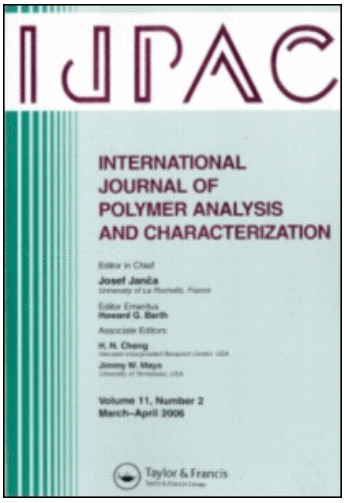

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Xiuli Wei[a]; Hui Yan[a]; Huinan Xu[a]; Wei Wu[a]
[a] School of Pharmacy, Fudan University, Shanghai, China

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Methoxypolyethylene Glycol Cyanoacrylate-Docosyl Cyanoacrylate Graft Copolymer: Synthesis, Characterization, and Preparation of Nanoparticles

Xiuli Wei, Hui Yan, Huinan Xu, and Wei Wu
School of Pharmacy, Fudan University, Shanghai, China

Abstract: Amphiphilic poly (MePEG-co-alkyl cyanoacrylate) copolymers have been studied as carriers for stealth nanoparticles. A promising area is nanoparticles prepared using copolymers with both high ratio of PEG blocks and sufficient hydrophobicity. In this study, poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) copolymer with longer alkyl chains has been synthesized by condensing MePEG cyanoacetate and docosyl cyanoacetate with formaldehyde in the presence of pyrrolidine. The hydrophobicity of the copolymer can be modulated by adjusting the MePEG/docosyl ratio. Characterization of the copolymers has been performed by FTIR, ^{1}H-NMR, and GPC analysis. PEGylated monodisperse nanoparticles were prepared by nanoprecipitation and characterized for particle size, morphology, and zeta potential. Preparation variables such as surfactant concentration in aqueous phase, copolymer concentration, and phase volume ratio influenced particle size. Negative surface zeta potential decreased with increased PEG content in the copolymer, which indicated that nanoparticles prepared with copolymer bearing larger number of PEG moieties provided better shield of the core surface. The newly synthesized copolymer is a potential material to be used to prepare stealth nanoparticles with high PEG density.

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Address correspondence to Wei Wu, Dept. of Pharmaceutics, School of Pharmacy, Fudan University, 138 Yi Xue Yuan Rd., Shanghai 200032, China. E-mail: wuwei@shmu.edu.cn

Keywords: Amphiphilic copolymers; Nanoparticles; Poly (alkyl cyanoacrylate); Poly (ethylene glycol); Poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate)

INTRODUCTION

One of the major objectives of advanced drug delivery today is drug targeting with colloidal drug delivery systems, e.g., nanoparticles. However, there are still a lot of unsolved problems, among which the first-pass uptake of nanoparticles after intravenous injection by the organs of the reticuloendothelial systems, especially liver and spleen,[1] appears to be the most serious, preventing drug-bearing nanoparticles from reaching other organs and tissues. Previous studies demonstrated that hepatic uptake of nanoparticles can be reduced by modifying their surface with hydrophilic, flexible, and nonionic polymers, such as polyethylene glycol (PEG), poloxamer, and poloxamine.[2–6] A covalently bound PEG coating is preferable, since an adsorbed coating layer is likely to be unstable and may desorb before reaching the target site.[7] It was recently shown that the synthesis of amphiphilic PEGylated polymers allowed the direct preparation of PEG-coated nanoparticles, commonly known as stealth or long-circulating nanoparticles, ensuring the stability of the PEG coating layer, as the PEG chains remained chemically linked to the nanoparticle core.[8–10]

