The preparation of phosphorylcholine-containing poly(L-lactide) nanoparticles with solvent evaporation method

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Abstract: Phosphorylcholine-containing poly(L-lactide) (PLLA-PC) is a kind of amphiphilic copolymer synthesized with L-lactide (LLA) as monomer and glycerophosphorylcholine as ring-opening reagent. In this paper, self-assembly nanoparticles of PLLA-PC were prepared with solvent evaporation method. The factors that affected the properties and stability of nanoparticles were investigated. Transmission electron microscope (TEM) indicated that PLLA-PC nanoparticles presented typical core/shell structure. The critical micelle concentration (CMC) was determined with fluorescent probe method. The results showed that the CMCs were quite low (< 1×10−3 g/l) and were dependent on LLA units in the copolymer. The size of the nanoparticles was detected by dynamic light scattering (DLS). The results indicated that the size could be affected by LLA units, the amount of solvent and water in the preparation process. On the other hand, the obtained nanoparticles were stable while being stored at 4°C, and hardly changed over the dilution with water, which was of great importance in venous injection. The solubility of clofazimine was better in aqueous solution of PLLA-PC nanoparticles than in pure water. This preliminary study suggested that PLLA-PC nanoparticles had a great potential as delivery system for hydrophobic drug.

Introduction

Biodegradable polymers, especially aliphatic polyesters such as polylactide (PLA), polyglycolide (PGA), and poly(ε-caprolactone) (PCL), have been investigated worldwide as biomaterials because of their biocompatibility and biodegradability. These polymers have been used for temporary therapeutic applications such as sutures, osteosynthetic devices, sustained drug delivery devices, and scaffolds in tissue engineering (e.g., Li, S et al. [1, 2, 3]). However, they suffer from some disadvantages and thus are largely limited in further application as drug delivery materials. For example, their strong hydrophobicity often results in protein adsorption and subsequent nonspecific uptake by the reticuloendothelial system after intravenous injection. In order to overcome the defect of polyesters, hydrophilic blocks have been incorporated into their backbones (e.g., Cai, Q; Chen, H.T; Okuda, T, Gillies, E.R; Guillaudeu, S.J et al. [4, 5, 6, 7, 8, 9, 10]). In drug delivery system, hydrophilic poly(ethylene glycol) (PEG) block is more frequently introduced into biodegradable polyesters. The introduction of PEG into polyesters can enhance hydrophilicity and reduce polyester particles to be uptaken by the mononuclear phagocytic system, which is sometimes referred to as the “stealth function” (e.g.,
Gref, R et al. [11, 12]). Meanwhile, the opsonization-inhibiting property of PEG enables long circulation times of drug in vivo (e.g., Li, X et al. [13]).

In addition to PEG modification, the introduction of phospholipid into biodegradable polyesters is one of the most notable strategies employed in recent years (e.g., Fredrik, N; Sheng, M; Iwasaki, Y; Beth, M.C; Junji, W; Fredrik, N et al. [14, 15, 16, 17, 18, 19]). Phosphoryl choline (PC) is a hydrophilic moiety in naturally occurring phospholipids presented in the cell membrane. By the introduction of PC into biodegradable polyesters, biocompatible and biodegradable copolymers can be obtained. Iwasaki, Y et al had firstly synthesized PC-containing copolymer by copolymerizing L-lactide with glycerophosphorylcholine (GPC) [16]. However, these copolymers have not been extensively investigated probably due to low yield of copolymerization (34%-69%). In order to solve the problem, our groups had prepared GPC by direct hydrolysis of lecithin from eggs and synthesized PC-containing PLLA, PLLA-PC, with yield up to 84%. (e.g. Wang, L.J et al. [20]). The results obtained by studying PLLA-PC showed that the hydrophilicity of PLLA was significantly enhanced due to the presence of PC. Furthermore, the protein adsorption and other biological responses such as the adhesion and activation of platelets were also suppressed (e.g., Chen, N.C et al. [21]. All these were consistent with the results reported by other researchers (Fredrik, N; Sheng, M; Iwasaki, Y; Beth, M. C; Junji, W et al. [14, 15, 16, 17, 18]). Meanwhile, PLLA-PC could support the adhesion and spreading of vascular endothelial cells, so the copolymer was cytocompatible e.g, Chen, Y.W et al. [22].

