Transactions of the Materials Research Society of Japan 26 [3] 897-900 (2001)

Blood compatible nanoparticles immobilized with 2-methacryloyloxyethyl phosphorylcholine polymer

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The purpose of this study is preparation of the blood compatible nanoparticles which can inject into the blood vessel and deliver drug to target organs. The poly(L-lactic acid) (PLA) nanoparticles immobilized with a phospholipid polar group, 2-methacryloyloxyethyl phosphorylcholine (MPC) units, which has excellent blood compatibility, were prepared by a solvent evaporation technique using the water-soluble amphiphilic MPC polymer as an emulsifier and a surface modifier. The diameter and ζ-potential of the nanoparticles strongly depended on the concentration of the MPC polymer. The X-ray photoelectron spectroscopic analysis indicated that the phospholipid polar groups of the MPC polymer were located at the surface of the nanoparticles, that is, the MPC polymer was immobilized on the PLA surface. The amount of protein, bovine serum albumin, adsorbed on the nanoparticles was significantly smaller than that on the conventional polystyrene nanoparticles. Moreover, the MPC polymer nanoparticles did not induce the platelet activation and could avoid the recognition reaction from macrophages. That is, the MPC polymer nanoparticles were "stealth" in a bloodstream for a long period and may be applied for drug carrier to target organs.
Key words: poly(L-lactic acid), phospholipid polymer, polymer nanoparticles, protein adsorption, drug carrier

1. INTRODUCTION

Biodegradable polymers represented poly (L-lactic acid) (PLA) have been investigated in the pharmaceutical field to release drug for long period or deliver it to target organs. However, when the PLA is contacted with living organisms or injected into blood vessel as a small particles, the proteins are immediately adsorbed and conformational change of them was induced on the PLA surface. And macrophages are activated and, finally, they are phagocyted by macrophages. Therefore, the surface modification of PLA particles with a blood compatible material is necessary in order to prolong the circulation period in the bloodstream. We have synthesized 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers which are inspired from surface structure of biomembrane. The MPC polymers show excellent blood compatibility, i.e., suppression of protein adsorption and cell adhesion. In this study, PLA nanoparticles immobilized with MPC polymer were prepared by solvent evaporation technique using water-soluble amphiphilic MPC polymer as both an emulsifier and a surface modifier. The characterization and function of PLA nanoparticles immobilized with MPC polymer were described.

2. EXPERIMENTAL

2.1 Materials

BMA was purified by distillation under a reduced pressure of 30 mmHg and fraction of b.p. 60 °C. MPC was synthesized by a previously reported method and purified by recrystallization from acetonitrile[1]. Water-soluble poly(MPC-co-BMA) with a 0.30 MPC unit mole fraction (PMB30W) was synthesized by a conventional radical polymerization technique using *t*-butyl peroxy neodecanoate as the initiator. The obtained PMB30W was purified by an ultrafiltration method from its aqueous solution[2]. The molecular weight of PMB30W was 5.0×10^4 which was determined by light scattering. The chemical structure of PMB30W is shown in Figure 1.

Poly (L-lactic) acid (Mn = 2 x 10^4) was purchased from Wako Pure Chemicals Industries, Ltd., Osaka, Japan, and used without further purification. Polystyrene nanoparticles were purchased from Polysciences, Inc., Warrington, PA, USA (average diameter = 202 nm). Bovine serum albumin (BSA) was purchased from Sigma Chemicals, St. Louis, MO, USA, and used without further purification. The other reagents were extra pure grade and used without further purification.

2.2 Preparation of PLA nanoparticles

Preparation of PLA nanoparticles in PMB30W aqueous solution was carried out by the solvent evaporation technique. A brief explanation is as follows. In a sample tube, 40 mL of aqueous solution containing a given amount of PMB30W was placed, and stirred at 400 rpm with cooling in an ice bath. PLA (20 mg) was dissolved in 2.0 mL of methylene dichloride. The PLA solution was then dropped into the PMB30W aqueous solution. The mixture was sonicated using a probe-type generator (Sonifier 250, Branson, USA) for 30 min and kept under reduced pressure for 2 h to evaporate the methylene dichloride. The formed nanoparticles (PMB30W/PLA nanoparticles) were fractionated by centrifugation at 10,300 g at 4 °C for 30 min. The nanoparticles as a precipitate were resuspended with distilled water and centrifuged again under the same conditions. This procedure was repeated three times to completely remove any free PMB30W. The nanoparticles were finally lyophilized.