Multiple grafted polymers with PEG as hydrophilic moiety, such as PEG-PLGA, PEG-PLA, and PEG-PCL, have been studied as stealth nanoparticle carriers.[9–12] Recently, Peracchia and coworkers[13–16] have synthesized poly (MePEG cyanoacrylate-co-hexadecyl cyanoacrylate) (MePEG-PHDCA) amphiphilic copolymer as an alternative carrier for PEG-coated nanoparticles. This polymer has been proven to facilitate evading of hepatic uptake for nanoparticles efficiently. However, the grafted copolymer is endowed with drawbacks, one of which is that only PEG chains at the surface can work as protective moieties because PEG blocks are distributed throughout the matrix of the nanoparticle. Both the PEG chain length and chain density affect in vivo behavior of stealth nanoparticles.[6,17] But for PEG chains consisting of more than 50 ethylene oxide units corresponding to a PEG molecular weight larger than 2200, adsorption of opsonins on the nanoparticle surface, the most important mechanism of hepatic uptake, seems to be independent of PEG chain length. If the PEG molecular weight exceeds the critical value, the adsorption is a function only of the surface density of the PEG chains.[18] One way to increase surface PEG density is through adjusting the ratio of PEG blocks to the hydrophobic counterparts during chemical synthesis. As for MePEG-PHDCA, for instance, a copolymer with MePEG/hexadecyl raito of 1/1 would be expected to meet with higher

PEG density than that with a ratio of 1/2 or less. In this respect, it would be desirable to prepare nanoparticles using copolymers with the highest MePEG/hexadecyl ratio. Unfortunately, when the ratio of PEG/hexadecyl increased, the synthetic yield of MePEG-PHDCA copolymer decreased, even down to 3.5% when the ratio of PEG/hexadecyl was 1/1, because of its high solubility in water.[13] Also, the recovery of nanoparticles prepared from polymers with higher water-solubility was limited. It is necessary to prepare nanoparticles using copolymers with both high ratio of PEG blocks and sufficient hydrophobicity, which is crucial for the formation of nanoparticles. One of the applicable approaches to preparing such copolymers is to replace hexadecyl with a longer-chain alkyl moiety such as docosyl during chemical synthesis of poly (MePEG cyanoacrylate-co-alkyl cyanoacrylate).

Thus, the aim of this work was to synthesize a novel poly (methoxypolyethylene glycol cyanoacrylate-co-docosyl cyanoacrylate) copolymer (MePEG-PDDCA) and prepared PEG-coated nanoparticles. Physicochemical measurements were carried out to characterize the copolymers and nanoparticles prepared by nanoprecipitation.

EXPERIMENTAL SECTION

Materials

Methoxypolyethlene glycol (MW 5000, $MePEG_{5000}$) was purchased from Sigma Chemical Co. (St. Louis, USA). Cyanoacetic acid was purchased from Fluka Chemical Co. (Steinheim, Switzerland). 1-Docosanol and pyrrolidine were also purchased from Fluka (Buchs, CH, Germany). N, N-dicyclohexylcarbodimide and 4-dimethylaminopyridine were from Shanghai Chemical Reagent Co. (Shanghai, China). Formaline (37%) was purchased from Shanghai Jianxin Chemical Co. (Shanghai, China). Aqueous dimethylamine (40%) was provided by Shanghai Linfeng Chemical Co. (Shanghai, China). Other reagents were of analytical purity and provided by local distributors.

Synthesis of Cyanoacetate Esters

Poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) was synthesized by condensation of $MePEG_{5000}$ cyanoacetate with docosyl cyanoacetate in ethanol, in the presence of formaline and dimethylamine.[15,16]

The cyanoacetate esters were prepared by esterification of the cyanoacetic acid with docosanol or $MePEG_{5000}$ in dichloromethane, in the presence of both 1,3-dicyclohexylcarbodiimide (DCC) as coupling agent and 4-dimethylaminopyridine (DMAP) as catalyst. In detail, for the

synthesis of docosyl cyanoacetate, cyanoacetic acid (44 mmol) and docosanol (22 mmol) were dissolved in dichloromethane (80 mL), then DMAP (2.2 mmol) was added, followed by DCC (44 mmol) as a solution in dichloromethane (20 mL). The reaction was carried out with magnetic stirring in a water bath maintained at 25°C with nitrogen. After 24 hours, hexane (100 mL) was added, and the solid product was filtered off and washed with more dichloromethane. The combined filtrates were concentrated under reduced pressure. The resultant solid was purified by recrystallizing from methanol. After filtration and drying under vacuum, the purified docosyl cyanoacetate was obtained as a buff amorphous solid.

The MePEG cyanoacetate was synthesized similarly, replacing docosanol with $MePEG_{5000}$. And before filtration, no hexane was added to the reacting solution. The purification step consisted in recrystallization of the crude ester from isopropanol. After filtering and drying under vacuum, the purified MePEG cyanoacetate was obtained as a yellow amorphous solid.

