Size separation of ultrafine PEG-coated polycaprolactone nanoparticle and morphological studies by AFM

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Novel ultrafine polycaprolactone nanoparticles (PCL NPs) were prepared using poly(ethyleneglycol)$_{2000}$-distearyl phosphoethanolamine (PEGPE) as the oily phase emulsifier in the emulsion process, resulting in PEG-coated PCL NPs (PEGPE-PCL), which could be reduced and confined in size to below 50 nm. Size separation of PEG-coated polymer NPs remain significant challenges in the preparation for fundamental studies and applications. We used a gel filtration chromatography method for the rapid size separation of PEGPE-PCL and studied the physicochemical characteristics. The morphology of the core-shell PEGPE-PCL on a mica surface followed by NP concentration was observed by atomic force microscopy (AFM). It is important to separate size and define PEGPE-PCL structure for further potential in drug delivery application.

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1. Introduction

Biodegradable and biocompatible polymers such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and poly(e-caprolactone) (PCL) nanoparticles (NPs) are of great interest because of their importance in biomedical applications, for example as carriers for drug delivery systems [1, 2]. Polymeric NPs have found many applications due to their attractive properties, which include simple transformations to versatile preparations (by mixing or covalent conjugation) and biocompatibility. To develop further applications of polymer NPs *in vivo*, it is important that the rate of NPs clearance by macrophages be reduced to achieve a greater ability to target the site of interest. This requires the NPs to have a diameter of 100 nm or less [3].

Numerous techniques have previously been developed to prepare polymer NPs [4-6]. The most commonly utilized techniques for polymer NP preparation are the emulsion solvent diffusion method, the emulsion solvent evaporation method, the nanoprecipitation method, and the salting out method [7]. The most important biological consequence of modifying nanoparticular carriers with protecting polymers is a sharp increase in their circulation time and a decrease in their reticuloendothelial system (RES) accumulation [8]. PEG chains are known to be highly water soluble, highly hydrated, and able to serve as efficient steric protectors for various NPs in biological media. PEGylation can be carried out by different methods such as physical adsorption [9], covalent grafting, or with PEG copolymers [10, 11]. We previously reported utilizing the emulsion-solvent evaporation preparation method to prepare ultrafine PEG-coated poly(lactic-co-glycolic acid) (PLGA) NPs using PEGPE as the oily emulsifier [12]. These PEGPE-PLGA NPs exhibited high drug loading content, reduced burst release, high serum stability, and an enhanced cell uptake rate compared with traditional PLGA NPs. The size of the prepared PLGA NPs was able to be reduced and confined to below 50 nm. Although small PLGA NPs with diameters below 100 nm can be prepared by nanoprecipitation [13] or microfluidic fabrication methods [14], these NPs have poor drug encapsulation and rapid drug release. However, the emulsion solvent evaporation method offers the advantages of high encapsulation efficiency and mass production, and enables the simple preparation of sub-100 nm PLGA NPs.

Atomic force microscopy (AFM) was applied to various polymer NPs to investigate the morphology and mechanical properties (e.g., viscoelasticity and adhesion) [15, 16]. AFM is one of the most powerful tools for studying the surface characteristics of nanophase materials [17, 18]. The nanophase properties of materials can be analyzed using AFM in three operating modes: contact mode, non-contact and tapping mode. In general, AFM for NP characterization is both cost and time effective as well as easier to use than transmission electron microscopy (TEM). The resolution of AFM is greater or comparable to that of TEM, and the major advantages of AFM for NP characterization include being able to directly measure height and volume and having a 3D display.

2. Experimental

2.1 Materials

Polycaprolactone (average Mn 60,000), ammonium thiocyanate (NH_4SCN), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), coumarin-6 and Sepharose® CL-2B were purchased from Sigma Aldrich and used as supplied.1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEGPE) was purchased from Avanti.

2.2 Preparation of ultrafine PEGPE-PCL

Preparation of PEGPE-PCL, 0.5 mL chloroform containing PCL (5 mg) and PEGPE (10 mg) with coumarin-6 (0.2 mM) was slowly added into 3 mL H_2O following the mixed solution was emulsified for 1 min using a microtip probe sonicator with appropriate energy output. The chloroform was removed from the colloidal suspension by rotary evaporator, and the residual suspension centrifuged at 14,000 rpm for 20 min and the pellets then removed. The NP were washed three times using an Amicon Ultra-4 centrifugal filter (Millipore) with a molecular weight cutoff of 30,000 to remove free PEGPE. The yield of ultrafine PEGPE-PCL was 62 ± 1.7% (n=4).

2.3 Transmission electron microscopy (TEM) measurements

TEM was performed on a Japan Hitachi, H-7650 instrument with an acceleration voltage of 80 kV. TEM samples were prepared by administering the NP suspension onto a copper grid followed by negatively staining for 10 seconds at room temperature with freshly prepared, and sterile-filtered 2% (w/v) uranyl acetate aqueous solution.

