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# New amphiphilic biodegradable β-cyclodextrin/poly(L-leucine) copolymers: Synthesis, characterization, and micellization

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ABSTRACT

A new amphiphilic biodegradable β-cyclodextrin/poly(L-leucine) (β-CD–PLLA) copolymer was synthesized by ring-opening polymerization of *N*-carboxy-L-alanine anhydride (LL-NCA) in *N*,*N*-dimethylformamide (DMF) initiated by mono-6-amino-β-cyclodextrin ($H_2N$-β-CD). The structures of the copolymers were determined by IR, $^1H$ NMR and GPC. The fluorescence technique was used to determine the critical micelle concentrations (CMC) of copolymer micelle solution. The diameter and distribution of micelles were characterized by dynamic light scattering (DLS) and its shape was observed by transmission electron microscopy (TEM). The results showed that LL-NCA could be initiated by $H_2N$-β-CD to produce the copolymer. These copolymers could self-assemble into nano-micelles in water. The CMC of copolymer solution and the size of micelle reduced with increasing proportion of the hydrophobic part. TEM images demonstrated the micelles are all spherical. Such block copolymers could be expected to find applications in drug delivery systems and other biomedical fields.



## 1. Introduction

Amphiphilic block and graft copolymers can self-assemble into micelles. The micelles have unique characteristics such as nanosize, a core–shell architecture, and a good thermodynamic stability in physiological condition because of their low critical micelle concentration (CMC) (Tian et al., 2005). The micelles formed by amphiphilic biodegradable block and graft polymers may be used for controlled release of drug implants, proteins, medical devices, etc. because they possesses both biodegradability and amphiphilicity (Bikram et al., 2004; Soppimath, Aminabhavi, Kulkarmi, & Rudzinski, 2001).

Poly(α-amino acid)s (PAA) are well known as very important synthetic biodegradable materials. Due to their polypeptide backbone, they have the potential to be degraded in biological environments. Moreover, since they have low immunogenicity, good biocompatibility and excellent mechanical properties, they may be widely used in pharmaceutical and other medical fields (Fukuoka, Uyama, & Kobayashi, 2004; Iwata, Matsuda, Mitsuhashi, Itoh, & Ikada, 1998; Rypáček, 1998). But it is well known that most commonly used PAAs, such as poly(α-leucine), poly(α-alanine), poly(γ-benzyl glutamate), etc. are rather hydrophobic and degrade very slowly by simple hydrolysis under human body conditions. In order to improve the hydrophilicity and to control the biodegradable rate of these poly(α-amino acid)s, hydrophilic functional groups may be introduced into the polymer chain. Poly(ethylene glycol) (PEG) was usually introduced into the copolymer as hydrophilic segment to enhance the copolymer's hydrophilicity because of its excellent hydrophilicity and biocompatibility (Lavasanifar, Samuel, & Kwon, 2002). There are many reports about copolymers of amino acids which were synthesized from PEG so as to regulate the hydrophilicity and biodegradable rate, such as poly(aspartic acid)/poly(ethylene glycol) block copolymer, poly(γ-benzyl L-glutamate)/poly(ethylene glycol) block copolymer, poly(L-lysine)/poly(ethylene glycol) block copolymer, poly(L-alanine)/poly(ethylene glycol) block copolymer and so on (Harada & Kataoka, 1995, 1998; Kugo, Ohji, Uno, & Nishino, 1987; Zhang, Ma, Li, & Wang, 2003). These amphiphilic copolymers could be self-assembled into nanoscaled micelles in suitable medium and some have been used as carriers of drug delivery systems (Kataoka et al., 2000; Li & Kwon, 2000). However, there are few reports about the completely biodegradable amphiphilic copolymers which were synthesized from hydrophobic poly(amino acid)s with hydrophilic and biodegradable products.