Synthesis of PEGylated and Non-PEGylated PDDCA Polymers

The MePEG-PDDCA copolymers with MePEG/docosyl ratios of 1/1, 1/3, and 1/5 were synthesized by condensation/polymerization of MePEG cyanoacetate and docosyl cyanoacetate. First the two esters were dissolved in a 1:2 mixture of ethanol and dichloromethane and 37% formaline was sequentially added. The mixture was left to react for 24 hours under magnetic stirring in 25°C water bath with nitrogen. The reaction mixture was concentrated under reduced pressure and the residue was mixed with distilled water and further extracted with dichloromethane three times. The combined organic phases were then purified by washing with 1 M HCl and distilled water. Finally, the organic phase was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was placed overnight under vacuum to give the copolymer as a yellow waxy compound.

PDDCA, used as a control non-PEGylated polymer, was also obtained by a condensation/polymerization reaction of docosyl cyanoacetate. Briefly, docosyl cyanoacetate (5 mmol) was dissolved in 1:2 mixture of ethanol and dichloromethane. Thirty-seven percent formaline (1.2 mL, 15 mmol) and 40% aqueous dimethylamine (0.4 mL, 3 mmol) were added. The reaction and the purification procedure were then carried out as described above for the copolymer.

Characterization of Copolymers

The infrared spectra were measured on a Fourier transform-infrared (FTIR) spectrometer Avatar™ 360 E.S.P.™ (Thermo Nicolet Corp. USA).

The ^{1}H-NMR (nuclear magnetic resonance) spectra were recorded on a Mercury Plus 400 MHz nuclear magnetic resonance spectrometer (Varian, USA) equipped with an 11.75 T magnet using a 5 mm ^{1}H probe, after dissolution of the samples in deuterated chloroform using 5 mm OD tubes. Gel permeation chromatography (GPC) was performed using a Waters liquid chromatograph equipped with a Waters 515 pump, 2414 differential refractometer, and sequentially connected Styragel® HR columns. The mobile phase was tetrahydrofuran (THF) flowing at 1.0 $cm^3 min^{-1}$. The GPC sample was prepared by dissolving 50 mg of copolymer in 5 mL of THF. Molecular weight and molecular distribution were calculated using polystyrene as the standard.

Determination of Solubility

The solubility at room temperature of 20 mg of the poly (MePEG-PDDCA) copolymer was tested in common organic solvents (1 mL) used in nanoparticle preparation, such as acetone and dichloromethane. The copolymer was considered very soluble (++) the in case of immediate dissolution, poorly soluble (+) in the case of a slower but complete dissolution under ultrasonication, and insoluble (−) in the case of partial dissolution.

Preparation of the Nanoparticles

Nanoparticles were prepared by a nanoprecipitation/solvent diffusion method.[19] Briefly, the MePEG-PDDCA copolymer or the PDDCA polymer (100 mg) was dissolved in 5 mL of THF, and the solution was added to 50 mL of demineralized water (Milli-Q water purifier, Millipore, USA) containing poloxamer 188 as surfactant under mechanical stirring (IKA, Labortechnik, Germany) at 1000 rpm. Particle precipitation occurred immediately. After evaporation of the organic solvent using a rotative evaporator (Büchi, Rotavapor R-200), the nanoparticle suspension was filtered (Millex® AP, Millipore, 1.2 μm) to remove particle agglomerates, and the nanoparticles were ultracentrifuged at 50,000 g for 60 min (MX series preparative ultracentrifuge, Hitachi) and washed twice with demineralized water to remove any residual surfactant. The concentrated nanoparticle suspensions were stored at 4°C.

Physicochemical Characterization of the Nanoparticles

Particle size and distribution were measured by quasi-elastic light scattering with a Nicomp™ 380/ZLS nanosizer (Santa Barbara, Calif., USA)

at 20°C in water. Nanoparticles were diluted to a concentration of $10\,mg\,mL^{-1}$ with demineralized water and sonicated to prevent particle aggregation before measurement. The particle size was measured in triplicate and recorded as an average with standard error.

The zeta potential was determined using a Nicomp™ 380/ZLS zeta potential analyzer (Santa Barbara, Calif., USA) by dipping a palladium electrode in the particle suspension. Each sample was analyzed in triplicate.

For transmission electron microscopy (TEM) study, a small drop of concentrated aqueous particle suspension was placed on Formvar®-coated copper grids. After negative staining with 2% (w/v) phosphothungstic acid, nanoparticles were visualized under a Hitachi H-600 transmission electron microscope.

