Synthesis of a Novel Poly(MePEG cyanoacrylate-*co*-alkyl cyanoacrylate) Amphiphilic Copolymer for Nanoparticle Technology‡

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ABSTRACT: In order to develop "stealth" injectable PEG-coated poly(alkyl cyanoacrylate) (PACA) nanoparticles, a novel poly(MePEG cyanoacrylate-*co*-alkyl cyanoacrylate) copolymer has been synthetized in a simple way by condensing MePEG cyanoacetate esters with formaldehyde in the presence of dimethylamine. This approach allowed the covalent linkage of PEG moieties to the cyanoacrylate polymer, thus avoiding the risk of desorption of PEG, as in the case of a PEG coating obtained by simple adsorption onto preformed nanoparticles. In addition, the condensation procedure allowed a better control of the polymerization process than in the case of anionic polymerization of alkyl cyanoacrylates. Finally, we have shown that the hydrophobicity of the copolymer can be perfectly modulated by adjusting the MePEG cyanoacetate/alkyl cyanoacetate ratio. Characterization of the copolymer has been performed by NMR, GPC, and FTIR analysis. Monodispersed nanoparticles were easily prepared by nanoprecipitation of this amphiphilic copolymer.

Introduction

Encapsulation of active compounds into polymeric carriers represents a very promising way of preventing drug degradation, avoiding any toxic effects, controlling its release, and achieving specific targeting. However, particles are recognized by the macrophages of the mononuclear phagocyte system (MPS) after adsorption of blood proteins (opsonins) onto their surface and accumulate in MPS organs, such as liver and spleen.[1] Thus, the therapeutic activity of compounds entrapped into injectable carriers is limited to MPS organs, due to the rapid elimination from the bloodstream.

The development of "stealth" long-circulating carriers is possible by modifying the surface of particulate carriers with hydrophilic, flexible, and nonionic polymers, such as poly(ethylene glycol) (PEG).[2,3]

A PEG coating covalently bound to the nanoparticles is desirable, in particular in the case of biodegradable nanoparticles, where an adsorbed coating layer is unstable *in vivo*.[4] The synthesis of amphiphilic PEG−R copolymers allows the preparation of nanoparticles in a single step by the classical procedures,[5] ensuring the stability of the PEG binding. In the literature, a number of PEG−R amphiphilic copolymers were reported,[6] such as PEG−polystyrene, PEG−poly(methyl methacrylate), PEG−polyisobutylene, or PEG−poly(dimethylsiloxane).[7] However, in nanoparticles technology, for the preparation of injectable carriers, biodegradable diblock or multiblock PEG−R copolymers have been developed only from polyesters [5,8] and polyanhydrides.[9]

More recently, the possibility to develop more rapidly biodegradable PEG-coated nanoparticles has been considered by using poly(alkyl cyanoacrylate) (PACA). PEG−PIBCA nanoparticles were formed by emulsion/polymerization of alkyl cyanoacrylate monomer in an aqueous medium in the presence of PEG.[10] PEG initiated the polymerization of the cyanoacrylate monomer and was chemically coupled to the growing PIBCA. Other authors reported a zwitterionic synthesis of block copolymers PEG−triphenylphosphine−PIBCA[11] and the preparation of nanoparticles from this preformed material. Indeed, polymerization of IBCA initiated by phosphines showed the near ideal living polymerization,[12] whereas anionic polymerization initiated by PEG alkoxide salts, due to the well-known high reactivity of the cyanoacrylate monomers and the high propagation rate during ionic polymerization,[13] did not allow us to obtain copolymers of definite structure.[11] However, the presence of the phosphine group in the polymer structure could determine toxicological effects.

Thus, the aim of this work was to synthetize in a simple way a novel PEG−polycyanoacrylate copolymer for the formation of PEG-coated PACA nanoparticles. We proposed a single step condensation of MePEG cyanoacetate with formaldehyde,[14] a classical procedure for the formation of poly(alkyl cyanoacrylate) oligomers, via the formation of the amino derivate. A fatty alkyl chain was inserted into the polymer structure during the condensation reaction in order to increase the hydrophobicity of the final compound, which needs to be water insoluble for allowing the preparation of nanoparticles.

