoacetate esters were prepared by esterification of the cyanoacetic acid with hexadecanol or $MePEG_{2000}$, in the presence of both 1,3-dicyclohexylcarbodiimide (DCC) as coupling agent and 4-(dimethylamino)pyridine (DMAP) as catalyst (6). An additional purification step was added to the synthesis, as described below.

For the synthesis of hexadecyl cyanoacetate, cyanoacetic acid (88 mmol) and hexadecanol (44 mmol) were dissolved in methylene chloride (50 ml) and ethyl acetate (5 ml). The mixture was cooled to 4°C. DMAP (catalytic amount) was added, followed by DCC (48.4 mmol) as a solution in methylene chloride (50 ml). The reaction was carried out under stirring at room temperature, under nitrogen. After 24 hours, hexane (50 ml) was added and the solid was filtered off and washed with more hexane. The combined filtrates were concentrated under reduced pressure. The crude ester was purified by chromatography on silica gel (Merck silica gel 60 (230-400 mesh ASTM), Darmstadt, Germany), using ethyl acetate–hexane 1:5 as eluent. After drying under vacuum, the purified hexadecyl cyanoacetate was obtained as a white amorphous solid.

For the synthesis of MePEG cyanoacetate, the same procedure was followed, except for the amounts of cyanoacetic acid (22 mmol) and DCC (22 mmol); hexadecanol was replaced by $MePEG_{2000}$ (11 mmol). The purification step consisted in recrystallization of the crude ester from isopropanol.

Preparation of the Poly(PEGCA-co-HDCA) Copolymer

The copolymer was synthesized as previously described by Peracchia *et al.* (6) with some modifications, at a MePEG to hexadecyl ratio of 1:4. The reaction is based on the condensation/polymerization of MePEG cyanoacetate (1 mmol) and hexadecyl cyanoacetate (4 mmol) which were both dissolved in pure ethyl alcohol (10 ml) and methylene chloride (20 ml). Formaline (25 mmol) and pyrrolidine (1.2 mmol) were then added and the reaction mixture was stirred at room temperature, under nitrogen. After 24 h, organic solvents were eliminated under reduced pressure and the residue was mixed with water and extracted 3 times with methylene chloride. The combined organic phases were then purified by washing with HCl 1N and 2 times with water. Finally, the organic phase was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The poly(PEGCA-co-HDCA) copolymer was obtained as a yellow waxy compound.

Preparation of the PHDCA and PPEGCA Polymers

These polymers, used as control, were also obtained by a condensation/polymerization reaction of the corresponding monomers. For PHDCA, hexadecyl cyanoacetate (10 mmol) was dissolved in pure ethyl alcohol (30 ml) and methylene chloride (5 ml). Formaline (15 mmol) and DMA (3 mmol) were added. The reaction and the purification procedure were then carried out as described before for the copolymer. For PPEGCA, the reaction involved MePEG cyanoacetate (1 mmol), formaline (1.5 mmol) and DMA (1.5 mmol) under the same conditions as described for PHDCA. After 24 h., the solvent was eliminated and the crude polymer was washed with ether before drying under vacuum.

Measurements

^{1}H-NMR

The ^{1}H-NMR (200MHz) spectra were recorded on a Bruker AC 200P spectrometer, after dissolution of the samples in $CDCl_3$. Measurements were performed on the monomer, polymer, and copolymer samples and also on the aqueous washing extracts of the copolymer.

Near Infrared Analysis

Near infrared analysis was performed on a Bühler–Nirvis interferometer based spectrometer with a 2 m optical fiber. Calculations were done using Nircal V3 (Bühler–Anatec, Uzwil, CH). Reflectance of the samples, in powder form, was recorded through the transparent glass wall of a flask in the near infrared region of 4008–9996 cm^{-1}, at 12 cm^{-1} full resolution. In order to appreciate measurement variability, five spectra were recorded for each sample. The number of batches of compounds assayed varied from 1 to 6.

Near Infrared: Qualitative Discriminant Analysis

The transformed NIR spectra were treated by PCA. The objective of PCA is to modelize the variability of each class of compounds and to represent each class as a unique cluster made up of the spectral data (3).

The principle of PCA calculation is to find new variables other than wavelength to describe the spectra. Considering the 500-dimension space where spectra are represented by their reflectance value at each wavelength, forming a cloud extending in several directions, PCA calculation will lead to the computation of a new axis that allows the description of this cloud starting by its main direction. After computation of this first axis (which corresponds to the first loading), and removal of its direction, the second main direction is described by a new axis and so on, until the remaining amount

of spectral information is of the same order of magnitude as the background noise of the apparatus. Loadings are thus independent variables—not correlated between each other—describing the wavelength space on the basis of the main cause of variance in spectra. So, spectra are characterized by the reflectance at each wavelength in the wavelength space, and by their score on each loading in the PC space. By plotting the 2 or 3 first loadings, similar spectra will cluster in the same region of this space, whereas compounds with spectral differences will cluster in other parts of this space (3).