Cyclodextrins (CDs) are a family of cyclic oligosaccharides that are composed of α-1,4-linked glucopyranose subunits. CDs are produced from starch by enzymatic degradation. The most common CDs are of three types: α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) (Girek, Kozlowski, Koziol, Walkowiak, & Korus, 2005). Among them, β-CD which contains seven glucopyranose units is the most accessible, the lowest-priced and

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generally the most useful. It has low toxicity, hydrophilicity, excellent biocompatibility and biodegradability (Gao, Wang, Fan, & Ma, 2005; Prabaharan & Mano, 2006). One of the most remarkable applications of β-CD is its use as drug carriers in controlled release systems. As drug carriers, β-CD allows the solubilization, stabilization, and transport of hydrophobic drugs together with several pharmacological benefits such as the reduction of unwanted side effects (Prabaharan & Gong, 2008). Poly(L-leucine) (PLLA) is one of the synthetic biodegradable polypeptides, if the PLLA chain is combined with β-CD to prepare an amphiphilic completely biodegradable polymers, its hydrophilicity and biodegradability can be regulated, and thus its applications may extend widely.

In this work, a new amphiphilic completely biodegradable β-cyclodextrin/poly(L-leucine) (β-CD–PLLA) copolymer was synthesized by ring-opening polymerization of *N*-carboxy-L-leucine anhydride (LL-NCA) using mono-6-amino-β-cyclodextrin ($H_2N$-β-CD) as a initiator. Their structures were characterized and some properties of the copolymers were also investigated.

## 2. Experimental

### 2.1. Materials

L-Leucine (biochemical reagent) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and dried in vacuum for 24 h before use. Triphosgene (chemical reagent) was obtained from Haining Zhonglian Chemical Reagent Co. Ltd. (Zhejiang, China), used without any treatment. β-Cyclodextrin (β-CD, biochemical reagent, >98%) was purchased from Aoboxing Biotech Company Ltd. (Beijing, China), used without any treatment. *p*-Toluenesulfonyl chloride (TsCl, chemical reagent) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and recrystallized in the mixed solvents of chloroform and *n*-hexane before use. Tetrahydrofuran (THF, analytical reagent) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), dried and distilled in the presence of sodium immediately before use. *N*,*N*-dimethylformamide (DMF, analytical reagent) was supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and dried over $CaH_2$ and distilled before use. Other chemicals are all analytical reagents made in China and used without further purification.

### 2.2. Preparation of mono-6-amino-β-cyclodextrin ($H_2N$-β-CD)

$H_2N$-β-CD was prepared according to a literature procedure (Matsumoto, Noguchi, & Yoshida, 1998). First, β-CD and *p*-toluenesulfonyl chloride (TsCl) reacted to obtain mono-6-(*p*-tolylsulfonyl)-β-cyclodextrin (TsO-β-CD). Anal. calcd. for $C_{49}H_{76}O_{37}S$ (%): C: 45.65, H: 5.94, O: 45.92. Found (%): C: 45.78, H: 5.75, O: 45.82. Then, TsO-β-CD reacted with sodium azide ($NaN_3$) to give mono-6-azido-β-cyclodextrin ($N_3$-β-CD). Anal. calcd. for $C_{42}H_{69}N_3O_{34}$ (%): C: 43.49, H: 5.99, N: 3.62. Found (%): C: 43.76, H: 6.02, N: 3.57. Finally $N_3$-β-CD reacted with ammonium hydroxide ($NH_3{\cdot}H_2O$) to give mono-6-amino-β-cyclodextrin ($H_2N$-β-CD). Anal. calcd. for $C_{42}H_{71}NO_{34}$ (%): C: 44.48, H: 6.31, N: 1.24. Found (%): C: 44.62, H: 6.54, N: 1.21. The reaction route is shown in Scheme 1.

### 2.3. Preparation of N-carboxy-L-leucine anhydride (LL-NCA)

*N*-carboxy-L-leucine anhydride (LL-NCA) was prepared by the reaction of L-leucine with triphosgene in dried THF at 50 °C according to a literature procedure (Daly & Poché, 1988), melting point 76–77 °C. Yield: 80%. The reaction route is shown in Scheme 1.