Experimental Section

Materials. MePEG 2000 (purity >95%) and cyanoacetic acid (purity >99%) were purchased from Fluka (Buchs, CH). All other reagents were of analytical grade.

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‡ *Abbreviations*: R, hydrophobic unit in an amphiphilic copolymer; PEG, poly(ethylene glycol); MePEG, methoxypoly(ethylene glycol); IBCA, isobutyl 2-cyanoacrylate; PIBCA, poly(isobutyl 2-cyanoacrylate); PACA, poly(alkyl cyanoacrylate); DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 1,4-(dimethylamino)pyridine; NMR, nuclear magnetic resonance; GPC, gel permeation chromatography; FTIR, Fourier transform infrared spectroscopy; QELS, quasi-elastic light scattering; THF, tetrahydrofuran.

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Preparation of the Cyanoacetate Esters. MePEG cyanoacetate was prepared by esterification of the cyanoacetic acid with MePEG in dichloromethane, in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) as catalyst. The reaction was carried out at room temperature under nitrogen. Cyanoacetic acid (11 mmol) and MePEG (5.5 mmol, molar ratio 2:1) were placed into a glass flask and dissolved in dichloromethane (30 mL). Then, DCC (11 mmol) and DMAP (catalytic amount) were added. After stirring during 6 h, the solid was filtered off and washed with three 20 mL portions of CH_2Cl_2. The combined filtrates were concentrated under reduced pressure to leave a viscous oil which solidified on standing. The crude MePEG cyanoacetate obtained was used directly in the polymerization step without further purification.

The cyanoacetate ester of hexadecanol was prepared according to the same procedure, except that hexane was added to the reaction before filtration.

Condensation/Polymerization. Condensation of MePEG cyanoacetate with *n*-hexadecyl cyanoacetate was carried out in ethanol, in the presence of formalin and dimethylamine. The PEG cyanoacetate (1 mmol) and hexadecyl cyanoacetate (1, 2, 3, 4, or 5 mmol) were placed into a glass flask fitted with a rubber septum, then dispersed in ethanol and dissolved with a few drops of dichloromethane. Formalin 37% w/v (7.5 mmol) and dimethylamine 40% w/v aqueous solution (7.5 mmol) were introduced with a syringe. The reaction was carried out at room temperature under nitrogen. After stirring for 8 h, the reaction mixture was concentrated under reduced pressure and the residue taken into water. The solution was extracted with dichloromethane, and the combined organic phases were dried over $MgSO_4$. The solvent was evaporated, and the residue was placed several hours under vacuum to give the copolymer as a pale yellow waxy material.

Attempted Synthesis of MePEG or Hexadecyl 2-[(phenylsulfinyl)methyl]cyanoacetate. To a solution of *n*-hexadecyl cyanoacetate (2 mmol) in acetonitrile (5 mL) was added thiophenol (3 mmol), formalin 37% w/v (3 mmol), and a drop of piperidine. After stirring overnight, the reaction mixture was concentrated under reduced presure, poured into water, and extracted with ethyl acetate. The combined organic phases were dried over $MgSO_4$, and the solvent was evaporated to give the crude product which was used directly in the next step. To a solution of hexadecyl 2-[(phenylthio)methyl]cyanoacetate (2 mmol) in dichloromethane was added at 0 °C a solution of peracetic acid (32% wt in acetic acid, 2.1 mmol). After stirring for 2 h at 20 °C, the reaction mixture was poured into a saturated sodium bicarbonate aqueous solution. Extraction and concentration afforded a crude product, which turned out to be a polymeric material.

Preparation of Nanoparticles. Nanoparticles were formed by the solvent exchange (nanoprecipitation) method.[15] In practice, the polymer (100 mg) was dissolved in THF, and the polymer solution was added, under mechanical stirring, to 100 mL of MilliQ water. Particle precipitation occurred spontaneously. After evaporation of the organic solvent, nanoparticles were collected by ultracentrifugation at 20.664 g for 20 min (Ultracentrifuge L7-55, Beckman) and lyophilized (Christ Liophilizer, D) over 24 h (−30 °C/+30 °C).