RESULTS AND DISCUSSION

Synthesis of the Copolymer

Copolymers of MePEG-PDDCA with MePEG/docosyl ratios of 1/1, 1/3, and 1/5 were synthesized employing a base-catalyzed condensation mechanism with high yields (Table I). Due to the water-washing procedure during the purification process to remove water-soluble impurities, yields of copolymers decreased with increased MePEG/docosyl ratio or, in other words, increased hydrophilicity. In a previous study,[20] we investigated the synthesis of MePEG-PHDCA and found that yields were much lower for copolymers with high MePEG moieties. The yields of MePEG-PHDCA copolymers with a MePEG/hexadecyl ratios of 1/1, 1/3, and 1/5 were 3.7%, 48.7%, and 64.2%, respectively, which increased to 55.8%, 75.2%, and 80.6% in this study after substituting hexadecyl with docosyl to increase the copolymer's hydrophobicity.

Table I. Composition and molecular weight of the copolymers obtained by condensation/polymerization of MePEG cyanoacetate with increasing amount of docosyl cyanoacetate

			Molecular weight	
Initial ratio	Polym. comp[a]	Yield (%)	Determined	Theoretical[b]
1/5	1/4.42	80.6	7535	7080
1/3	1/3.12	75.2	6379	6294
1/1	1/1.06	55.8	5446	5508

[a]Calculated from ^{1}H-NMR spectra.
[b]Calculated by considering the constitutional repeating units according to the initial ratio.

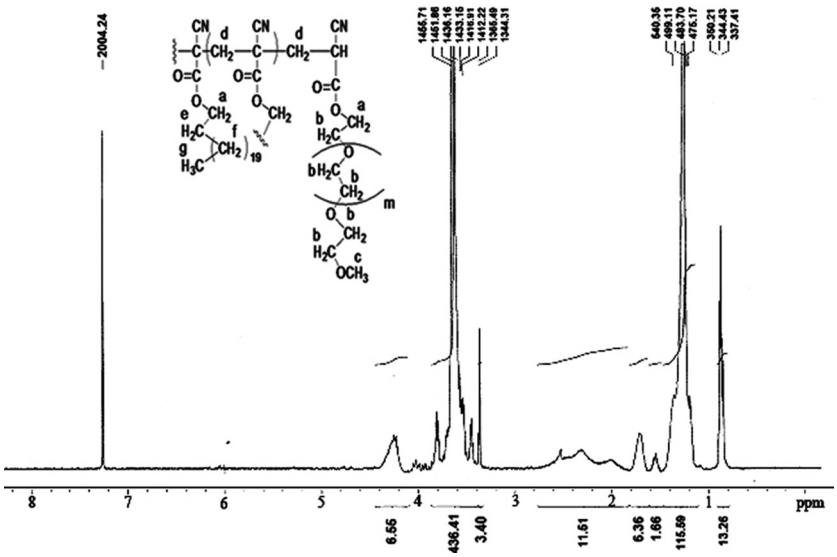


Figure 1. ^{1}H-NMR spectrum of the poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) 1/5 copolymer.

Figure 1 shows the ^{1}H-NMR spectrum of the copolymer synthesized from MePEG cyanoacetate and docosyl cyanoacetate at a molar ratio of 1:5. The shift at 4.26 ppm was attributed to the methylene in the α-position to the ester groups. The broad signal between 2.2 and 2.8 ppm and the shift at 3.65 ppm were assigned to the methylene protons of poly (cyanoacrylate) and the PEG backbone, respectively, which was indicative that polymerization had taken place. The ^{1}H-NMR spectrum also allowed calculation of the experimental ratio of MePEG to docosyl, by comparing the integrated surfaces of the "c" and "g" peaks attributed to the resonance signals of the methyl groups belonging to the two ester moieties.[13]

The FTIR spectrum of poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) (Figure 2) shows absorption bands related to CN stretching vibration at 2267 cm^{-1}, ester carbonyl at 1730 cm^{-1}, and C-O stretching of PEG at 1196 cm^{-1}.

Table I summarizes the results of synthesis yields, MePEG/docosyl molar ratios and molecular weight. The theoretical molecular weight was obtained by considering the molecular weight of the constitutional repeating units, which was 5067 for MePEG cyanoacetate, 393 for docosyl cyanoacetate, and 30 for formaldehyde. Molecular weight determined by gel permeation chromatography agreed with the theoretical calculations.