Finally, in order to select the minimum number of PCs needed to model spectra without any loss of valuable spectral information, one part of the spectra (2/3) was used to build the model (calibration set) while the rest (1/3) (validation set) was used to test its validity with regard of unknown samples.

Near Infrared Spectrum Transformation

Spectral transformation is usually applied to spectra before PCA, in order to minimize the intra-class variability of a compound and to focus on inter-class variability (3). The goal of this transformation is to correct the variability arising from particle size and/or from measurement-to-measurement reproducibility. For polymer analysis, spectrum transformation was carried out by applying Multiplicative Scatter Correction (MSC), a special function designed to minimize the effect of light scattering (12).

Preparation of the Nanoparticles

Nanoparticles were prepared by the methods of nanoprecipitation (10) and emulsion–solvent evaporation (11).

Nanoprecipitation

30 mg of poly(PEGCA-co-HDCA) was dissolved in acetone (6 ml) under magnetic stirring and gentle warming. This organic phase was poured through a syringe into stirred water (12 ml); nanoparticle precipitation occurred instantaneously. Acetone was then evaporated by Rotavapor®. The suspension was finally filtered on a sintered glass membrane (Millex® AP 20, Millipore).

Emulsion–Solvent Evaporation

20 mg of poly(PEGCA-co-HDCA) dissolved in methylene chloride (2 ml) was pre-emulsified in water (30 ml) by vortexing (1 min). The coarse o/w emulsion was then sonicated 3 min. (Sonics & Materials Inc., Danbury, USA) and immediately subjected to a high-pressure homogenizer (Microfluidizer Sodexim S.A., Watts Fluidair, USA), operating at 4 bars (5 min, in an ice bath). The organic solvent of the emulsion was then evaporated under stirring at room temperature and the resulting nanoparticle suspension was filtered through a sintered glass membrane (Millex® AP 20, Millipore).

Physicochemical Characterization of the Nanoparticles

Particle Size

The mean diameter and the particle size distribution were measured by quasi-elastic light scattering (90°) with a nanosizer (Coulter® N4MD, Coulter Electronics, Hialeah, USA) at 20°C in water.

MePEG Content of Nanoparticles

MePEG concentration was measured by a colorimetric assay (13,14). Briefly, standards (0–20 μg MePEG /ml) and samples were diluted at 3.0 ml in water and mixed with 75 μl of a reagent composed of I_2 (10 g/l) and KI (20 g/l); the samples hydrolyzed by NaOH (see below) were diluted in phosphate buffer (0.5M, pH = 7.4). Absorbance was read at 525 nm, against a solution containing 3.0 ml of water and 75 μl of the reagent.

MePEG was measured in the following poly(PEGCA-co-HDCA) nanoparticle samples: the whole nanoparticle suspension in which both MePEG available at the surface of the nanoparticles and MePEG free in solution were measured (optical density OD1), the supernatant obtained after ultracentrifugation (145000 g, 1 h, 4°C, Beckmann L7-55 ultracentrifuge, USA) of the colloidal suspension which accounted only for MePEG free in solution (OD2) and the degraded nanoparticle suspension after digestion by NaOH 2N (50 °C, 5 days) which determined the total amount of MePEG (in the core of the nanoparticles, at their surface and free in solution) (OD3). It was then possible to calculate MePEG at the surface of the nanoparticles (S = OD1-OD2) or entrapped in the core of the particles (T = OD3-OD1).

No difference in optical density was observed between MePEG bound to the nanoparticle or free in solution, as compared with the dosage of MePEG and MePEGMe (polyethylene glycol dimethylether). Moreover, the absorbance of iodine with PHDCA nanoparticles (without MePEG) was negligible. Finally, the turbidity of the supernatant was checked at 400 nm to ensure the absence of nonsedimented nanoparticles.

The presence of MePEG at the surface of the nanoparticles was also investigated through zeta potential measurements, in KCl 1 mM at 14.6 mV (Zeta Sizer 4, 7032 Multi 8 Correlator, Malvern Instruments).

Near Infrared Spectroscopy and Spectrum Treatment for Nanoparticles

Nanoparticle suspensions, crude nanoparticles, and their dispersion medium (obtained after ultracentrifugation) were analyzed by near infrared spectroscopy, as described above for the polymers. All the samples were freeze dried (24h, –30°C / +30 °C, Christ lyophilisateur loc-1, Bioblock Scientific) before analysis.

The first derivative of the spectra were used, in order to improve spectral differences and to correct baseline effects (3). The transformed spectra were then treated by PCA, as for the polymers.

RESULTS

Synthesis of the Monomers, the Polymers, and the Copolymer

From the purified monomers (hexadecyl cyanoacetate and MePEG cyanoacetate), we obtained the PHDCA and PPEGCA polymers, respectively, as confirmed by ^{1}H-NMR data (not presented), and the poly(PEGCA-co-HDCA) copolymer.

