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Nanoparticle Processing in the Solid State Dramatically Increases the Cell Membrane Permeation of a Cholesterol-Lowering Drug, Probucol

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Abstract: High cholesterol levels (or hypercholesterolemia) are linked with many diseases, particularly with the risk of coronary heart diseases. Probucol is commonly used to reduce cholesterol in blood. While the effectiveness of this drug highly depends on its solubility, unfortunately, it is nearly insoluble (solubility is 5 ng/mL in water). Therefore, it is essential to develop approaches to increase its solubility and bioavailability and to enhance the efficiency of the drug. Here we show that a new method increases the solubility of probucol in water and its ability to permeate cell membranes. This new method of processing the drug in a nanoparticle utilizes the grinding of PBC probucol together with sodium dodecylsulfate and methacrylic copolymer. Solid-state NMR experiments reveal the polymorphic state of probucol and the conversion of this drug from crystalline to the amorphous state, and determine its nearness to the copolymer due to the grinding process that enables the formation of nanoparticles.

Keywords: Cogrinding; drug delivery; nanoparticles; NMR spectroscopy; solid-state reactions

Introduction

High cholesterol levels (or hypercholesterolemia) are linked with many diseases, particularly with the risk of coronary heart diseases.[1,2] Antihypolipidemic drugs based on statin are commonly used to inhibit cholesterol synthesis and lower its serum level. However, a number of side effects, such as hepatic failure, thrombocytopenia and skeletal muscle abnormalities including myopathy and rhabdomyolysis, caused by these drugs have been reported.[3−5] Therefore, there is significant current interest in developing alternative compounds to overcome these difficulties.

Probucol (PBC) is commonly used to reduce cholesterol in blood. The main advantages of using PBC over other drugs are its better acceptance and tolerance by patients and ease of administration. It is also very less expensive than other antihypolipidemic drugs available in the market. PBC lowers serum cholesterol level by increasing the fractional rate of low density lipoprotein (LDL) catabolism in the final metabolic pathway for cholesterol elimination from the body.[6,7] Studies have shown that PBC inhibits dietary cholesterol absorption, biosynthesis of cholesterol, and

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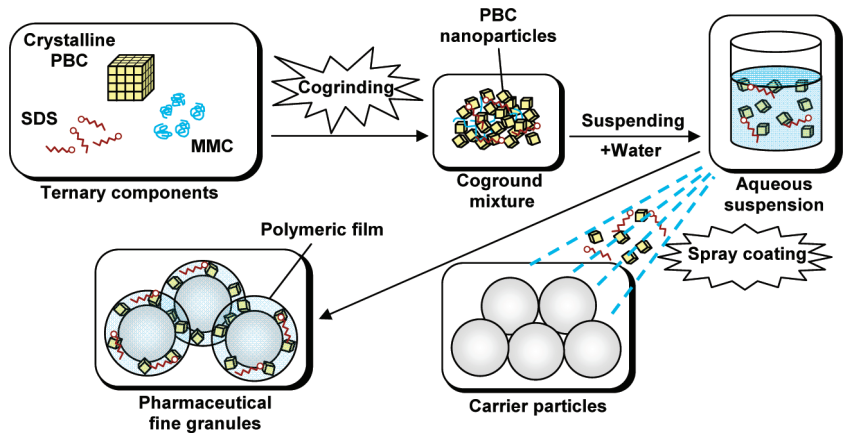

Figure 1. A schematic representation of the preparation of pharmaceutical fine granules containing probucol nanoparticles.

oxidation and tissue deposition of LDL cholesterol.[8,9] However, unfortunately, its extremely poor solubility considerably lowers its efficiency to suppress cholesterol,[10,11] and therefore development of new approaches to increase the solubility of PBC would be valuable. Since PBC functions via a different mechanism than the antihyperlipidemic agents based on statin, such new approaches would enable PBC and its derivatives to suppress the level of degenerate pathogenic LDL in blood. In this study, we show that a nanoparticle processing approach enhances the bioavailability of PBC, and design a solid dosage form for practical use. This concept is schematically shown in Figure 1.

Experimental Section

Materials. Probucol (MW 516.84), 4,4′-{(1-methylethylidene)bis(thio)}-bis{2,6-bis(1,1-dimethylethyl)}phenol, was supplied by Dai-ichi Sankyo Co., Ltd. (Tokyo, Japan). Methacryclic acid-methylmethacrylate copolymer (1:1) (MMC, MW ca. 135000) was provided from Evonik Degussa Japan Co., Ltd. (Tokyo, Japan). Sodium dodecyl sulfate (SDS, MW 288.38) was purchased from Sankyo Co., Ltd. (Tokyo, Japan). Ammonium hydroxide, triethyl citrate, Tween 80, and glycerol monostearate were purchased from Wako Pure Chemical Industries Ltd. All other chemicals used in this study were of reagent grade.