Measurements. The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, unless otherwise stated, on a Bruker AC 200P (200 MHz) spectrometer. Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C NMR spectra rests on the *J*-modulated spin-echo sequence.

GPC of poly(MePEG cyanoacrylate-*co*-hexadecyl cyanoacrylate) was performed in THF with a Waters liquid chromatograph equipped with a Waters 501 pump, 712 WISP, 745 data module, and R401 differential refractometer. Molecular weight and molecular weight distribution were calculated by using polystyrene (Polysciences, Inc.) as the standard.

The FTIR spectrum (Fourier transform infrared spectrometer Impact 420, Nicolet, Madison, WI) was obtained from a neat film cast from the chloroform copolymer solution between NaCl tablets.

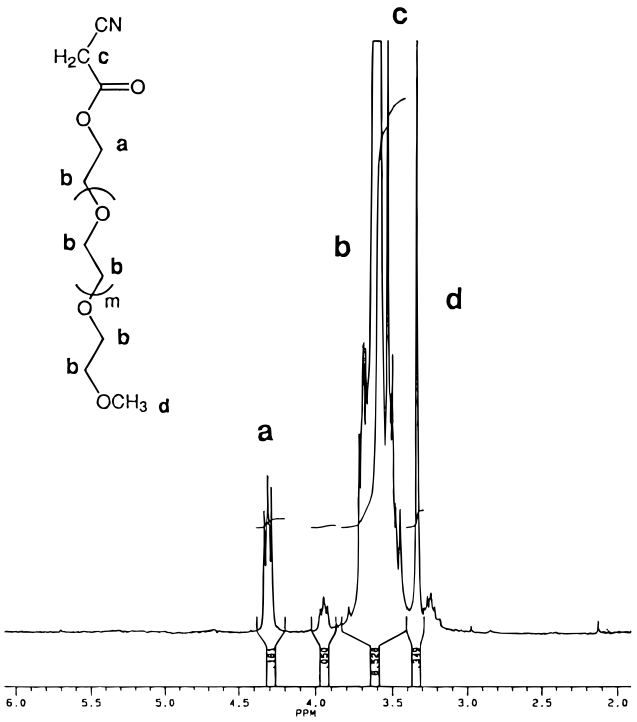
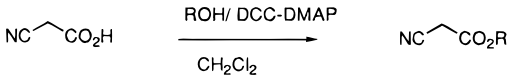


Figure 1. ^{1}H-NMR spectrum of PEG cyanoacetate.

Scheme 1. Esterification of the Cyanoacetic Acid with PEG or Hexadecanol



R = $MeO(CH_2CH_2O)_nCH_2CH_2$-

The size of nanoparticles prepared from the obtained copolymer has been determined at 20 °C and analyzed by Quasi-Elastic Light Scattering (QELS), with a nanosizer (Coulter N4MD, Coulter Electronics, Inc., Hialeah, FL).

Results and Discussion

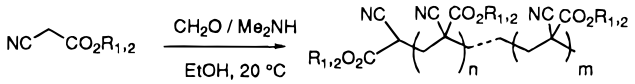
In order to obtain in a simple way a novel PEG−poly(cyanoacrylate) polymer, we proposed to use the base-catalyzed condensation of alkyl cyanoacetates with formaldehyde, described in the literature for the synthesis of oligomers of poly(alkyl cyanoacrylates).[14] The nature of oligomer formation in cyanoacetate−formaldehyde condensation involved a Michael addition as the step-growth mechanism, with chain lengths governed by steric hindrance.[16] Other authors proposed an anionic polymerization mechanism, in which polymer chain lengths are determined by chain transfer to the alkyl cyanoacetate.[17]

The advantage of poly(alkyl cyanoacrylate) synthesis from alkyl cyanoacetates instead of alkyl cyanoacrylate monomers is clearly that it is possible to better control the polymerization process, avoiding the extremely fast anionic polymerization of alkyl cyanoacrylates initiated by bases and other nucleophilic agents.[13]

The esterification of cyanoacetic acid with MePEG led to the formation of the corresponding esters in almost pure form and high yield (Scheme 1).