Following a similar procedure, PDDCA was also synthesized with high yield.

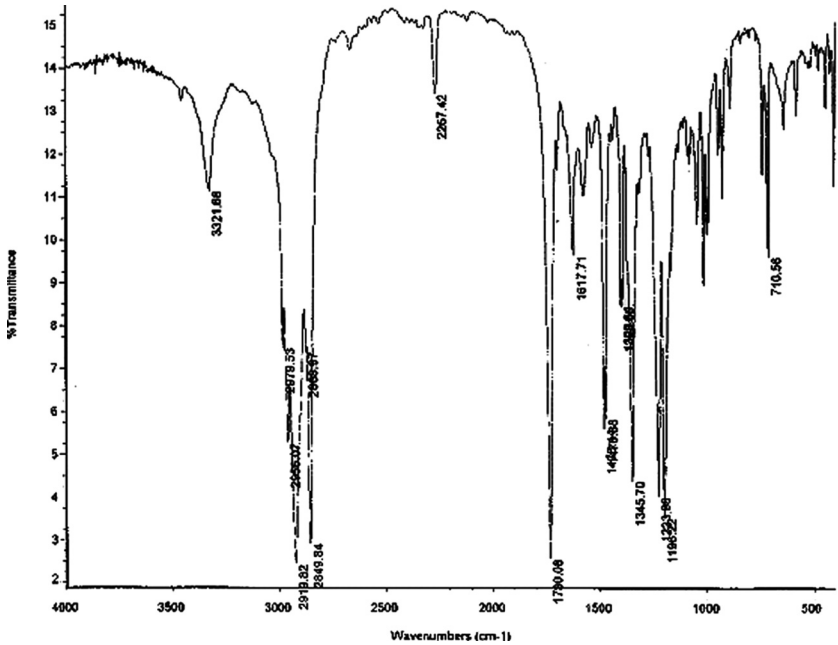


Figure 2. FTIR spectrum of the poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) 1/5 copolymer.

Table II. Solubility of the synthesized copolymer in different PEG/docosyl ratios in organic solvents employed for the preparation of colloidal nanoparticles

	Copolymer in different PEG/docosyl ratios		
Solvent	1/1	1/3	1/5
Acetone	−	−	−
Ethanol	−	−	−
Ethyl acetate	−	−	−
Methanol	−	−	−
Tetrahydrofuran	+	+	+
Methylene chloride	++	++	++
Chloroform	++	++	++
Methylene chloride/acetone	++	++	++
Methylene chloride/ethanol	++	++	++
Methylene chloride/ethyl acetate	++	++	++
Chloroform/ethanol	++	++	++
Chloroform/acetone	++	++	++
Chloroform/ethyl acetate	++	++	++

++: very soluble; +: soluble; −: insoluble.

Solubility of the Copolymer

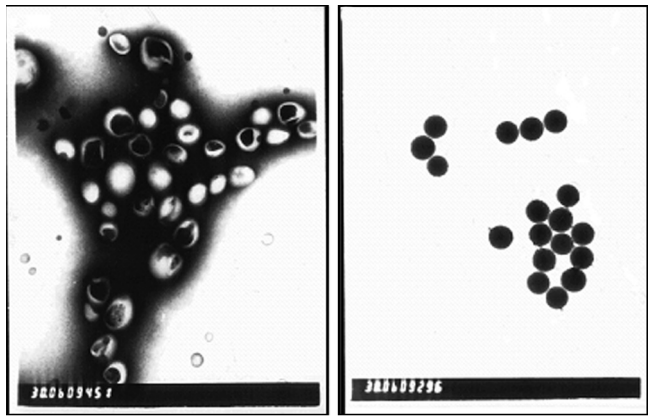
Copolymer solubility was determined in the organic solvents employed in nanoparticle production. As illustrated in Table II, the copolymer (1/1, 1/3, and 1/5 MePEG/docosyl ratio) was found to be insoluble in most of the organic solvents miscible with water, such as ethanol and acetone, except THF. This can be explained by the presence of the highly hydrophobic fatty alkyl chains in the copolymer structure. On the other hand, the copolymer was found to be soluble in all chlorinated solvents, as well as in their 1/1 mixture with acetone or ethyl acetate. No obvious difference existed between copolymers with different MePEG/docosyl ratios.