The copolymer was prepared according to the initial protocol of Peracchia *et al.* described elsewhere (6). However, difficulties were encountered during the synthesis. The com-

position of the copolymer obtained was quite different from that theoretically expected according to the respective amounts of the two monomers (hexadecyl cyanoacetate and MePEG cyanoacetate) used in the synthesis. Moreover, the MePEG/hexadecyl ratios were not only significantly different between the different copolymer batches but also before and after purification. Indeed, analysis of the aqueous extracts obtained after purification showed ^{1}H-NMR spectra corresponding to highly pegylated copolymers (data not shown).

In order to obtain a copolymer batch with a reproducible 1:4 ratio between MePEG and hexadecyl (corresponding to the initial amounts of the two monomers used in the synthesis), we modified the synthesis process of Peracchia *et al.* (6) as follows: the catalyst DMA was replaced by pyrrolidine, formaline was added in large excess and the ratio between the solvents (ethyl alcohol and methylene chloride) was changed, so that the copolymerization took place in a homogenous medium.

Analysis of the Monomers, the Polymers, and the Copolymer

Figure 1 (top) shows the ^{1}H-NMR spectrum of the copolymer synthesized from MePEG cyanoacetate and hexa-

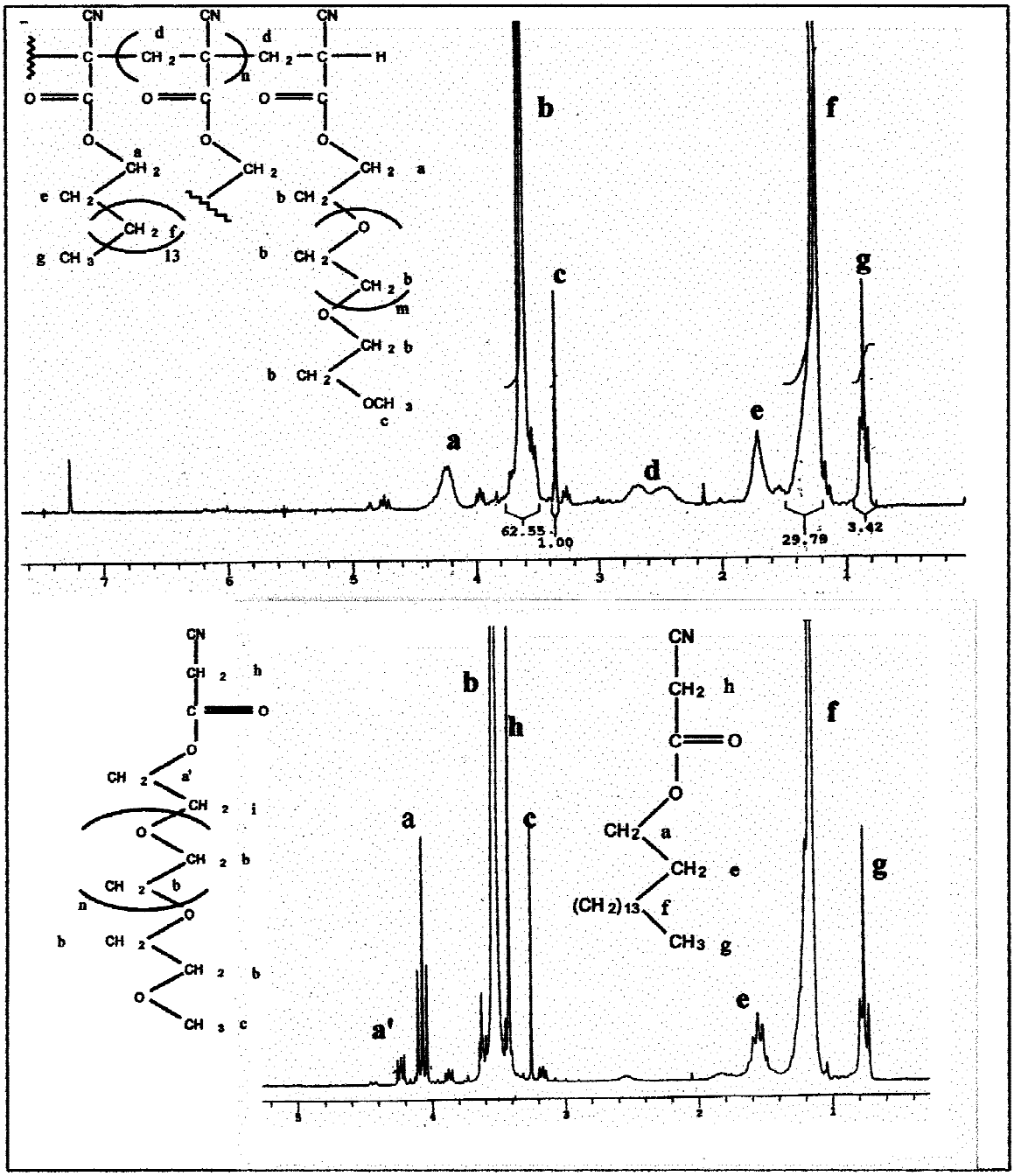

Fig. 1. ^{1}H-NMR spectra of the poly(PEGCA-co-HDCA) 1:4 copolymer (top) and of a mixture of the MePEG cyanoacetate and hexadecyl cyanoacetate monomers (bottom).