2.4 Quantitative determination of PEGPE.

PEGPE was measured colorimetrically with ammonium ferrothiocyanate. Samples were dissolved in 1 mL chloroform and mixed with 1 mL of ammonium ferrothiocyanate reagent (30 mg/mL NH_4SCN, 27 mg/mL $FeCl_3 \cdot 6H_2O$). The mixed solution was shaken for 3 minutes and centrifuged at 3,000 rpm and the red lower layer (chloroform) collected. The PEGPE derivative was determined at 470 nm absorbance.

2.5 Determination of encapsulation efficiency of coumarin-6

Coumarin-6 encapsulation efficiency (EE) was determined by applying the following equation: EE (%) = $M_{encapsulated} / M_{feed} \times 100\%$, where $M_{encapsulated}$ is the mass of Coumarin-6 entrapped within the PCL NPs and M_{feed} is the mass of the coumarin-6 feed in the preparation of PCL NP. The lyophilized NPs were dissolved in chloroform and Coumarin-6 fluorescence at 450 nm, with excitation wavelength of 495 nm, was measured using a fluorescence reader (infinite M200, TECAN).

3. Results and discussion

In this study, we utilized the oil-in-water (o/w) emulsion solvent evaporation technique to prepare a PEG-coated PCL NPs as shown in Fig. 1. It was assumed that the PEG segment of PEGPE would be exposed to the outer surface of the PCL NP because of its hydrophilicity, which would generate a PEG shell surrounding the PCL core. The size of the prepared PEGPE-PCL could be reduced and confined to below 50 nm, allowing for the simple preparation of ultrafine PEG-coated polymer NPs. Fig. 2a shows the negative stain morphology of the ultrafine PEGPE-PCL observed by TEM. The size distribution measured from the TEM images of PEGPE-PCL were in the range of 15-50 nm. The zeta potential PEGPE-PCL was -38.5±1.2 mV. Further investigation revealed the PEGPE content to be about 85.5 % of the prepared PEGPE-PCL (Table 1). Purification by centrifugation can remove large and unstable NPs from the colloidal solution, but this method lacks precise control over particle size. To lower the variance in the NP size distribution, we used Sepharose® CL-2B gel to separate the PEGPE-PCL 15-30 nm (Fig. 2b) and 30-50 nm size ranges. Coumarin-6 is a commonly fluorescent dye, being encapsulated into PEGPE–PCL for size separation. Three size distribution of PEGPE–PCL had Coumarin-6 encapsulation efficiency and zeta potential were shown in Table 1. 15-30 nm of PEGPE-PLGA had higher encapsulation efficiency than 30-50 nm size distribution. For a PEGPE-PCL size 15-30 nm, the PEGPE content was about 90.6 % more than for the 30-50 nm size (66.7 %). It was assumed that more PEGPE would be exposed to the outer surface of PCL, which would generate a smaller PEGPE-PCL size. The PEGPE shell of polymer NPs may inhibit the escape of small molecules and burst release. The TEM image of PEGPE-PCL showed a PCL core of spherical shape but the surface morphology of the PEPEG shell was not clearly observable.

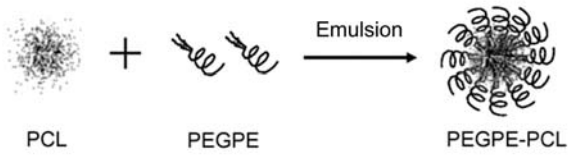

Fig. 1. Ultrafine PEGPE-PCL preparation by emulsion-solvent evaporation method.

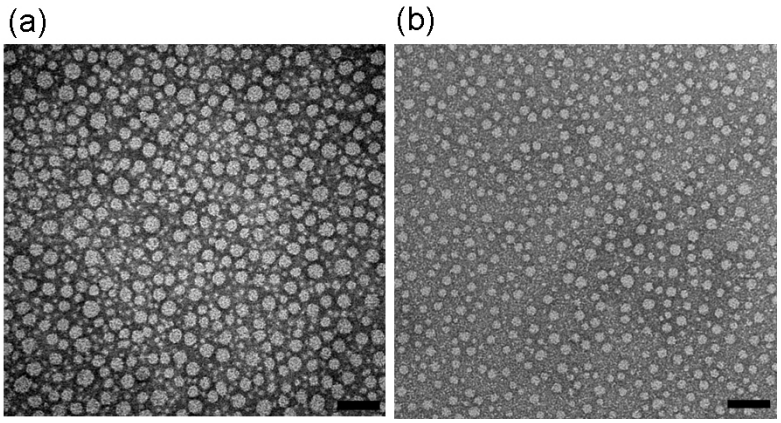

Fig. 2. Negative stain TEM images of (a) ultrafine PEGPE-PCL and (b) 15-30 nm PEGPE-PCL. (Scale bars: 100 nm).