Nanoparticle Size and Morphology

Nanoparticles with a mean diameter in the size range of 150–500 nm were prepared by nanoprecipitation. Nanoparticle size and distribution were

Table III. Effect of preparation variables on the diameter of the nanoparticles

Preparation variable	Diameter (nm)
Concentration of poloxamer 188 in aqueous phase (%, w/v)	
0.2	116.3 ± 29.4
0.5	129.4 ± 33.6
1	124.6 ± 18.2
2	197.9 ± 24.4
3	253.5 ± 18.6
4	318.3 ± 19.7
5	429.9 ± 19.4
Concentration of copolymer in organic phase (mg/mL)	
2.5	170.4 ± 23.1
5	197.9 ± 14.4
10	266.1 ± 19.9
15	303.2 ± 22.8
Aqueous/organic phase volume ratio	
3.3	206.3 ± 19.6
5	197.9 ± 24.4
10	91.2 ± 16.0
Stirring rate (rpm)	
700	205.5 ± 28.0
1000	197.9 ± 14.4
1300	192.8 ± 20.5



(a) (b)

Figure 3. Transmission electron microscopy photographs of MePEG-PDDCA (a) and PDDCA (b) nanoparticles, at a magnification of 3×10^4.

influenced by several factors such as the type of stabilizing surfactant, its concentration in the aqueous phase, polymer concentration in the organic phase, and volume ratio of organic to aqueous phase (Table III). As we were specifically interested in the copolymer with highest MePEG/docosyl (1/1) ratio, nanoparticles prepared with this copolymer were studied in detail. Monodisperse nanoparticles were obtained using poloxamer 188 as surfactant, while stabilizing agents like PVA, Myij59, and Myij45 failed to produce monodisperse nanoparticles. Nanoparticles prepared with MePEG-PDDCA or PDDCA were spherical in shape and uniform in size, about 200 nm (Figure 3). From Figure 3, we can see that the configuration of PEGylated nanoparticles differs greatly from the non-PEGylated nanoparticles. Diameter of nanoparticles calculated from TEM pictures was in good accordance with laser diffraction nanosizer determinations.

Surfactant concentration in aqueous phase of 1% (w/v) had no effect on particle size. When surfactant concentration increased from 1% to 5%, increase in particle size was observed. It seems that the size of nanoparticles prepared through interfacial nanoprecipitation does not simply subject to interfacial tension, and other mechanisms remain to be deciphered. Copolymer concentration in organic phase played an important role in nanoparticle formation, with higher concentration leading to increased particle size, which may be interpreted by increased viscosity of copolymer in tetrahydrofuran. Moreover, the volume of organic phase also affected the size of the nanoparticle; decreasing the volume of organic phase led to an increase in particle size. However, the speed of stirring had no obvious effect on the particle size, conflicting with common knowledge that faster stirring

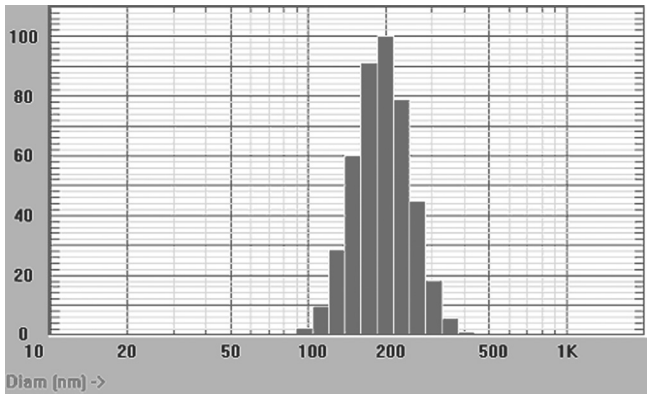


Figure 4. Size distribution of nanoparticles prepared from poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) 1/1 copolymer.

rate produces smaller particle size. Scrutinizing the nanoprecipitating process, we found that formation of nanoparticles took place simultaneously as the copolymer solution was dropped into the aqueous phase, even without mechanical stirring. The purpose of mechanical stirring was only to keep newly formed nanoparticles from forming agglomerates. This is why stirring rate has no obvious effect on particle size.

From the results, we concluded that nanoparticles can be prepared with optimal size distribution and the size was tunable through adjusting preparation variable levels. A size distribution graph of nanoparticles with a mean diameter of 200 nm prepared with MePEG/docosyl ratio of 1/1 is shown in Figure 4.