### 2.4. Synthesis of β-cyclodextrin/poly(L-leucine) (β-CD–PLLA) copolymer

Certain amounts of $H_2N$-β-CD and LL-NCA were dissolved together in anhydrous *N*,*N*-dimethylformamide (DMF) and kept at 25 °C for 72 h in nitrogen atmosphere under stirring. The reaction mixture was poured to 200 ml of distilled water to give white precipitate. The precipitate was filtered off and washed with distilled water and THF, respectively, then dried in vacuum at room temperature for 24 h to give the desired β-CD–PLLA copolymers. The reaction scheme is shown in Scheme 1. Different molar ratios of the feeding NCA to $H_2N$-β-CD resulted in the corresponding copolymers with various compositions as listed in Table 1.

### 2.5. Characterization of the copolymers

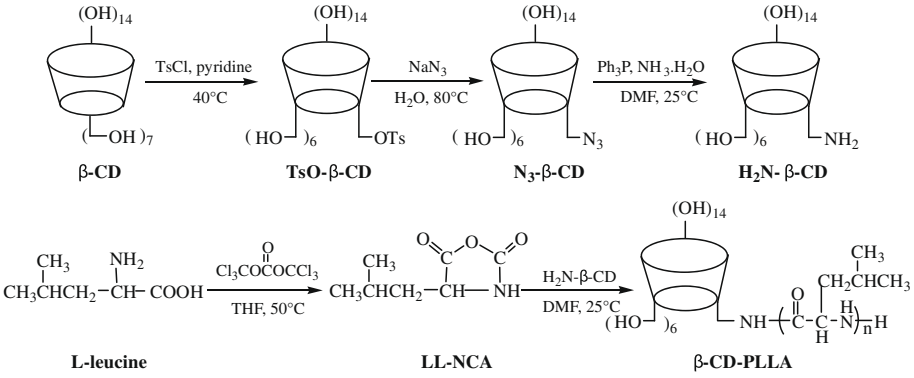
The IR spectra were collected by a Perkin-Elmer FT-IR spectrometer using KBr disks. Elemental analysis was performed on Thermo Electron Flash EA 1112 instrument. $^1$H NMR spectra were measured on a Varian Mercury-300 NMR spectrometer at room temperature, using $CDCl_3$ as solvent. Chemical shifts ($\delta$) were given in ppm using tetramethylsilane (TMS) as an internal reference. The gel permeation chromatography (GPC) measurement was conducted with a Waters 1515 GPC instrument equipped with a HT 3 column (effective molecular-weight range: 500–30,000) and a 2414 differential refractive index detector. THF was used as eluent at the flow rate of 1.0 ml/min at 30 °C, and the molecular weights were calibrated with polystyrene standards.

### 2.6. Preparation of micelles

The micelles were prepared using a solvent displacement method with a tetrahydrofuran/water (THF/$H_2O$) system (Wilhelm et al., 1991). A copolymer (50 mg) was first dissolved in 5 ml of THF in a 100 ml round bottomed flask, and then 40 ml of doubly distilled water was slowly added into the copolymer solution. The THF was removed using rotary evaporator at 25 °C for 2 h. The solution was transferred into a 50 ml volumetric flask after dilution to the calibration line with doubly distilled water to get 1 g/l micelles.

### 2.7. Micelle characterization

The critical micelle concentrations (CMC) of the copolymers were measured by fluorescence technique using pyrene as a probe. Pyrene solution (0.1 mg/l in acetone) was added into a series of volumetric flasks in such an amount that the final concentration of pyrene in each sample solution was $2.47 \times 10^{-7}$ mol/l after dilution to the calibration line, and then the acetone was removed completely. The polymer solution and doubly distilled water were added into the volumetric flasks containing the pyrene to obtain desired copolymer concentrations from $5 \times 10^{-5}$ to 1.0 mg/ml. The samples were sonicated for 15 min, heated at 50 °C for 2 h, and cooled to room temperature for overnight to equilibrate the pyrene and micelles. Steady-state fluorescence spectra were obtained on a Varian Cary Eclipse fluorescence spectrophotometer. For emission spectra, $\lambda_{ex}$ = 339 nm, and for excitation spectra, $\lambda_{em}$ = 390 nm. The scan rate was 120 nm/min and the slit opening was 2.5 nm. The size distribution of micelles was determined by dynamic light scattering (DLS) using Malvern Nano ZS instrument at 25 °C. The morphology of the micelles was investigated by transmission electron microscopy (TEM), carried out on a JEM-100SX electron microscope, operating at an accelerating voltage of 80 kV. Specimens were prepared by dipping copper grids into aqueous solutions of copolymers. The grids were then left to stand on a piece of filter paper and air dried before measurements.