Moreover, Hisue et al reported poly(2-methacryloyloxyethyl phosphorylcholine)-block-poly(D, L-lactide) (PMPC-PLA) could form nanoparticles with the dialysis method, predigating that polylactides containing phosphorylcholines have the ability to self-assemble into aggregates due to their amphiphilicity (e.g., Gingho, H et al. [23]). In our previous work, self-assembly micelles of PLLA-PC (molar LLA: PC=5:1) were prepared with film rehydration method. The properties and capacity to encapsulate hydrophobic drugs of PLLA-PC micelles were preliminarily investigated. The micelles exhibited a capacity to improve the solubility of hydrophobic drug in aqueous environment (e.g., QIU, Z et al. [24]). Thus PLLA-PC copolymer appears very promising as a novel material for drug delivery systems. However, it hardly formed micelle with film rehydration method because the longer the chains of LLA exist in the PLLA-PC copolymer, the stronger the hydrophobicity is in copolymer. So, in this study, self-assembling nanoparticles of PLLA-PC with longer chains of LLA were prepared with solvent evaporation method, which is frequently used for hydrophobic polymers to prepare nanoparticles in drug delivery system (e.g., Sunghar, K et al. [25]). The morphology of the nanoparticles, the factors that affected the properties and stability of nanoparticles were investigated.

Results and discussion

The preparation and morphology of PLLA-PC nanoparticles

In this study, LLA: PC molar ratio in the copolymer was higher than 10, according to the integration ratio between signals at 5.15 ppm from methine protons of LLA and at 3.25 ppm from the –N⁺ (CH₃)₃ of PC units in the ¹HNMR spectrum. Owing to the existence of more hydrophobic LLA units in PLLA-PC, film rehydration method was thus not suitable for the preparation of nanoparticles.
For materials which are not dissolvable in water, their nanoparticles can be prepared with the aid of organic solvents, namely, oil/water solvent evaporation method (e.g., Sunghar, K et al. [25]). The preparative method is fit either for totally hydrophobic polymers or for amphiphilic polymers. In these cases, the solvent evaporation method is used to prepare particles. The specific procedure of solvent evaporation method is that polymers are first dissolved in an appropriate organic solvent and then mixed with water under mechanical stirring or ultra-sonication, finally, the organic solvent is excluded with an appropriate technique. In this study, self-assembly PLLA-PC nanoparticles were prepared with solvent evaporation method using acetone as the organic solvent. The reason is that acetone has good solubility for amphiphilic PLLA-PC.

Figure 1 shows TEM image of the nanoparticles of PLLA-PC copolymers prepared with solvent evaporation method. The nanoparticles exhibited typical core/shell structure with sizes ranging from 36 to 156 nm, which was close to the average size 136 nm estimated by DLS.

**Fig. 1.** TEM photograph of PLLA-PC (LLA: PC = 30:1, 0.5 mg/ml) nanoparticles.

*The critical micelle concentration of various ratios of PLLA-PC nanoparticles*

Amphiphilic block copolymers could self-assemble to form micelles in aqueous media. The critical micelle concentrations of the copolymers were determined from pyrene excitation spectra (e.g., Guosen, H; Subbu, S.V et al. [26, 27]). A red shift of pyrene was observed in the excitation spectrum when the concentration of copolymer was increased. Specifically, pyrene in water at 334 nm shifted to 336 nm upon addition of copolymer. This red shift resulted from the transfer of Pyrene molecules from a water environment to the hydrophobic micellar core and thus provided information on the location of the pyrene probe in the system. Plots of the intensity ratio I\textsubscript{336}/I\textsubscript{334} from the excitation spectra versus copolymer concentration are shown in Figure 3. At low concentrations of copolymer, the total fluorescence intensity ratio remains nearly unchanged. As the concentration of copolymer increased, the intensity ratio started to increase dramatically at a certain copolymer concentration, where the CMC was determined from the intersection of two straight lines, as shown in Figure 3. As summarized in Table 1, the CMC values of the block copolymers depended upon the LL/ PC ratios, especially the more hydrophobic blocks (LLA) in the copolymer, the less CMC values, indicating that micelle formation becomes easier as the number of hydrophobic in copolymer increased. The CMC decreased in the order 20:1, 30:1, 50:1 block copolymer, this probably resulted from the fact that
the hydrophobic interactions increased as the hydrophobic blocks increased. But, the CMC of the molar ratio of the LLA: PC (LLA: PC=10:1) was smaller than that of above. It may be explained by the hypothesis that the self-assembly particles of PLLA-PC (molar LLA: PC = 10:1) compose fewer molecules due to the better hydrophilicity, which makes it easier to form micelles.