The structure of the obtained ester was confirmed by ^{1}H-NMR (Figure 1). The most striking feature was the singlet at 3.51 ppm, corresponding to the "active" methylene of the cyanoacetate moiety, whereas the methylene groups of the PEG showed a large peak at 3.57 ppm.

Scheme 2. Condensation/Polymerization of the Cyanoacetates in the Presence of Formaldehyde and Dimethylamine



R_1, R_2 = *n*-$C_{16}H_{33}$ or $MeO(CH_2CH_2O)_nCH_2CH_2$-

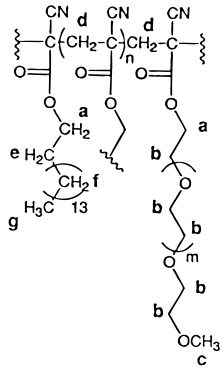
In the attempt to obtain poly(MePEG cyanoacrylate), we condensed MePEG cyanoacetate with formaldehyde (resulting from the thermal decomposition of paraformaldehyde) with a catalytic amount of piperidine as catalyst. These previously reported conditions led easily to the desired poly(MePEG cyanoacrylate). However, this material was completely water-soluble and therefore unusable for the preparation of a suspension of nanoparticles.

Thus, in order to increase the hydrophobicity of the polymer, we proposed to insert into the polymer structure an alkyl chain derived from a fatty alcohol (Scheme 2). To this aim, the hexadecyl cyanoacetate was first prepared by using the same conditions to form the MePEG cyanoacetate. The two esters were then combined at different ratios (1:1, 1:2, 1:3, 1:4, and 1:5, mole ratio between MePEG cyanoacetate and hexadecyl cyanoacetate) and condensed with formaldehyde. A thick suspension formed immediately. The use of formalin and dimethylamine in protic media allowed a smooth polymerization at room temperature. Unreacted MePEG cyanoacetate was eliminated by washing with water. Evaporation of the solvent left a yellow waxy material.

The ^{1}H-NMR spectrum is consistent with the structure of the expected copolymer. Figure 2 shows the spectrum related to a copolymer formed from MePEG cyanoacetate and hexadecyl cyanoacetate at the molar ratio 1:5. The peak at 4.26 ppm was attributed to the methylene in the α-position to the ester groups. The broad signal at 2.10−2.70 ppm and the resonance at 3.61 ppm were assigned to the methylene protons of poly(cyanoacrylate) and the PEG backbone, respectively. The singlet at 3.37 ppm was attributed to the MeO terminal group of the PEG chain. Signals at 1.72, 1.25, and 0.84 ppm were assigned to the methylene and methyl protons of the hexadecyl chain. Integration data showed a good correlation between the initial molar ratios and the final copolymer composition. Since peaks "c" and "g" could be assigned to the resonance signals of methyl groups belonging to both ester moities, the ratio of their respective surfaces gave direct access to the copolymer composition. Thus, for instance, in the case of the 1:5 copolymer, this calculation was consistent with an average of 5.6 hexadecyl subunits to 1 PEG (Figure 2). Data related to the other molar ratios between the two cyanoacetates are reported in Table 1, which sumarizes the results for the copolymerization.

The ^{13}C-NMR spectrum is consistent with the proposed structure of the copolymer backbone (Figure 3). Ester carbonyl and cyano groups show broad positive peaks at 165.2 and 114.2 ppm, respectively. The signal at 42.8 ppm was assigned to the quaternary carbon atoms of the poly(cyanoacrylate) chain, whereas the methylene carbons bearing the oxygen atoms of the side chains appear at 68 ppm. The terminal MeO group of the MePEG chain shows a negative peak at 55.4 ppm, whereas the methylene presented a large signal at 70.3 ppm. The hexadecyl chain exhibits several methylene groups between 34 and 25 ppm and a methyl group at 18.13 ppm.