2.3 Characterization of PLA nanoparticles

One drop of suspension containing the PMB30W/PLA nanoparticles, which were prepared in the 1.0 mg/mL PMB30W solution, was spread on a mica plate. It was dried in air at room temperature. The morphology of the PMB30W/PLA nanoparticles was observed using atomic force microscopy (AFM, SPI-3800, Seiko, Chiba, Japan). The particle size, size distribution and ζ-potential of the PMB30W/PLA nanoparticles were determined using an electrophoretic light scattering (ELS, ELS-8000, Otsuka Electronics, Ltd., Tokyo, Japan) with 60 degrees for the particle size distribution and 0 degrees for the ζ-potential as the scattering angle. These experiments were carried out in phosphate-buffered saline (Dulbecco's PBS, pH7.4; ionic strength, 0.14 M).

The density of the MPC unit on the surface of the nanoparticles was analyzed with an X-ray photoelectron spectroscope (XPS, ESCA-200, Scienta, Uppsala, Sweden). Preparation of the sample was carried out in the same way as that of the AFM observations except for using a poly(ethylene terephthalete) plate as the substrate.

2.4 Adsorption of protein on PMB30W/PLA nanoparticles

BSA was dissolved in Dulbecco's PBS and the concentration was adjusted to 20 mg/mL. Polystyrene nanoparticles or PMB30W/PLA nanoparticles suspended in PBS were added to the BSA solution. The final concentration of nanoparticles in the suspension was 0.05 wt%. After the suspension was incubated for 30, 60, and 180 min at 37 °C, the nanoparticles were precipitated by centrifugation (10,300 g, 30 min, 4 °C). The concentration of BSA in the supernatant was measured using a protein analysis kit (microBCA protein assay reagent kit, Pierce, Rockford, IL, USA). The amount of adsorbed BSA on the PMB30W/PLA nanoparticles or polystyrene nanoparticles was calculated by comparison of the BSA concentration in the supernatant with that of the original BSA solution.

2.6 Phagocyte test with macrophages

Endoceliac macrophages were recovered from 6 weeks female ddy mouse. The obtained macrophages were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10 mg/mL penicillin, 25 mg/mL streptomycin, and amphotericin B in 0.85 % saline, at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. After 1 h incubation, the media was withdrawn and the nonadherent cells removed by washing with RPMI-1640. Subsequently, 0.5 mL of media containing PMB30W/PLA nanoparticles and polystyrene nanoparticles loaded fluorescence probe in a concentration of 0.1 wt% were added into each well and allowed to co-incubate with the cells. After 1 h incubation, the macrophages were washed with RPMI-1640 and phosphate buffer solution. The nanoparticles phagocyted by macrophages were observed with a fluorescence microscope.

3. RESULTS AND DISCUSSION

3.1 Characterization of PMB30W/PLA nanoparticles

The PMB30W/PLA nanoparticles could be prepared by a solvent evaporation technique from oil in water emulsion system using the water-soluble amphiphilic phospholipid polymer, PMB30W, which served as both an emulsifier and surface modifier. The PMB30W forms stable polymer aggregate when the concentration of it is above 1.0 mg/mL[2]. Thus, in the PMB30W aqueous solution with 1.0 mg/mL, the PLA nanoparticles were formed effectively and stored for more than 1 month at room temperature

without aggregation. It is suggested that the surface is covered with hydrophilic phosphorylcholine groups. On the contrary, the dispersion ability of the nanoparticles was poor when they prepared at a lower PMB30W concentration (below 0.01 mg/mL) . They gradually aggregated during the storage. Figure 1 is a schematic representation of the ideal structure of the PMB30W/PLA nanoparticle. The PMB30W chains were considered to be stably immobilized on the surface of the PLA nanoparticles, because both PLA and PMB30W chains interlocked each other.

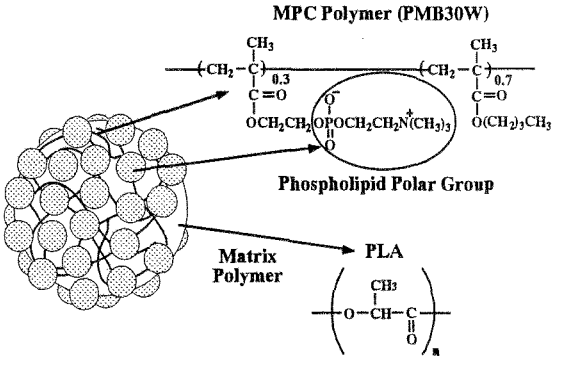

Figure 1 Chemical structure of PMB30W and poly(L-lactic) acid (PLA) and shematic representation of PMB30W/PLA nanoparticles.