Size and distribution variation was also studied after one month of storage at 4°C. From Table IV, we can see that nanoparticles after storage did not show any significant change in nanoparticle size. For nanoparticles prepared out of PDDCA and bearing no surface PEG moieties, the particle

Table IV. Particle size of PEGylated and non-PEGylated nanoparticles prepared by nanoprecipitation using copolymers with different PEG/docosyl ratios and that after one-month storage

	Particle size (nm)	
Polymer	Initial	After one-month storage
$MePEG_{5000}$-PDDCA (1/1)	197.9 ± 24.4	205.1 ± 19.2
$MePEG_{5000}$-PDDCA (1/3)	205.6 ± 29.3	210.2 ± 20.9
$MePEG_{5000}$-PDDCA6 (1/5)	210.5 ± 18.8	215.1 ± 19.2
PDDCA	307.0 ± 24.0	312.0 ± 21.7

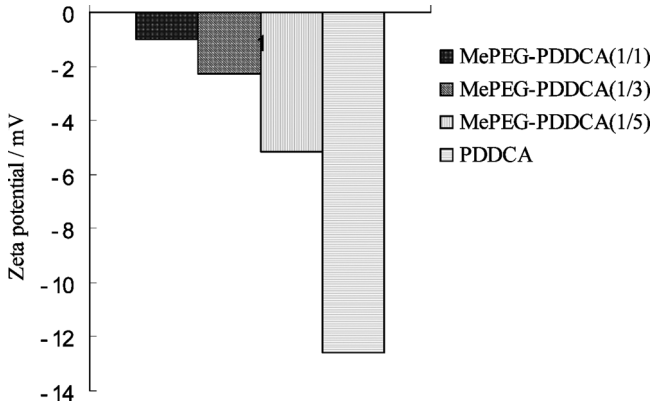


Figure 5. Zeta potential of PEGylated and non-PEGylated nanoparticles prepared with different PEG/docosyl copolymer ratios.

size was bigger than that prepared with MePEG-PDDCA. Reduction in particle size with increasing amounts of MePEG in the copolymer molecule was also observed and the reason may be the increased PEG surface density stabilizing the formation of smaller particles.

Particle Surface Charge

Because of the electrically negative nature of polycyanoacrylate polymers, nanoparticles prepared with them always charge negative.[21,22] In this study, the zeta potential of nanoparticles prepared with PDDCA was about −13 mV. Upon coating with PEG chains, a "corona"-like hydrophilic shell forms and shields the negative surface of the core.[12] PEG coating seemed to decrease negative charge of the nanoparticles,[16,23] and it is reasonable to assume that a dense and stable coating would provide the nanoparticles with near-neutral surface charge. In good agreement with our assumption that nanoparticles made from copolymers with more PEG moieties show denser surface PEG coating, the zeta potential of MePEG-PDDCA nanoparticles with MePEG/docosyl ratios of 1/5, 1/3, and 1/1 decreases stepwise (Figure 5). In contrast the nearly neutral surface charge with equal molar of MePEG and docosyl moieties was indicative of dense PEG coating and perfect shielding.

CONCLUSIONS

An amphiphilic poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) copolymer was synthesized simply by condensation/polymerization of

MePEG and docosyl cyanoacetate with formaldehyde. The hydrophobicity/hydrophilicity of the copolymer can be easily modulated by adjusting the MePEG/docosyl ratio. Copolymer with MePEG/docosyl ratios of 1/1, 1/3, and 1/5 was obtained with high yields. Physicochemical characterization proved the formation of the copolymers, and findings were in good correlation with theoretical calculations.

Monodisperse nanoparticles were easily prepared by nanoprecipitation, especially for the more hydrophilic copolymer with MePEG/docosyl ratio of 1/1. Thus, nanoparticles with denser surface PEG coating would be expected. Preparation variables such as surfactant concentration in aqueous phase, copolymer concentration, and phase volume ratio affected particle size. Scanning electron microscopy observation showed different morphologies of PEGylated and non-PEGylated nanoparticles. Surface zeta potential decreased with increased PEG content in the copolymer, which indicated that nanoparticles prepared with a copolymer bearing a larger number of PEG moieties provided a better shield of the core surface.

Poly (MePEG cyanoacrylate-co-docosyl cyanoacrylate) is a potential material to be used to prepare stealth nanoparticles with high PEG density.

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