protons	chemical shift (ppm)	intensity
a	4.26	1.0
b	3.61	19.8
c	3.37	0.3
d	2.70-2.10	1.5
e	1.72	1.1
f	1.25	15.1
g	0.84	1.7



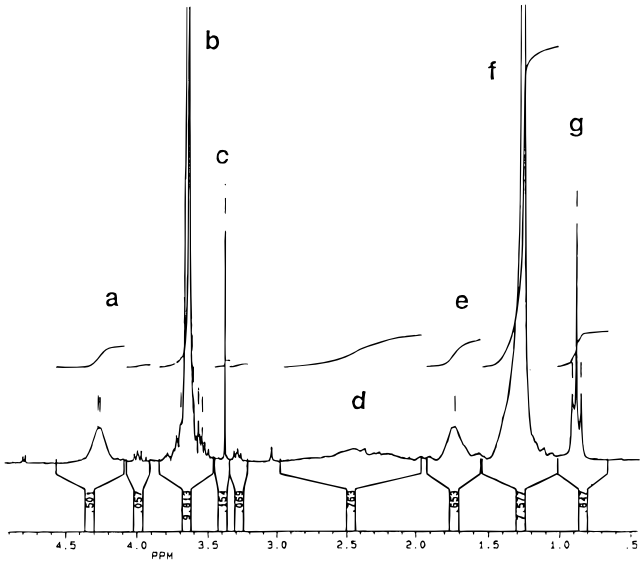


Figure 2. ^{1}H-NMR spectrum of poly(PEG cyanoacrylate-*co*-hexadecyl cyanoacrylate).

Table 1. Results of the Condensation/Polymerization of MePEG Cyanoacetate with Hexadecyl Cyanoacetate and Formaldehyde

mole ratio PEGCA to HDCA			weight-av MW	
feed	polym comp[a]	yield (%)	GPC	calcd
1:5	1:5.6	46	3719	3780
1:4	1:4	40	3506	3441
1:3	1:2.5	35	3410	3102
1:2	1:2	30	2962	2763
1:1	1:0.6	3.5	2373	2424

[a] Calculated from ^{1}H-NMR spectra.

The hydrophobicity of the polymer was easily modulated by adjusting the MePEG cyanoacetate/alkyl cyanoacetate ratio (molar ratio from 1:1 to 1:5). In this respect, it would be desiderable to use for nanoparticles preparation the copolymer with the highest MePEG cyanoacetate/alkyl cyanoacetate ratio, in order to ensure a high PEG surface density. Indeed, it is expected that, due to the amphiphilic properties of the obtained copolymer, the PEG chains migrate to the interface with the aqueous phase during nanoparticles preparation, disposing at the surface of the formed particles. However, it should be noted that the copolymer (MePEG cyanoacetate/alkyl cyanoacetate at the ratio 1:1) was obtained in only low yield (3.5%, as reported in Table 1), since most of it was lost during the washing with water. By increasing the molar ratio between the two

carbons	type	chemical shift (ppm)
a	C	165.2
b	C	114.2
c	CH_2	70.3
d	CH_2	68.0
e	CH_3	55.4
f	C	42.8
g	CH_2	34 to 22
h	CH_3	13.8