**Scheme 1.** The route for the synthesis of β-cyclodextrin/poly(L-leucine) copolymer.

**Table 1**
Related data on β-CD–PLLA copolymers.

| Sample | $H_2N$-β-CD/NCA[a] | $W_{β\text{-CD}}/W_{PLLA}$[b] | Yield (%) | $M_n$[b] | $M_n$[c] | $M_w/M_n$ | CMC (g/l) | Micelle size (nm)[d] |
|---|---|---|---|---|---|---|---|---|
| β-CD–PLLA10 | 1/10 | 53/47 | 44.6 | 2150 | 2079 | 1.26 | $2.63 \times 10^{-3}$ | 201 |
| β-CD–PLLA30 | 1/30 | 27/73 | 50.0 | 4185 | 3986 | 1.25 | $1.77 \times 10^{-3}$ | 168 |
| β-CD–PLLA50 | 1/50 | 17/83 | 45.3 | 6558 | 6159 | 1.21 | $1.23 \times 10^{-3}$ | 142 |

[a] Molar ratio of $H_2N$-β-CD to NCA in feed.
[b] Determined by $^1H$ NMR in $CDCl_3$ solution.
[c] Determined by GPC in THF at 30 °C.
[d] Determined by dynamic light scattering in aqueous solution at 25 °C.

## 3. Results and discussion

### 3.1. Synthesis of β-cyclodextrin/poly(L-leucine) (β-CD–PLLA) copolymer

It is well known that primary amines, being more nucleophilic than basic, can be used as initiators for the ring-opening polymerization of NCA to prepare poly(α-amino acid)s, undergoing a nucleophilic addition to the carbonyl group of the NCA (Gotsche, Keul, & Höcker, 1995). Because $H_2N$-β-CD contains primary amine group, it can initiate ring-opening polymerization of LL-NCA to form copolymer. A series of the copolymers with various molecular weights were synthesized and the results were summarized in Table 1. It was found that the total molecular weights of the copolymers increased with the molar ratio of the feeding monomer NCA to the initiator.

The IR spectra of β-CD–PLLA copolymers were shown in Fig. 1. The absorption peak at 3297 $cm^{-1}$ was assigned to $\nu_{NH}$ stretch vibration, and the typical amido absorption bands I and II were observed at 1655 and 1554 $cm^{-1}$, respectively, indicating the formation of the polypeptide block. No carbonyl absorptions of NCA at 1820 and 1758 $cm^{-1}$ appeared in the spectra of all products, which indicated that no NCA residue co-existed in the polymer samples.

The $^1H$ NMR spectra of β-CD–PLLA were shown in Fig. 2. The peaks at 0.82 and 0.93 ppm were assigned to protons of the $-CH_3$ units of the PLLA segment. The peaks at 0.98–1.82 ppm were assigned to protons of the β-CD. The peak at 2.65 ppm was assigned to protons of the $-CH_2$ units of the PLLA segment. The peaks at 2.88–2.96 ppm were assigned to protons of –OH group in the β-CD. The peak at 4.21 ppm was assigned to protons of the –CH group in the –COCHNH– units of the PLLA segment. 8.03 ppm was assigned to proton of N–H group in the copolymer.

The GPC traces of the copolymers were shown in Fig. 3. The three copolymers showed unimodal molecular weight distribution. This further indicated that the copolymerization was completed successfully and there was no homo-polymer in the reaction product. GPC data of the copolymers are listed in Table 1.