**Effects of the sizes of the PLLA-PC nanoparticles**

DLS was employed to evaluate the size of the nanoparticles. All nanoparticles were prepared by the same stirring rate. Table 1 showed the average sizes of PLLA-PC nanoparticles in solution with various molar ratios. It is obvious that the sizes shrank over the increase in molar ratio of LLA: PC except 10:1. This was assigned to stronger hydrophobic interactions between the hydrophobic chains of PLLA-PC molecules. However, the average size of PLLA-PC (molar LLA: PC = 10:1, 1 mg/ml) nanoparticles was detected to be 106 nm, which was the least and smaller than those of PLLA-PC in molar ratios. Meanwhile, the polydispersity of nanoparticles is measured to be 0.123 which was larger than that of PLLA-PC (molar LLA: PC >10:1, 1 mg/ml) nanoparticles, which might be explained by the hypothesis that the self-assembly particles of PLLA-PC (molar LLA: PC = 10:1) composed fewer molecules due to the better hydrophilicity, which was also accorded with the CMC value.

**Tab. 1.** The CMCs and the particle sizes of nanoparticles.

<table>
<thead>
<tr>
<th>LLA: PC</th>
<th>10:1</th>
<th>20:1</th>
<th>30:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC (mg/ml)</td>
<td>$1.97 \times 10^{-4}$</td>
<td>$4.75 \times 10^{-4}$</td>
<td>$3.57 \times 10^{-4}$</td>
<td>$2.30 \times 10^{-4}$</td>
</tr>
<tr>
<td>The average sizes (nm)*</td>
<td>106±2.52</td>
<td>167±1.53</td>
<td>158±2.89</td>
<td>136±3.79</td>
</tr>
<tr>
<td>Polydispersity*</td>
<td>0.123±0.01</td>
<td>0.097±0.02</td>
<td>0.091±0.01</td>
<td>0.087±0.01</td>
</tr>
</tbody>
</table>

*The concentration of the copolymer was 20 mg/ml, the concentration of the nanoparticles was 1 mg/ml. Three measurements were repeated for each polymer.

As described in the experimental section, the self-assembly nanoparticles were prepared by the injection of polymer/acetone solution into stirred water. Thus the amount of solvent, namely the concentrations of polymer in acetone, was of importance and it might influence the aggregation of the copolymer. In Table 2 row 3, 2 and 1 are shown the average sizes of PLLA-PC nanoparticles to be 92 nm, 136 nm and 175 nm, while the amount of the organic solvent was 1.0, 0.25 and 0.1 mL, respectively. It meant the size was closely correlated with the amount of the organic solvent or concentration of the polymer. It is well-known, the viscosity of the copolymer increases with the increase in the concentration. Thus the dispersion of the polymer solution in water became more difficult and consequently led to aggregation in large sizes. Additionally, the influences of the amount of the water on the aggregation of the copolymer were also investigated (row 3, 4, 5). As shown in Table 2, the copolymer solutions were injected in various amount of water. The average size of the copolymer was 92 nm, 141 nm and 136 nm, respectively, increasing with the increase of the amount of the water.

The change of the average size is shown in Figure 4 for the prepared nanoparticle solution diluted with water by various dilution times. The average size of
nanoparticles hardly changed with increased dilution times (a). The phenomena were also found with molar ratio of LLA: PC as 10:1 (b) and the concentration of nanoparticles was 5 mg/ml (c). These results indicated that the sizes of PLLA-PC particles could be adjusted by injecting the solution of PLLA-PC into different amount of water in the preparation process, and dilution had no effect on particle sizes. This character was of key importance for intravenous injection.