decyl cyanoacetate at a molar ratio of 1:4. The spectrum of a mixture of the two monomers was also recorded for comparison (Figure 1, bottom). The broad signal between 2.2 and 2.8 ppm for the copolymer was attributed to the methylene protons of the poly(cyanoacrylate), which proves that polymerization took place. Moreover, the singlet at 3.45 ppm in the monomer mixture, corresponding to the methylene of the cyanoacetate groups, has disappeared in the copolymer spectrum, which confirms both the involvement of the methylene function in polymer formation, and that there was no residual monomer left. The ^{1}H-NMR spectrum also allowed the experimental ratio of MePEGcyanoacrylate to hexadecylcyanoacrylate to be determined, by comparing the integrated surfaces of the peaks at 3.37 and 0.84 ppm assigned to the resonance signals of the methyl groups belonging to the two ester moieties (6). During different replicate syntheses with an initial monomer ratio MePEG cyanoacetate to hexadecyl cyanoacetate of 1:4 respectively, we obtained the following purified copolymer compositions: 1:3.4 - 1:3.7 - 1:3.8 - 1:4.2 and 1:4.6. These values were well centered around 1:4.

Near infrared analysis was performed on the following products: MePEG cyanoacetate and hexadecyl cyanoacetate, PHDCA and PPEGCA, poly(PEGCA-co-HDCA) 1:6 (catalyst DMA, Peracchia *et al.* (6)) and 1:4 (catalyst pyrrolidine), and a physical mixture of MePEG cyanoacetate and PHDCA to represent a case in which copolymerization did not occur. Figure 2 shows typical results of NIR experiments. Before discriminant analysis, near infrared spectra were treated by MSC in order to minimize the intra-class variability of a compound and to focus on inter-class variability (3,12). The MSC-transformed spectra were then treated as explained in the Materials and Methods section by PCA to obtain the loadings. The data obtained after PCA showed that only two principal components (loading values 1 and 2) were necessary to separate the different classes of compounds, which were represented by distinct clusters. Different batches of samples clustered in the same area of principal component space, showing a good batch to batch reproducibility. PHDCA and PPEGCA were correctly synthesized, because their clusters did not overlap those of the starting monomers. Poly(PEGCA-co-HDCA) 1:4 represented—except for one batch—a distinct zone which was relatively well separated from the 1:6 copolymer. Moreover, the copolymer clusters did not overlap with those of the physical mixture of MePEG cyanoacetate and PHDCA. Finally, principal component 2 separated the compounds according to their hydrophobic (score on loading 2 > 0.2) or hydrophilic (score < –0.2) character. Intermediate loading 2 values (between 0.2 and –0.2) corresponded to amphiphilic compounds, such as the 1:4 and 1:6 copolymers or the mixture. Figure 3 shows the typical spectra of hexadecyl and MePEG cyanoacetate, the hydrophobic and hydrophilic moieties of the copolymer, respectively, and the factors which are the projection of the two first loadings in the wavelength space. While factor 1 represented the main structural variations between compounds, factor 2 exhibited variations in the wavelength area where hexadecyl and MePEG cyanoacetate presented maximal spectral differences (4200 to 6000 cm^{-1}, 6600 to 7200 cm^{-1}, and 7800 to 8400 cm^{-1}). Thus loading 2 allows to discriminate the compounds on the basis of their alkyl (hydrophobic) to oxyethylene (hydrophilic) ratio.