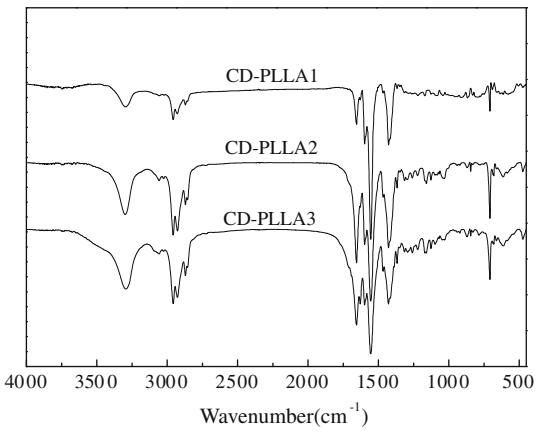



**Fig. 1.** IR spectra of β-CD–PLLA copolymers.

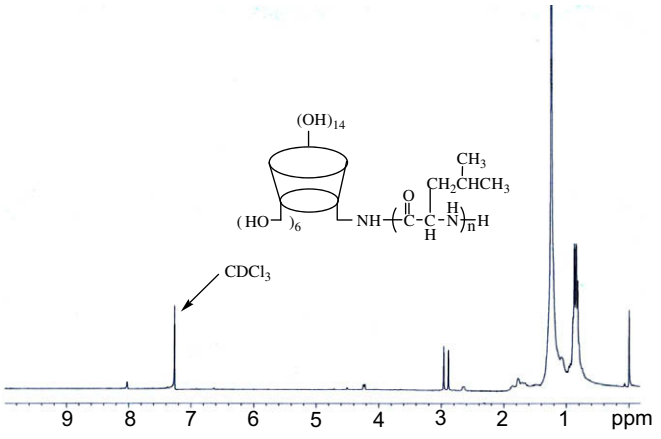



**Fig. 2.** $^1H$ NMR spectrum of β-CD–PLLA10 copolymer in $CDCl_3$.

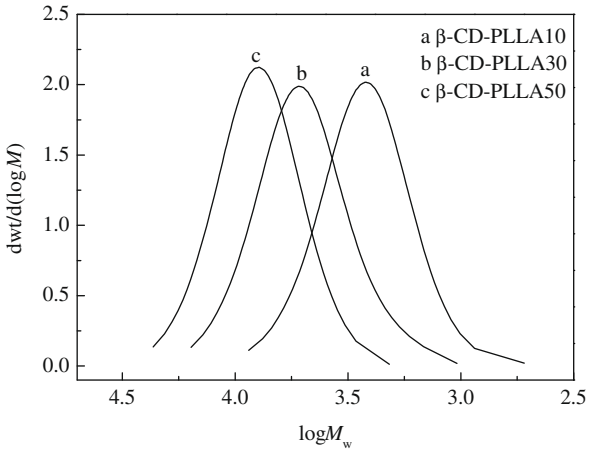



**Fig. 3.** GPC traces of β-CD–PLLA copolymers.

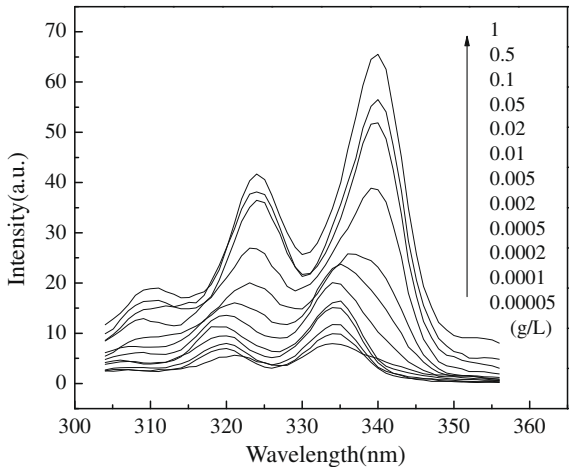



**Fig. 4.** Excitation spectra of pyrene as a function of β-CD–PLLA10 concentration in water.

### 3.2. Formation of micelles

Pyrene has been widely used as a probe to monitor the association and micellization of macromolecules in solutions because its photophysical character changes with the variation of the existing environment (Li, Lin, Chen, & Zhang, 2006). The micellar structures of β-CD–PLLA are confirmed by fluorescence technique using pyrene as a probe. The fluorescence excitation spectra of pyrene of $1.98 \times 10^{-7}$ mol/l in the presence of β-CD–PLLA10 at various concentrations are shown in Fig. 4. A red shift from 334 to 339 nm is observed with increasing concentration of β-CD–PLLA10, indicating that micellization takes place for the β-CD–PLLA copolymer. Such results can be attributed to the transfer of pyrene molecules from water to a hydrophobic environment within the micelle core.

The onset of micellization and the critical micelle concentrations (CMC) can also be obtained from the studies of excitation spectra (Deng et al., 2007). For the copolymer β-CD–PLLA10, 334 and 339 nm are chosen as the peak wavelength of the (0, 0) band in the pyrene excitation spectra in the aqueous phase and in the entirely hydrophobic core of polymeric micelle, respectively. The pyrene fluorescence intensity ratios ($I_{339}/I_{334}$) are plotted against the logarithm of copolymer concentration. The plots are shown in Fig. 5. Below a certain concentration, $I_{339}/I_{334}$ is constant, above this concentration, $I_{339}/I_{334}$ increases with increasing lgC and finally