Table 1. Physicochemical characteristics of the PEGPE-PCL.

PEGPE-PCL (n=4)	Zeta potential (mV)	PEGPE content (%)	Entrapment (%) (coumarin-6)
Size < 50 nm	-38.5 ± 1.2	85.5 ± 2.8	65.1 ± 1.8
15-30 nm	-39.2 ± 1.8	90.6 ± 1.5	76 ± 2.3
30-50nm	-32.7 ± 2.2	66.7 ± 3.2	48.2 ± 3.6

To demonstrate the possibility of PE-PEG coated PCL NPs by image, the PEGPE-PCL solution was dropped onto the mica slide for 10 minutes. The three samples were not washed, washed once and washed twice with H_2O, respectively. The mica was freshly cleaved just before use and then air-dried to be observable under AFM (D3100, Nanoscope IVa controller, Veeco) in tapping mode. The tip used in this study was a silicon nitride tip with a spring constant of about 0.7-3.8 N/m and a resonance frequency of about 50-90 kHz. All measurements were performed with a scan rate of 0.9 Hz. AFM analysis revealed the three morphologies in Fig. 3. Fig. 3a shows a multilayer PEGPE-PCL image; the high PEGPE-PCL concentration caused the NP surface of PEPEG to fuse together in the drying process. The concentration of PEGPE-PCL in mica was important. When PEGPE-PCL was dropped onto the mica that was washed once, we observed the core/shell shape of PEGPE-PCL shown in Fig. 3b. The core of PCL was encapsulated in the shell of PEGPE. In Fig. 3c, for PEGPE-PCL on mica that was washed twice, superfluous PEGPE was washed from the PCL surface. Therefore, it was only possible to observe the PCL core and not the PEGPE shell. Employing AFM was an effective method to study the morphology of PEGPE-PCL. The morphology of a well-defined three NP concentration of PEGPE-PCL was observed by AFM. Fig. 3d and Fig. 3e show the cross-sectional height profiles from Fig. 3b and Fig. 3c, respectively. The PEGPE shell of the PEGPE-PCL in Fig. 3b was completely flattened, forming layers of two different heights. The height of PEGPE-PCL was about 25 nm and that of the PEGPE shell about 10nm in Fig. 3d. The PCL NP height was about 15 nm in Fig. 3e. This PEGPE-PCL surface may correspond to a single-layered structure and the thickness of the PRGPE layer was about 5 nm.

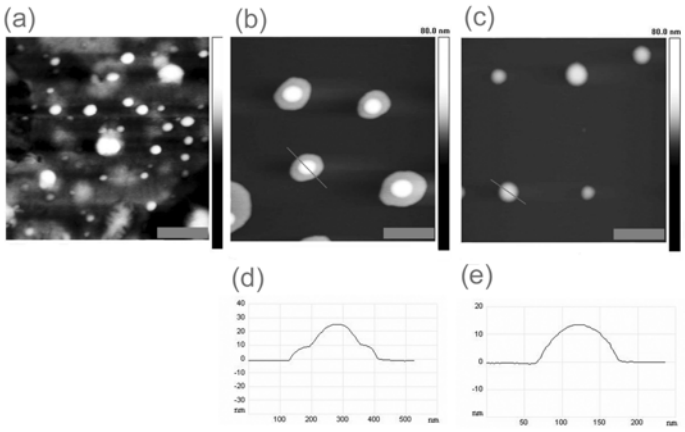

Fig. 3. Three AFM topographic images of different PEGPE-PCL concentrations. When PEGPE-PCL was dropped onto the mica (a) unwashed (b) washed once (c) washed twice. (Scale bars: 500nm) The height profiles of (d) and (e) were from the image of a cross-section line (b) and (c).

4. Conclusions

In summary, we have presented a simple and convenient method to prepare PEG-lipid base polymer NPs of ultrafine size and high drug loading content. The ultrafine PEGPE-PCL NPs have sub-50 nm diameter and PEG surface, which had not been previously achieved in polymer NP preparation. Moreover, we utilized the facile method of gel filtration chromatography to separate PEGPE-PCL into 15-30 nm and 30-50 nm sizes. The discrimination between the 15-30 nm and 30-50 nm sizes has great potential for drug delivery and cellular uptake applications. AFM analysis is a rapid and powerful technique that can provide information on morphology, size and size distribution. This study demonstrates that AFM can efficaciously differentiate three morphologies of different PEGPE-PCL NP concentrations. The clear morphology of lipid-based polymer NPs by AFM will be useful in other microscopic observations.

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