Preparation of Coground Mixture (GM). Blends of PBC, MMC and SDS (weight ratio was expressed as 1:*X*:*Y*) were physically mixed at a desired ratio in a glass vial using a vortex mixer for 5 min. A physical mixture (PM) of 2.5 g was ground in a vibrational rod mill (TI-200, CMT Co., Ltd., Fukushima, Japan, see Figure S1 in the Supporting Information) for 10 min under ambient conditions. Grinding the ternary mixture for 10 min resulted in a maximum fraction of nanoparticles (Figure S9 in the Supporting Information). The grinding cell and rod were made of aluminum oxide.

Particle Size Analysis. The GM was dispersed into distilled water, and then sonicated for 5 min to make the suspension. The particle size was determined by the dynamic light scattering method using NICOMP 380ZLS (Agilent Technologies Inc., Santa Clara, CA) under the following conditions: 532 nm wavelength, 25 °C temperature, 0.89 cp viscosity, and 1.333 diffractive index.

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Film Coating. Formulation of suspension for film coating is outlined in Table S1 in the Supporting Information. A fluidized bed coater (FL-Mini, Freund Corporation, Tokyo, Japan, Figure S2 in the Supporting Information) was used to prepare the film-coated pharmaceutical fine granules. Core particles (Nonpareil 103, 355−500 μm, Freund Corporation, Tokyo, Japan) were used as carrier particles. The processing parameters were as follows: batch size, 100 g; inlet air temperature, 28−30 °C; outlet air temperature, 28−30 °C; inlet air volume, 0.6 m^3/min; spray rate, 0.5 g/min; and atomizing air pressure, 0.1 MPa.

Caco-2 Permeability Experiments.[12,13] The permeability of presuspended PBC in PM and GM was studied across Caco-2 cell monolayers in an apical-to-basolateral (AP−BL) direction at an apical pH of 6.8, and basolateral pH of 7.4. Transepithelial electrical resistance (TEER) was measured using a Millicell ERS voltohmmeter (Millipore Corp., Bedford, MA). Powder samples (0.25 mg of PBC per monolayer) were suspended immediately in HBSS prior to experiments and then applied to apical buffer solution. Basolateral samples were withdrawn from the receiving chamber with designed interval and immediately replaced by an equivalent volume of fresh HBSS. The permeated amount of PBC was quantitatively determined by HPLC.

Solid-State NMR. Solid-state NMR experiments were initially performed on a 400 MHz Chemagnatics/Varian spectrometer at the University of Michigan and a 900 MHz Bruker (Lansing, MI) spectrometer was used to obtain final spectra presented in this paper. A 4 mm triple-resonance MAS probe was used. Experimental parameters, conditions, sample conditions, and other details are given in the Supporting Information. Resonance assignments were aided by previous 1D NMR studies on some of the components[14] and by the 2D NMR data given in Figures S3, S4, S5 and S6 in the Supporting Information.

Results and Discussion

The solubility, bioavailability and efficiency of a drug could be enhanced by reducing the particle size and also by formulating the drug in lipids or detergents.[15−17] In this study, nanoparticles were prepared by cogrinding PBC, a methacrylic acid-methyl methacrylate copolymer (1:1) (MMC) and sodium dodecyl sulfate (SDS) detergent (Figure S7 in the Supporting Information). Our results show that the particle size depends on the weight ratios of these three compounds (Figure S8 in the Supporting Information). Analysis of the particle size distribution revealed the existence of drug nanoparticles. Our results also infer that a maximum number of nanoparticles was obtained for a combination of 1:5:1 PBC:MMC:SDS (Figure S9 in the Supporting Information), which after cogrinding for 10 min resulted in only two different types of particles as indicated by the two peaks around 72 and 290 nm (Figure 2a). Since the larger peak ∼290 nm was similar to the mean particle size of MMC (Figure S8 in the Supporting Information), the smaller size particles (∼72 nm) are considered as drug nanoparticles generated by the cogrinding process. A procedure to filter out the large (∼290 nm) particles from the mixture is outlined in the Supporting Information. These results suggest that PBC exists as colloidal nanoparticles of ∼72 nm size in water. We have also confirmed that a two-component mixture does not produce nanoparticles (Figure S10 in the Supporting Information).

The solubility in water and in buffer and the membrane permeation of the nanoparticles were examined as described below. While ground nanoparticles were directly used in these experiments, they are sticky and difficult to handle for practical use by patients. Therefore, care was taken to uniformly coat the carrier particle with the nanoparticle mixture for oral administration as outlined in the Supporting Information. Scanning electron micrographs showed the formation of a polymeric nanoparticle film on the surface of the carrier particle (Figure S11 in the Supporting Information), and a polarized micrograph indicated that the crystalline component, consisting of PBC and/or SDS, was contained in the film due to the colored area by refraction of polarized light (Figure 2b and Figure S11 in the Supporting Information). In addition, excellent fluidity of the fine carrier particles was achieved by preparing pharmaceutical granules which were coated with polymer film containing drug nanoparticles as shown in Table S2 in the Supporting Information.