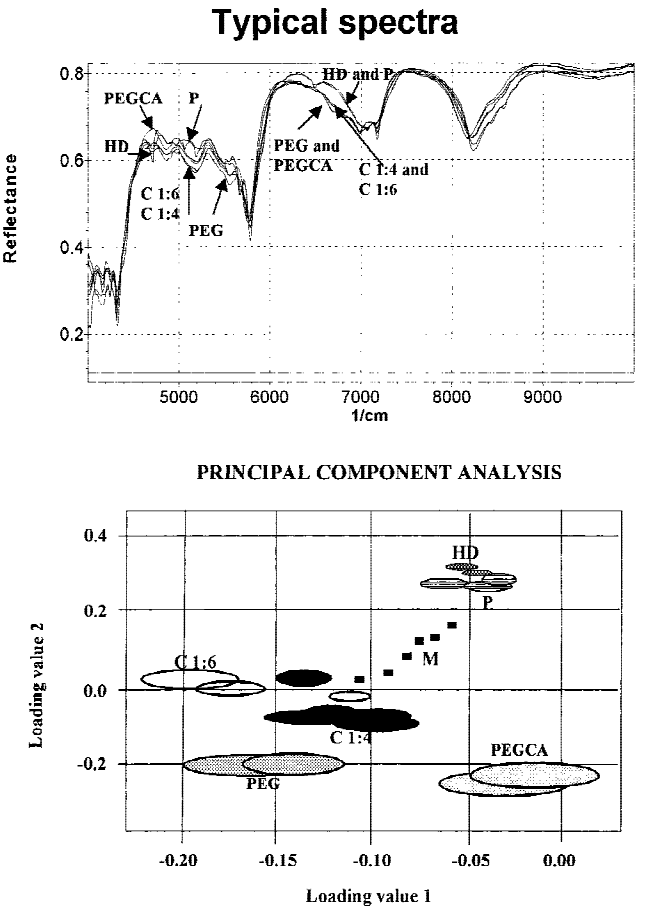

Fig. 2. MSC pretreated typical NIR spectra (top) and the corresponding principal component analysis (bottom). **HD**, hexadecyl cyanoacetate; **PEG**, MePEG cyanoacetate; **P**, poly(hexadecylcyanoacrylate); **PEGCA**, poly(MePEGcyanoacrylate); **C 1:4** and **C 1:6**, poly(PEGCA-co-HDCA) 1:4 and 1: 6 copolymers and **M**: mixture of poly(hexadecylcyanoacrylate) and MePEG cyanoacetate.

Physicochemical Characterization of Nanoparticles

Poly(PEGCA-co-HDCA) nanoparticles prepared by nanoprecipitation displayed a size of 127 nm, with a standard deviation of 27 nm and a polydispersity index of 0.07. Nanoparticles formed by emulsion–solvent evaporation were slightly larger, with a diameter of 140 nm, a standard deviation of 45 nm and a broader polydispersity index of 0.21. All batches showed a unimodal size distribution.

The results of the measurement of MePEG in the nanoparticle samples are shown in Figure 4. MePEG analysis revealed a large amount of pegylated material in the aqueous phase, not associated with the nanoparticles (84% and 90% of the total MePEG amount, according to the nanoparticles preparation method). This free MePEG fraction was in the form of a polymer or copolymer, and did not result from the hydrolysis of the ester bond, as indicated by ^{1}H-NMR in which no MePEG-OH was distinguishable. The method of emulsion–solvent evaporation gave rise to nanoparticles with a higher MePEG content: 16% (7% at the surface and 9% in the core) compared with 10% (4% at the surface and 6% in the core) for nanoprecipitation. Zeta potential measurements also confirmed that part of MePEG was present at the surface of the poly(PEGCA-co-HDCA) nanoparticles, since the

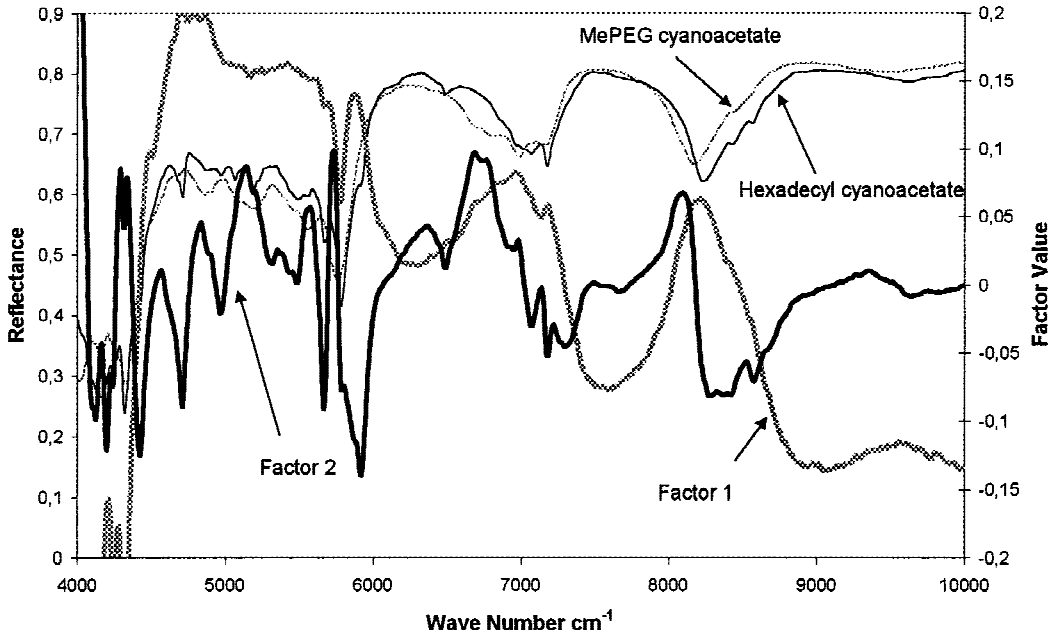

Fig. 3. Typical MSC pretreated spectra and the factors 1 and 2 of MePEG cyanoacetate and hexadecyl cyanoacetate.