An AFM view of the PMB30W/PLA nanoparticles prepared in 1.0 mg/mL of PMB30W aqueous solution is shown in Figure 2. The PMB30W/PLA nanoparticle had a spherical shape in the diameter range of 100-300 nm.

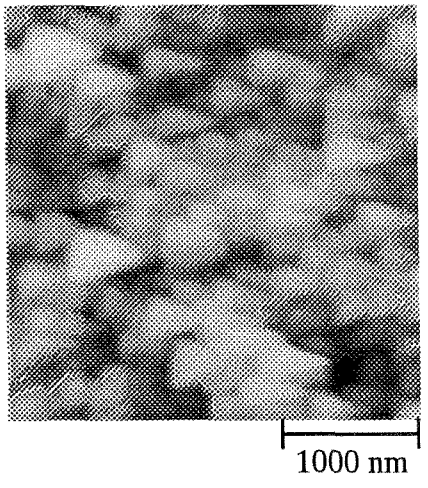

Figure 2 AFM view of PMB30W/PLA nanoparticles prepared in 1.0 mg/mL of PMB30W.

The relation between the PMB30W concentration and size of the nanoparticles is shown in Figure 3. The mean diameter of the nanoparticles strongly depended on the PMB30W concentration. The mean diameter became progressively smaller as the concentration of PMB30W increased. For the nanoparticles prepared in 1.0 mg/mL of PMB30W aqueous solution, it was 221 nm. This result was in good agreement with those of the AFM observation. The PMB30W concentration dependence of the ζ-potential of the PMB30W/PLA nanoparticles is also summarized in Figure 3. The nanoparticles prepared in a lower aqueous PMB30W concentration solution had a highly negative ζ-potential in PBS. On the other hand, the ζ-potential of the PMB30W/PLA nanoparticles prepared in a 1.0 mg/mL PMB30W aqueous solution was -2.5 mV in PBS. Although, the ζ-potential is nearly neutral, the surface of nanoparticles were covered with hydrophilic MPC unit. Therefore, the PMB30W/PLA nanoparticles were stable dispersed in aqueous media. Generally speaking, the biocomponents including the plasma protein have a slightly negative charge. The positive charged nanoparticles can significantly interact with proteins. On the other hand, the nanoparticles with a large negative ζ-potential induce unfavorable immunological reactions. That is, the PMB30W/PLA nanoparticles prepared in the 1.0 mg/mL PMB30W aqueous solution, which have a lightly anionic surface ζ-potential, satisfied one of the requirements for obtaining good blood compatibility.

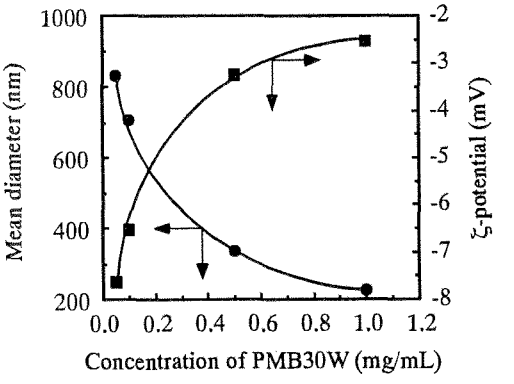

Figure 3 Dependence on the PMB30W concentration of the mean diameter and the ζ-potential of PMB30W/PLA nanoparticles. Measurement condition:Solvent : Dulbecco PBS (including 136 mM NaCl) Temperature : room temperature (25 °C).

The XPS spectra of C_{1s} and P_{2p} on the surface of the PMB30W/PLA nanoparticles are shown in Figure 4. In the case of nanoparticles prepared in a 0.01 mg/mL PMB30W aqueous solution, the phosphorous peak at 133 eV could not be observed, and the carbon in CH_3-, -COC-, -C(=O)- were similar to that of only PLA. On the other hand, those prepared in a 1.0 mg/mL PMB30W aqueous solution had a phosphorous peak and a strong carbon peak attributed to the methyl or methylene carbons (285 eV) present. These results indicated that the PMB30W was

existed on the surface of the PLA nanoparticles and stably immobilized on the surface of PLA nanoparticles when the PLA nanoparticles were prepared in a 1.0 mg/mL PMB30W aqueous solution. The PMB30W effectively

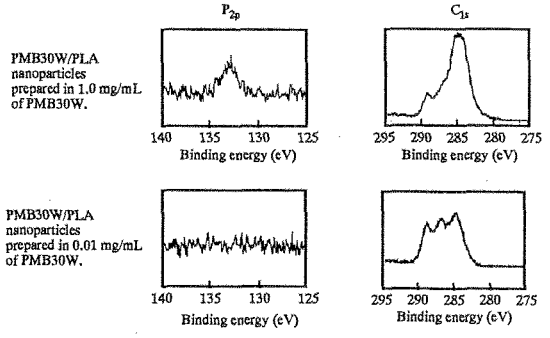

Figure 4 XPS spectra of PMB30W/PLA nanoparticles prepared in 1.0 mg/mL and 0.01 mg/mL of PMB30W concentration, respectively.

functioned as both an emulsifier and a surface modifier to prepare the nanoparticles due to its amphiphilic nature. In the following study, PMB30W/PLA nanoparticles prepared in a 1.0 mg/mL PMB30W aqueous solution were used.