reaches a plateau. From this plot, the critical micelle concentration (CMC) of $2.63 \times 10^{-3}$ g/l was obtained from the intersection of two straight lines: the base line and the rapidly rising $I_{339}/I_{334}$ line. The CMC of β-CD–PLLA30 and β-CD–PLLA50 were also obtained from the same methods and listed in Table 1. The CMC values were in the magnitude of $10^{-3}$ g/l and reduced with the increasing of the proportion of hydrophobic parts, indicative of the amphiphilic nature of β-CD–PLLA copolymers.

### 3.3. Size and size distribution of β-CD–PLLA micelles

The size and size distribution of micelles were measured by DLS. As shown in Fig. 6, the mean diameter of micelles formed by β-CD–PLLA10, β-CD–PLLA30, and β-CD–PLLA50 were about 201, 168 and 142 nm, respectively. The size of micelle was reduced with the increasing of the proportion of hydrophobic part, so the size of the copolymer micelle could be adjusted by changed the proportion of hydrophobic part of the copolymer. The PLLA chain in the copolymer was a poly(α-amino acid) like protein. Therefore, it would form a α-helix by intramolecular hydrogen bonding or a β-sheet by intermolecular hydrogen bonding in aqueous solution. When PLLA content increases, the intermolecular hydrogen bonding is enhanced. The micellar aggregation number increases, then the density increases and the particle size decreases (Liu, Zhu, Zhao, Jiang, & Wu, 2000).

### 3.4. Morphology of β-CD–PLLA micelles

The morphology of the micelles was examined by TEM. Fig. 7 presents the TEM images of the micelles. They were all spherical. The micelle size formed by β-CD–PLLA10, β-CD–PLLA30, and β-CD–PLLA50 were about 160, 120 and 90 nm, respectively. The diameter data were smaller than that determined by DLS, because the micelle diameter determined by DLS represents their hydrodynamics diameter while that obtained by TEM is related to the collapsed micelles after water evaporation.