**Tab. 2.** The effects of the preparation conditions on the sizes of PLLA-PC nanoparticles.

<table>
<thead>
<tr>
<th>The amount of the organic solvent (mL)</th>
<th>The amount of the water (mL)</th>
<th>The average sizes (nm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>5</td>
<td>175±1.41 a)</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
<td>136±5.29 a)</td>
</tr>
<tr>
<td>1.00</td>
<td>5</td>
<td>92±2.52 a)</td>
</tr>
<tr>
<td>1.00</td>
<td>50</td>
<td>136±4.02 a)</td>
</tr>
<tr>
<td>0.10</td>
<td>50</td>
<td>141±4.58 b)</td>
</tr>
</tbody>
</table>

a) PLLA-PC (LLA :PC = 50:1), the weight of the copolymer was 5 mg.  
b) PLLA-PC (LLA:PC=50:1), the weight of the copolymer was 0.5 mg.

**Fig. 2.** The effects of dilution on the particle sizes of PLLA-PC nanoparticles. (a, •): LLA: PC = 50:1, 1 mg/ml; (b, ■): LLA: PC = 12:1, 1 mg/ml; (c, ▲): LLA: PC = 50:1, 5 mg/ml.

The store property of PLLA-PC nanoparticles was also tested. Nanoparticle solutions (molar LLA: PC = 50:1, 1 mg/ml) were stored at 4 °C and 37 °C, respectively. The results of pH test presented that the pH value hardly changed after 18 days when the store temperature was 4 °C. In contrast, when the temperature was 37 °C, the pH decreased from 6.70 to 3.85 in the same period, and even to 2.75 after 50 days, as
shown in Table 3. This might be explained by the degradation of PLLA-PC copolymer which resulted in soluble monomers or oligomers during the storage period. This hypothesis was confirmed by GPC examination. Beyond 9 days, the value of number-average molecular weight (Mn) was 3893. They were 3613, 3313 and 1842, respectively, when the particles were kept at 37 °C for 18, 30 and 50 days. These results are difficult interpreted via erosion mechanism of PLA-PEG degradation reported by Stefani M et al (e.g., Muriel Stefan et al. [28]), But the Mn change of PLLA-PC degradation are consistent with the results of a continuous decrease of molecular weight of MeO-PEG-PLGA-based nanoparticles with time at 37 °C (e.g., Zweers M.L.T et al. [29]) studied. These results indicated that the nanoparticles were stable at 4 °C. Its degradation at 37 °C also implied that further investigation should be made on the influence of the degradation on the encapsulation and the release of the drugs in the particles.

Tab. 3. The effects of store time on the pH values and the molecular weight of PLLA-PC nanoparticles.

<table>
<thead>
<tr>
<th>Days (d)</th>
<th>0 d</th>
<th>9 d</th>
<th>18 d</th>
<th>30 d</th>
<th>50 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH**</td>
<td>6.70±0.15</td>
<td>4.71±0.22</td>
<td>3.85±0.07</td>
<td>3.18±0.04</td>
<td>2.76±0.05</td>
</tr>
<tr>
<td>Mn**</td>
<td>-</td>
<td>Mₙ=3836</td>
<td>Mₙ=3614</td>
<td>Mₙ=3276</td>
<td>Mₙ=1844</td>
</tr>
</tbody>
</table>

**PLLA-PC (LLA:PC = 50:1, 1mg/ml).

Encapsulation of hydrophobic drug
Clofazimine is a substituted iminophenazine derivative with anti-mycobacterial and inflammatory activity (e.g., Isam, I.S et al. [30]).

Fig. 3. The solubility of the clofazimine in the various ratios of PLLA-PC nanoparticles.
As most iminophenazine derivatives, clofazimine has the disadvantage of being a highly hydrophobic drug with inherent water solubility below 0.19 μg/ml. Various attempts have been made to enhance the solubility and bioavailability of clofazimine. Hernandez-Valdepena et al. (Hernandez-Valdepena et al. [31]) previously have studied polymeric nanosized aggregates formed by hydrophobized poly(methyl vinyl ether-alt-maleic acid) to solubilize clofazimine in neutral aqueous media. In the study, we used PLLA-PC nanoparticles as the carriers of clofazimine by solvent evaporation method. Figure 5 shows a higher solubility of clofazimine in PLLA-PC nanoparticles than that in pure water. This was because the hydrophobic interactions between the hydrophobic core of the nanoparticles and the clofazimine increased the solubility of clofazimine, which was a potential drug delivery alternative for hydrophobic drugs.