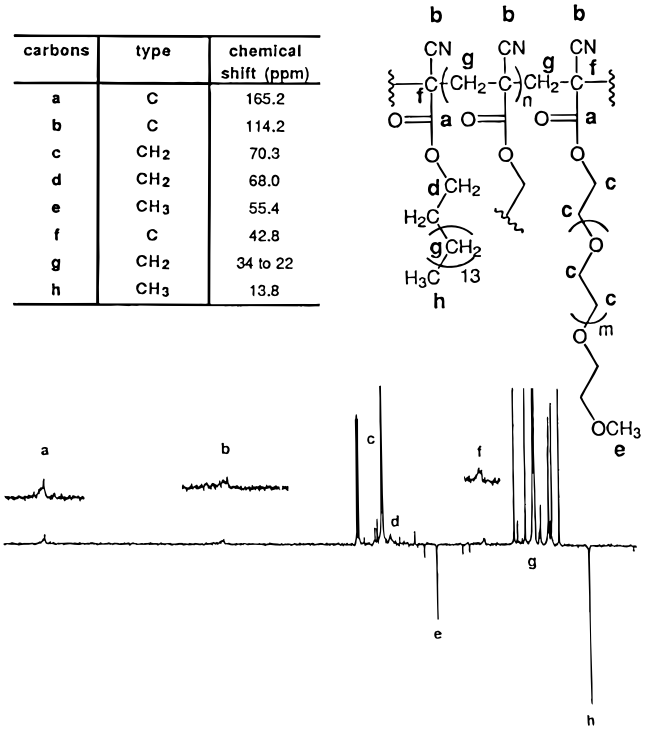


Figure 3. ^{13}C-NMR spectrum of poly(PEG cyanoacrylate-*co*-hexadecyl cyanoacrylate).

cyanoacetates, it was possible to increase the yield up to 46% (Table 1).

The analysis of the product suggested that the condensation of the growing chain was governed by steric hindrance of the MePEG cyanoacrylate monomer. We hypothesized that it is the sterically less demanding alkyl cyanoacetate which initiated the reaction and that the polymerization stopped when one PEG cyanoacetate linked at the end of the growing material. To support this hypothesis, we condensed PEG cyanoacetate with increasing amounts of hexadecyl cyanoacetate (molar ratio 1:1, 1:2, 1:3, 1:4, 1:5). Indeed, the turbidity of the reaction medium showed an increment of the kinetics depending on the alkyl cyanoacetate/PEG cyanoacetate ratio (Figure 4, A and B), indicating that the copolymerization rate is a function of the initial amount of alkyl cyanoacetate moieties and suggesting their major role in the polymerization process. For example, in the case of an initial PEG cyanoacetate/alkyl cyanoacetate ratio of 1:5, the polymer solution took only a few seconds for turning to a thick suspension, whereas in the case of an initial PEG cyanoacetate/alkyl cyanoacetate ratio of 1:1 the requested time was several minutes (Figure 4A,B).

In our initial efforts it was anticipated that copolymerization of PEG and alkyl cyanoacrylates might utilize "masked forms" of both species, able to deliver the corresponding monomers in a controlled manner. In the event, we chose to explore 2-[(phenylsulfinyl)-methyl]cyanoacetate, which could produce cyanoacrylate through thermal *syn*-elimination (Scheme 3). Condensation of cyanopropionates with formalin and thiophenol, catalyzed by piperidine, afforded the 2-[(phenylthio)-methyl]cyanoacetate in high yields. However, oxidation at the sulfur atom by means of sodium periodate, or peracetic acid, or *m*-chloroperbenzoic acid, led directly to the polymers, spoiled by sulfur-containing impurities. This extremely fast elimination thus precludes the use of this type of derivative as a stable masked form of cyanoacrylate monomers (data not shown).