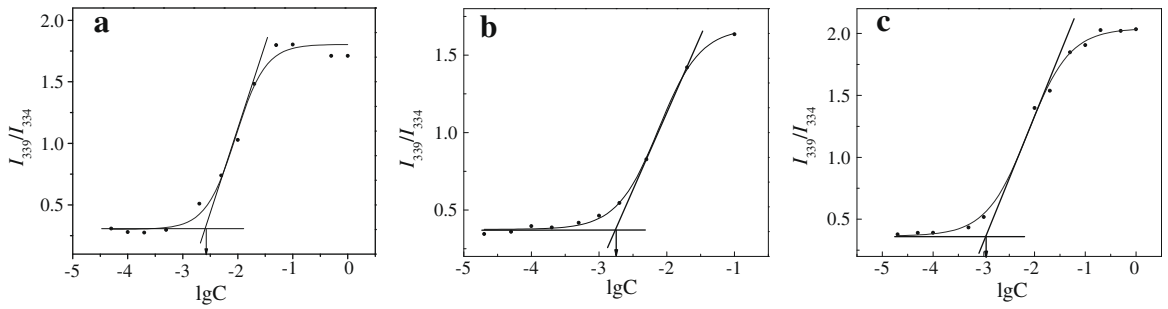



**Fig. 5.** Plots of $I_{339}/I_{334}$ versus logarithm of β-CD–PLLA10 (a), β-CD–PLLA30 (b) and β-CD–PLLA50 (c) concentrations.

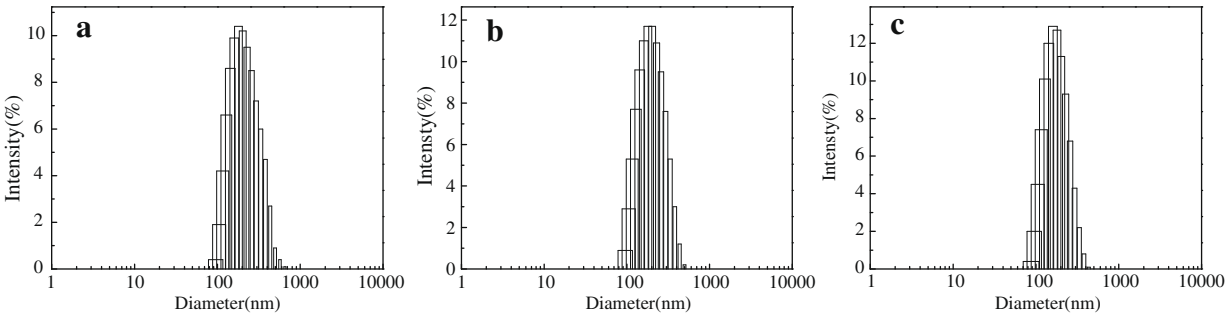



**Fig. 6.** The size distributions of β-CD–PLLA10 (a), β-CD–PLLA30 (b) and β-CD–PLLA50 (c) copolymer micelles in water measured by DLS.

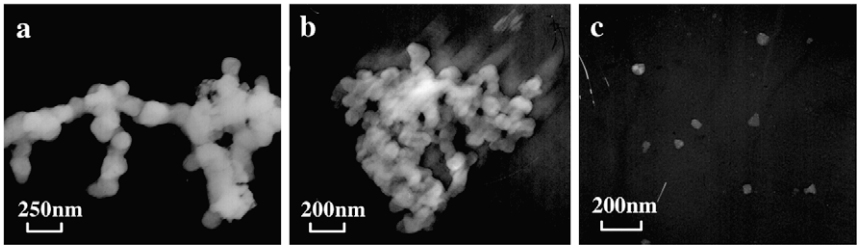



**Fig. 7.** TEM images of β-CD–PLLA10 (a), β-CD–PLLA30 (b) and β-CD–PLLA50 (c) copolymer micelles.

## 4. Conclusion

In this study, a new amphiphilic completely biodegradable β-CD–PLLA copolymer was synthesized by ring-opening polymerization of *N*-carboxy-L-leucine anhydride (LL-NCA) using mono-6-amino-β-cyclodextrin ($H_2N$-β-CD) as a initiator. Characterizations using IR, $^1H$ NMR and GPC had confirmed the designed structure. It could self-assemble into nano-micelles in water. The CMC value measured by fluorescence spectroscopy was in the magnitude of $10^{-3}$ g/l and decreased with the increasing of the proportion of hydrophobic parts. The average micelle size determined by DLS reduced with the increasing of the proportion of hydrophobic parts. TEM images demonstrated that they were all spherical. This amphiphilic property would be useful in drug delivery systems and other biomedical fields.

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