Conclusions
Self-assembly nanoparticles of PLLA-PC were prepared with solvent evaporation method. It presented a typical core/shell structure. The CMCs were quite low (<1×10^−3 mg/ml). The sizes of nanoparticles were regulated by the LLA units and the amount of organic solvent. They were also regulated by the injection of copolymer solution in different amount of water, but the obtained nanoparticles were stable over dilution with water. The PLLA-PC nanoparticles were stable while being stored at 4 °C. But they degraded at 37 °C. Moreover, the solubility of clofazimine was better in the PLLA-PC nanoparticles than that in pure water. These results provided basic data for further application of PLLA-PC self-assembly nanoparticles as novel drug delivery alternative.

Experimental part
Materials
L-Lactide (LLA) (Fushun Tianyuan Bioabsorbable Materials CO. LTD) was recrystallized in ethyl acetate and toluene. Glycerophosphorylcholine was obtained by hydrolysis of lecithin extracted from eggs. Stannous octoate (Aldrich) was distilled before use. Tetrabutylammonium hydroxide (approx. 20%) was purchased from Ke Long Co. Sichuan. Toluene was dried in sodium and distilled before use. All the other reagents and solvents were analytical grade and used as received.

Preparation of nanoparticles
PLLA-PC copolymers were synthesized by ring-opening polymerization using glycerophosphorylcholine as initiator and stannous octoate (0.1 wt %) as catalyst (e.g., Wang, L.J et al. [20]). Nanoparticles were prepared with solvent evaporation method. 50 mg of PLLA-PC copolymer was dissolved in 2.5 mL of acetone, and the copolymer solution was added dropwise to 50 mL of distilled water with stirring to yield nanoparticles. After the acetone was removed by evaporation, the dispersion was filtrated with a filter (diameter = 0.45 μm in pore size).

Measurements
Proton nuclear magnetic resonance (1HNMR) spectra were recorded at room temperature with a Bruker spectrometer operating at 400 MHz by using CDC13 as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference.
Gel permeation chromatography (GPC) measurements were performed on a Waters 717 apparatus equipped with an RI detector. THF was used as the mobile phase at a flow rate of 1.0 ml/min. A 0.1% (w/v) solution (50 μL) was injected for each analysis. Calibration was accomplished with polystyrene standards (Polysciences, Warrington, PA).

Transmission electron microscopy (TEM) was carried out on a JEM-100CX electron microscope, operating at an accelerating voltage of 80 kV. Specimens were prepared by dipping a copper grid into aqueous solutions of copolymers dyed with phosphotungatic acid (PTA). The grid was then left to stand on a piece of filter paper and air dried before observation.

Dynamic light scattering (DLS) measurements on nanoparticle solutions were performed on a NANOSIZE 3600, after the sample solutions were filtered using a Millipore 0.45 μm filter. The scattered light of a vertically polarized He-Ne laser (395 nm) was measured at an angle of 90° at room temperature. Three measurements were repeated for each polymer.

The critical micelle concentration (CMC) was performed on a fluorospectrophotometer (F-7000FL220-240V). The pyrene was used to measure the critical micelle concentrations of copolymers and the CMCs were determined from pyrene excitation spectra. The emission and excitation wavelength were 395 nm and 300 nm, respectively.

**Hydrophobic drug encapsulation**

Clofazimine was used as a model hydrophobic drug to test the encapsulation capacity of PLLA-PC nanoparticles. Drug-loaded nanoparticles were prepared by adding drugs into the PLLA-PC acetone solution and adding it dropwise to distilled water with stirring. After the acetone was removed by evaporation, the system was then filtrated with a filter (diameter = 0.45 μm in pore size). Acetone-water mixed solvents were then added into the filtrate to dissolve the encapsulated clofazimine. The absorption value of the acetone solution was determined on a spectrometer (U.V./Visible Lambda 15/Perkin Elmer) at 480 nm. The corresponding drug concentration was calculated with the standard equation obtained at 480 nm. Acetone-water served as a blank control.

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**References**