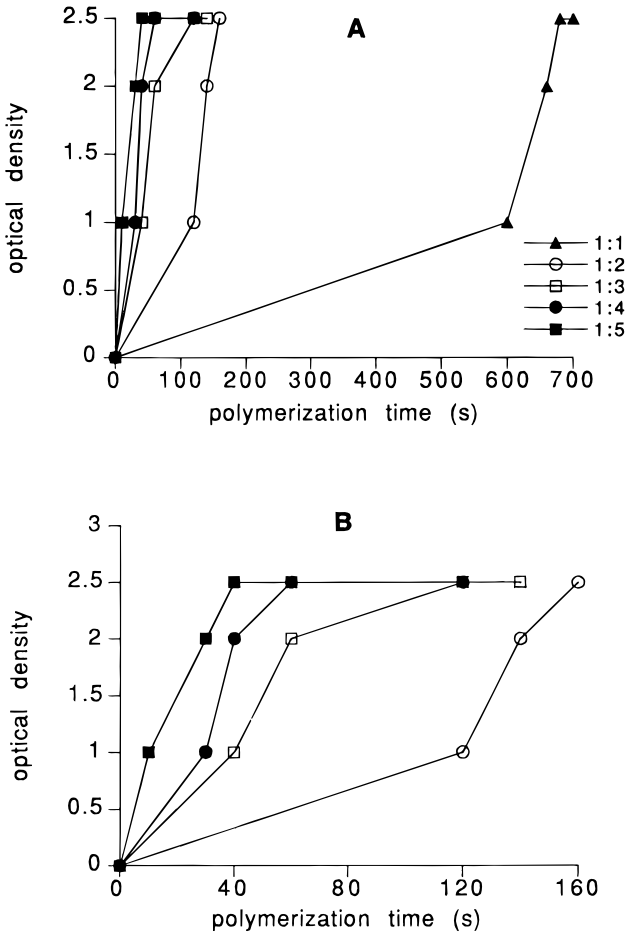
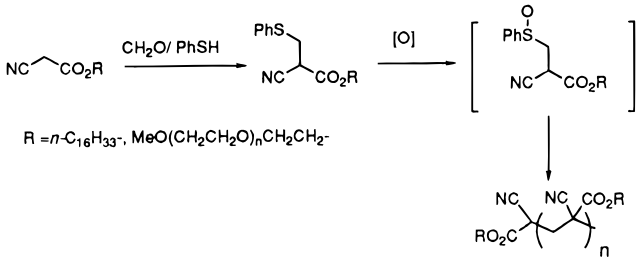


Figure 4. Turbidity (optical density) during the polymerization of different initial molar ratios of MePEG cyanoacetate and hexadecyl cyanoacetate: 1:1, 1:2, 1:3, 1:4, 1:5, time scale 0−700 s (A); 1:2, 1:3, 1:4, 1:5, time scale 0−160 s (B).

Scheme 3. Attempted Synthesis of MePEG or Hexadecyl 2-[(Phenylsulfinyl)methyl]cyanoacetate



GPC allows us to calculate the MW of the obtained copolymer (Figure 5), as well as the index of polydispersity. The average MW is 3720 for an initial ratio 1:5 between PEG cyanoacetate and hexadecyl cyanoacetate), with a low index of polydispersity (1.3). The calculated MW would be 3780; thus the method allowed us to obtain a very good correlation between the initial feed of the starting esters and the final copolymer composition. Table 1 reports all the data related to the experimental and theoretical MW for all the different molar ratios, showing in all the cases a good approximation in the definition of the copolymer structure, according to NMR data. The unimodal mass distribution excluded the presence of poly(MePEG acrylate) or poly(hexadecyl acrylate). The formation of low MW poly(alkyl cyanoacrylate)s was expected, since the condensation of alkyl cyanoacetates with formaldehyde is a method described for the synthesis of alkyl cyanoacrylate monomers. Furthermore, the interest of using

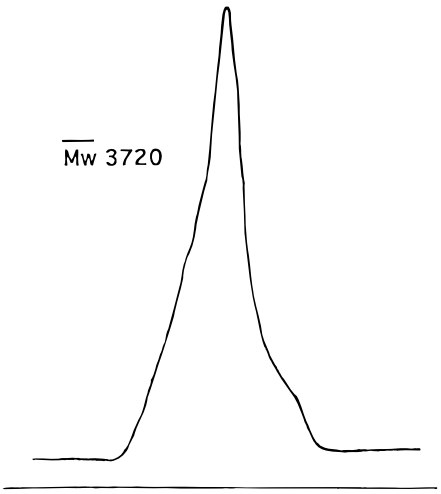


Figure 5. GPC chromatogram the poly(PEG cyanoacrylate-*co*-hexadecyl cyanoacrylate) copolymer.