strong negative surface charge of PHDCA nanoparticles (–45 mV) was found to be partially shielded when this polymer was replaced by its pegylated counterpart (surface potential: –27 mV). Finally, the oxyethylene/alkyl balance of nanoparticles and lyophilized supernatants was estimated using the NIR-PCA model built from polymers (Figure 2). As expected, supernatants lead to loading 2 scores of –0.2, in the hydrophilic region. Nanoparticle scores were between 0.0 and 0.2, in the amphiphilic region. The score for the more pegylated nanoparticles obtained by emulsion–solvent evaporation was about 0.06, while the nanoparticles obtained by nanoprecipitation scored at 0.12. This confirms the suitability of the NIR-PCA technique for rapid characterization of the nanoparticle's hydrophilic/hydrophobic balance.

Near infrared analysis with first derivative as pretreatment was performed on PHDCA and poly(PEGCA-co-HDCA) nanoparticles prepared either by nanoprecipitation or emulsion–solvent evaporation. In the case of nanoparticles prepared with poly(PEGCA-co-HDCA) 1:4 copolymer, the sediment and the supernatant obtained after ultracentrifugation were also analyzed. The data presented in Figure 5 demonstrate the following. Firstly, the analyzed compounds were found to be reproducible, because a well-defined zone was obtained for each of them (except for one copolymer batch). Secondly, nanoparticles of a different physicochemical nature were obtained from the same poly(PEGCA-co-HDCA) 1:4 when the two different methods of preparation (nanoprecipitation or solvent evaporation) were used; [this difference was probably neither the result of the different particle sizes (the pretreatment reduced the variability due to the size), nor the result of the MePEG density or conformation,] because similar observations were made for PHDCA nanoparticles. Furthermore, organic solvent residues (acetone or methylene chloride) could not explain these differences, because when polymers were spiked with solvents, the zones obtained were close to the products without solvent treatment. Finally, when either the nanoprecipitation or the emulsion–solvent evaporation method was employed for preparing nanoparticles from poly(PEGCA-co-HDCA) 1:4, it was obvious that precipitation concerned only a fraction of the molecular species of the polymer, since distinct clusters were obtained in near infrared for nanoparticle supernatant and sediment.

DISCUSSION

The question of whether the poly(PEGCA-co-HDCA) 1:4 copolymer (as a MePEG/hexadecyl ratio) was a unique molecule with the ideal structure depicted in Figure 6 and described elsewhere (6), or was composed of a mixture of individual oligomers with variable MePEG and alkyl chain contents (mean ratio value of 1:4) has been answered. Indeed, ^{1}H-NMR analysis performed on the aqueous phases used for the purification of the polymer showed the presence of poly(PEGCA-co-HDCA) molecules with a hydrophilic character arising from the same batch of synthesis. Moreover, it was observed by MePEG measurement and NIR that only the more hydrophobic fraction of the purified copolymer was in-

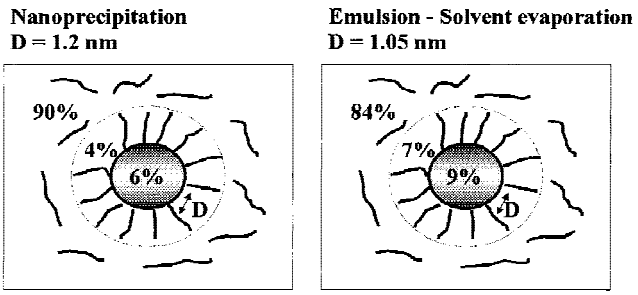

Fig. 4. Model based on MePEG measurement, showing the MePEG content and the distance between two grafted MePEG chains at the colloid surface, for nanoparticles obtained from poly(PEGCA-co-HDCA) 1:4 by nanoprecipitation or emulsion–solvent evaporation.