Figure 5 shows the amount of BSA adsorbed on polystyrene nanoparticles and PMB30W/PLA nanoparticles. The amount of BSA adsorbed on the polystyrene nanoparticles was higher than that on the PMB30W/PLA nanoparticles and increased with an increase in the incubation time. The adsorption of BSA hardly occurred on the PMB30W/PLA nanoparticles prepared in both concentrations of the PMB30W aqueous solutions. Protein adsorption is one of the important phenomena for evaluating the functionality of nanoparticles for application in the various fields of bioengineering. In this study, we used the BSA as a model protein for evaluating protein adsorption on the

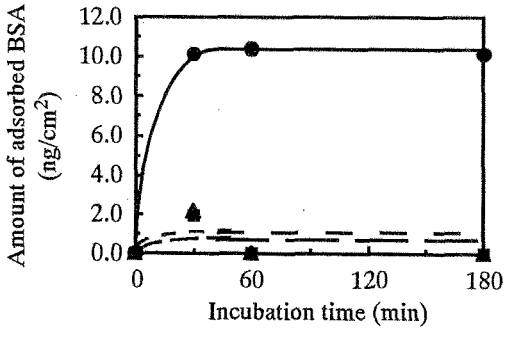

Figure 5 The time dependence of BSA amount adsorbed on the various nanoparticles. Initial concentration of BSA is 20 mg/mL, ● :Polystyrene nanoparticles(d = 202 nm), ▲ :PMB30W/PLA nanoparticles prepared in 1.0 mg/mL of PMB30W (d=225 nm), ■ :PMB30W/PLA nanoparticles prepared in 0.1 mg/mL of PMB30W (d=396 nm).

nanoparticles. That is, it is very important to inhibit the thrombus formation when the nanoparticles are introduced into the bloodstream to carry a drug. Therefore, it is a suitable moiety for modifying the nanoparticles which come in contact with blood.

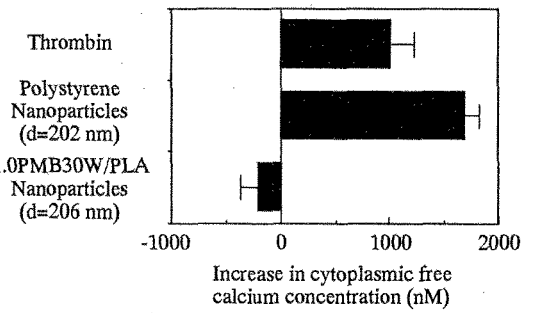

Figure 6 Change in cytoplasmic calcium levels in platelets in contact with thrombin or nanoparticles.

Figure 6 shows the change in cytoplasmic calcium levels in platelets in contact with various stimulations. The activation of platelets could not be observed even when the PMB30W/PLA nanoparticles.

Therefore, the PMB30W/PLA nanoparticles were expected to have weak interactions with the blood components, and not cause the opsonization which is one of the phagocytosis systems. Indeed, the polystyrene nanoparticles were obviously phagocyted by macrophage. On the other hand, PMB30W/PLA nanoparticles could avoid from phagocyted by macrophage (Figure 7).

This phenomenon showed that the PMB30W/PLA nanoparticles were not recognized as foreign body by

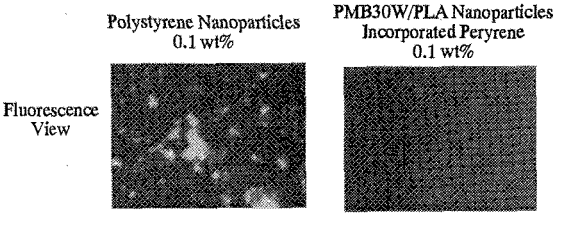

Figure 7 The fluorescence microscopic view of macrophages after contact with the polystyrene nanoparticles and PMB30W/PLA nanoparticles. Those nanoparticles loaded fluorescence probe.

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(Received December 7, 2000; Accepted February 23, 2001)