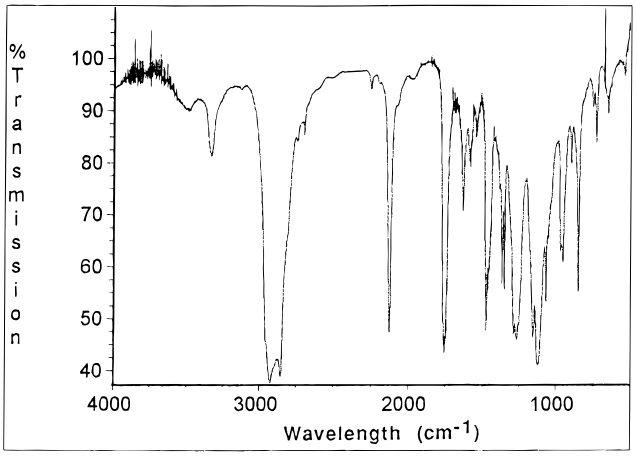


Figure 6. FTIR spectrum of poly(PEG cyanoacrylate-*co*-hexadecyl cyanoacrylate).

poly(alkyl cyanoacrylate) in controlled drug delivery is due to their rapid degradability by erosion, after hydrolysis of the ester lateral chain and the formation of the water soluble poly(cyanoacrylic acid),[18] eliminated by renal excretion. Thus, a low MW is an important issue for the degradability properties of the obtained material. Finally, the MW of the "hydrophobic" alkyl cyanoacrylate part of the copolymer was of the same order as that observed in nanoparticles prepared by emulsion/polymerization of IBCA, well-known to rapidly degradade *in vivo*.[19]

The FTIR spectrum of poly(MePEG cyanoacrylate-*co*-hexadecyl cyanoacrylate) (Figure 6) showed absorption bands related to the CN stretching vibration at 2120.6 cm^{-1} and the ester carbonyl at 1747.2 cm^{-1}. The C−O strechting of the PEG appeared at 1114.4 cm^{-1}.

Nanoparticles were prepared in a single step by nanoprecipitation, without using any further surfactant in the aqueous phase. As shown in Figure 7, nanoparticles showed a unimodal size distribution and a mean diameter of 81(±17) nm. Furthermore, due to the presence of PEG in their structure, the nanoparticles were easily redispersable in water after lyophilization. Surface chemical analysis and characterization of surface properties will allow us to verify the presence of PEG on the nanoparticles surface and the PEG capability to confer "stealth" properties to these injectable polymeric carriers.

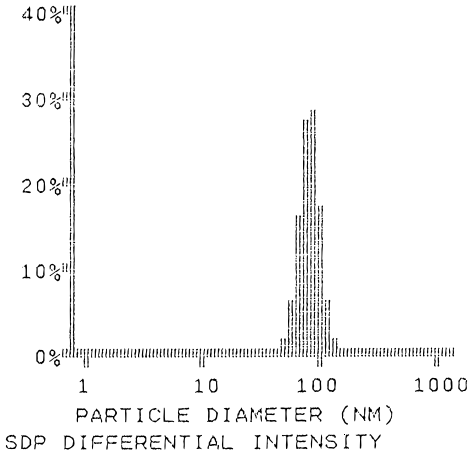


Figure 7. QELS of nanoparticles prepared from poly(PEG cyanoacrylate-*co*-hexadecyl cyanoacrylate) copolymer.

Conclusions

This work proposed the synthesis of a novel amphiphilic PEG cyanoacrylate copolymer for the single step preparation of sterically-stabilized nanoparticles. The desired copolymer was obtained in a simple way by condensation/polymerization of PEG and hexadecyl cyanoacetate esters with formaldheyde, the method for obtaining poly(alkyl cyanoacrylate) oligomers, in the presence of dimethylamine. The hydrophobicity/hydrophilicity of the copolymer can be perfectly modulated by adjusting the MePEG cyanoacetate/alkyl cyanoacetate ratio. Chemistry investigations allowed us to determine the chemical structure and the molecular weight of the obtained material.

Nanoparticles with unimodal size distribution and a size of less than 100 nm were prepared by nanoprecipitation. The advantage of preparing PEG-coated injectable nanoparticles from PEG−R copolymers is clearly that a covalent PEG binding would ensure a stable coating, potentially able to prevent any interaction with blood components *in vivo*. Current studies include the characterization and the optimization of the procedure for the preparation of PEG-coated nanoparticles starting from the proposed amphiphilic copolymer.

In conclusion, this novel copolymer, due to the ease of preparation, represents a promising material for the preparation of biodegradable particulate carriers for pharmaceutical application.

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