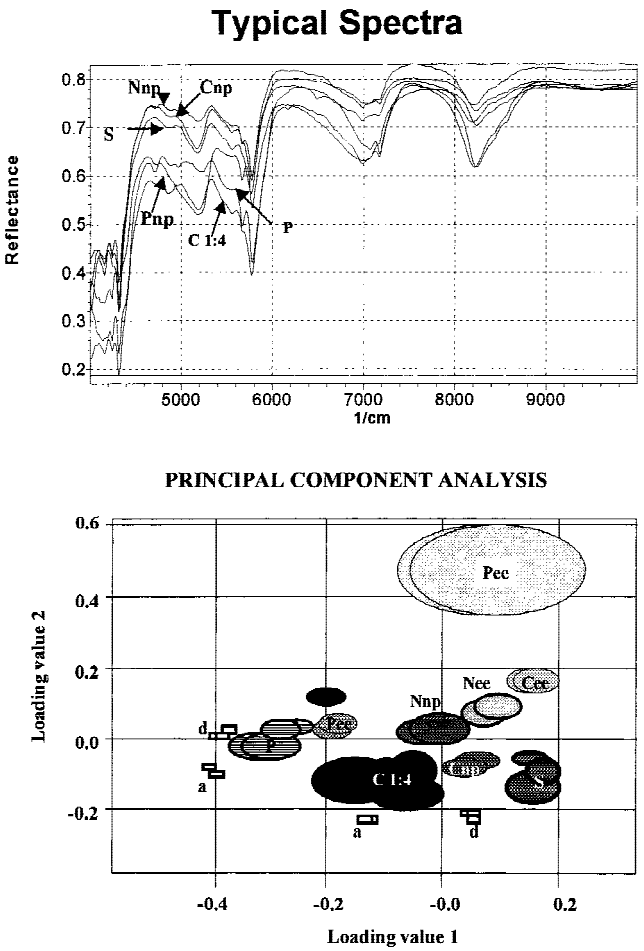

Fig. 5. Typical NIR spectra prior pretreatment (top) and PCA computed from first derivative pretreated spectra (bottom). **P**, PHDCA; **C 1:4**, poly(PEGCA-co-HDCA) 1:4; **Pnp**, nanoparticle suspension formed with PHDCA by nanoprecipitation; **Pee**, nanoparticle suspension formed with PHDCA by emulsion–solvent evaporation; **Cnp**, nanoparticle suspension formed with poly(PEGCA-co-HDCA) 1:4 by nanoprecipitation; **Cee**, nanoparticle suspension formed with poly(PEGCA-co-HDCA) 1:4 by emulsion–solvent evaporation; **Nnp and Nee**, crude poly(PEGCA-co-HDCA) 1:4 nanoparticles formed by nanoprecipitation and emulsion–solvent evaporation respectively; **S**, dispersion medium of poly(PEGCA-co-HDCA) 1:4 nanoparticles; **a** and **d**, polymer or copolymer spiked with acetone or methylene chloride respectively.

volved in nanoparticle formation, which again suggests the presence of several molecular entities. This molecular dispersion of the copolymer was probably due to the synthesis process (6), which presents some critical points: the character of the monomers is very different (hexadecyl cyanoacetate is hydrophobic while the MePEG cyanoacetate is very hydrophilic) and MePEG cyanoacetate is a monomer displaying steric hindrance. Furthermore, the copolymerization does not occur directly between the two monomers, but needs formaline as a coupling agent. Finally, the copolymer obtained is amphiphilic with tensio-active properties and concentrates at the interface during extraction and purification procedures. Thus, in order to obtain a purified copolymer displaying a constant MePEG/hexadecyl molar ratio of 1:4, we modified the synthesis process (6) after identification of the critical parameters. Under these conditions, reproducible batches of

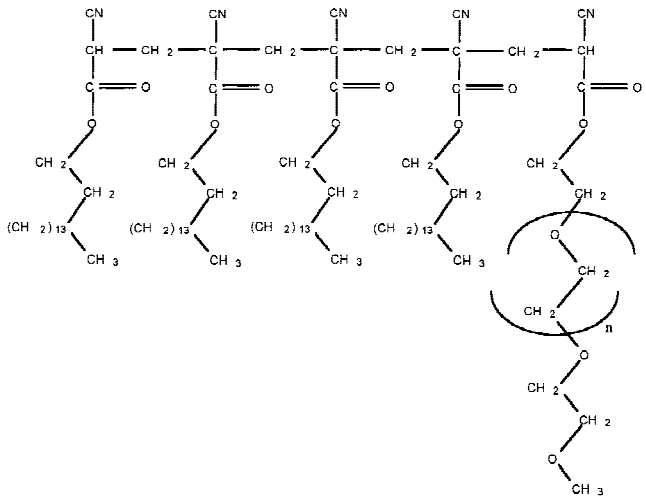

Fig. 6. Ideal structure of a poly(PEGCA-co-HDCA) copolymer at a molar ratio of 1:4.

poly(PEGCA-co-HDCA) 1:4 were obtained with a constant hydrophilic/hydrophobic ratio. This was proved by ^{1}H-NMR analysis and near infrared spectroscopy. The latter technique also allowed the different products to be separated according to their hydrophilic/hydrophobic character, which is a new way of identifying them.

As far as poly(PEGCA-co-HDCA) nanoparticles were concerned, it was possible to determine the nanoparticle surface available for one MePEG chain from nanoparticle size and MePEG measurements, and thus to calculate the mean distance between two grafted MePEG and to propose the model shown in Figure 4. It is well established that nanoparticles sterically stabilized by PEG coating provide enhanced stability in the blood compartment due to their ability to prevent the adsorption of various blood components (opsonins) onto their surface. This leads to a reduced uptake by the MPS and an extended blood circulation time (15,16). Jeon *et al.* (17,18) provided the theoretical basis for the rejection of opsonins, and proposed a model for the repulsion of protein from solid surfaces. According to this model, it is suggested that PEG should have a molecular weight above 2000 Da (19) and an optimal grafting density close to 0.7 nm^2 for one $MePEG_{5000}$ (16). Moreover, it has been demonstrated with $MePEG_{2000}$-poly(lactic) acid (PLA) nanoparticles that the threshold above which phagocytosis is no longer prevented is between 1.5 and 2 nm^2/MePEG molecule, corresponding to a distance of 1.2 to 1.4 nm between two grafted $MePEG_{2000}$ chains (14). Other authors suggest that 2.2 nm was the maximum distance for avoiding complement consumption (20).

In the proposed model of poly(PEGCA-co-HDCA) nanoparticles (Figure 4) based on the measurement of MePEG in different nanoparticle fractions, a grafting density of 1.5 nm^2/MePEG was found for nanoparticles obtained by nanoprecipitation and 1.1 nm^2/MePEG for those obtained by emulsion–solvent evaporation, which corresponds to a distance between two MePEG chains of 1.2 nm and 1.05 nm, respectively, and in both cases to a "brush" conformation of PEG. These values are thus quite compatible with a long circulating carrier, as verified by Peracchia *et al.* (9), who showed that poly(PEGCA-co-HDCA) nanoparticles remained for a longer time in the blood circulation after intra-

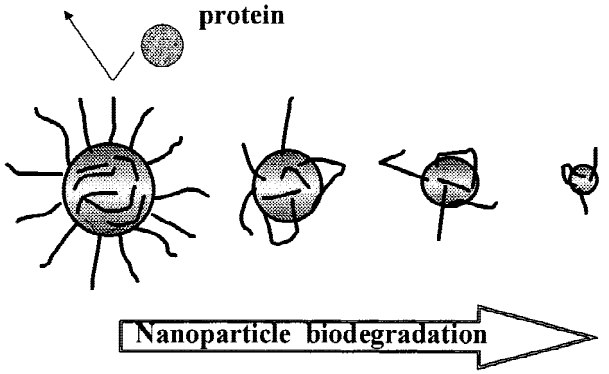

Fig. 7. Model for continuous unfolding of MePEG chains during the biodegradation of poly(PEGCA-co-HDCA) sterically stabilized nanoparticles.

venous administration to mice than the nonpegylated PHDCA nanoparticles. However, these authors noted that the initial degree of pegylation of the copolymer (hydrophilic/hydrophobic ratio 1:2 and 1:5) did not seem to affect the *in vivo* behavior of the nanoparticles. This observation is now quite understandable at the light of our experimental data demonstrating copolymer selection during nanoparticle precipitation with a lower, but probably constant amount of MePEG, independent of the initial copolymer ratio. Furthermore, in the proposed model, the presence of MePEG in the core of the particles as well as at the surface would present the advantage of maintaining the "stealth" properties of the nanoparticles during biodegradation, since new chains of MePEG would continuously become available during this process, which is not the case for other biodegradable long-circulating nanoparticles, such as PLA (Figure 7).

Finally, to our knowledge, there is no publication describing the use of NIR followed by PCA for characterizing colloidal suspensions. The data obtained have shown different clusters for the nanoparticles and their dispersion medium, which again proves that the copolymer was composed of macromolecular poly(alkylcyanoacrylate) entities bearing side-chains of different nature (MePEG or hexadecyl) (Figure 5). However, the most interesting result was the presence of distinct zones with both the PHDCA and poly(PEGCA-co-HDCA) nanoparticles depending on the method of preparation used (nanoprecipitation or emulsion–solvent evaporation). Since the differences in particle size distribution had been corrected by the pretreatment, the differences in spectra location came from chemical and/or physical properties of the samples. This shows for the first time that when starting from the same polymeric material (PHDCA or PEGCA-co-HDCA), the preparation method may dramatically influence the physicochemical properties of the colloids obtained. It may be supposed that the entanglement of the polymeric chains and the supramolecular organization may be influenced by the method used. This point, however, deserves further investigation.

CONCLUSIONS

This paper has approached the molecular characterization of the poly(PEGCA-co-HDCA) 1:4 copolymer as well as the nanoparticles formed from it, in an original way. The results obtained suggested that the copolymer is composed of a complex mixture of various poly(alkylcyanoacrylate) oligomers, and that only the more hydrophobic oligomers are able to form solid pegylated nanoparticles. Finally, near infrared spectroscopy with principal component analysis of spectral data has contributed efficiently to the characterization of these nanoparticles, for which a model is proposed. Similar approaches should be used to check the reproducibility of other pegylated colloidal drug delivery systems (i.e., PLA).

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