As mentioned earlier, the solubility of the drug plays an important role in the efficiency of the drug. Therefore, the dissolving behavior of nanoparticles was examined in buffer at different pH values. Results given in Figure 2c suggest that the solubility of nanoparticles is higher at neutral pH and low in acidic pH. Therefore, the nanoparticles will be quite stable in acidic conditions like in stomach while rapid drug release can be expected in intestine for better absorption (Figure 2c). On the other hand, no drug release was observed from physically mixed ternary components without grinding (Figure S12 in the Supporting Information). These results suggest that the grinding process is crucial in promoting

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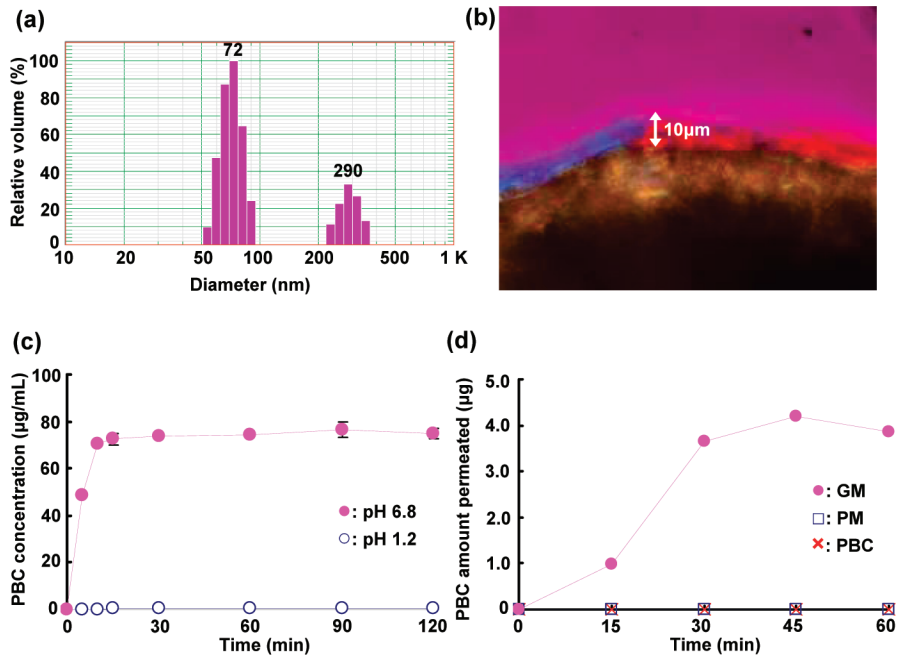

Figure 2. Formation of drug nanoparticles and encapsulation of carrier particles into polymer coated nanoparticles for an oral administration. (a) Particle size distribution of coground 1:5:1 PBC:MMC:SDS mixture suspended in water measured by dynamic light scattering experiment. (b) Cross-sectional polarized micrograph showing a layer of the carrier particle containing crystalline components (such as SDS and PBC) due to their refraction. (c) pH dependence of the drug release in the dissolution test. Drug nanoparticles were released in pH 6.8 buffer depending on the erosion of MMC copolymer, which was coated over the surface of the carrier particle, whereas it was not released in an acidic condition (pH 1.2). (d) Permeation of PBC drug across one layer of living Caco-2 cells at 37 °C at pH 6.8.

intermolecular interactions, leading to the formation of nanoparticles, and increasing the solubility of nanoparticles containing PBC.

Since the cholesterol-lowering efficiency of PBC is directly correlated with its ability to permeate membranes, it is important to examine the ability of the newly developed nanoparticles to permeate cell membranes. In this study Caco-2 cells were used to accomplish this task as explained in the Supporting Information. These cells are of colonic origin, but, unlike normal colonocytes, they show similar drug transport to the human small intestine.[12,13] Because of these reasons Caco-2 cells are commonly used to test the absorption of drugs in human. Permeated amounts of PBC from a mixture of 1:5:1 PBC:MMC:SDS with (i.e., nanoparticles of size ∼72 nm) and without grinding were measured (Figure 2d). Our experimental results suggest that nanoparticles permeate the cell membrane while the drug PBC alone or a physical mixture of 1:5:1 PBC:MMC:SDS without grinding did not permeate the cell membrane even after an hour. The detergent SDS perhaps opens the junction between Caco-2 cells, and the drug can transit from between cells. Indeed, the TEER value decreased to ca. 70% from the initial condition after the examination (Figure S13 in the Supporting Information). Current studies in our laboratories are being carried out to address the effect of SDS on the transmembrane permeation of PBC and *in vivo* study to investigate the drug absorption.

Since our results suggest that grinding the 1:5:1 PBC:MMC:SDS mixture is absolutely essential for membrane permeation and for the function of PBC, it is important to understand the underlying molecular interactions in the formation of nanoparticles. Solid-state NMR experiments were performed on nanoparticles in ground mixture (GM), physical mixture (PM) without grinding, and the pure forms of all three individual components of the mixture. Spectra of PBC-I and PBC-II are different, and differences in the spectra of GM and PM can be noted (Figure 3). A combination of ^{1}H and ^{13}C chemical shift spectra (Figure 3), 2D ^{1}H/^{1}H chemical shift correlation under MAS (Figure 4), and 2D ^{1}H/^{13}C chemical shift correlation under MAS (Figure S5 in the Supporting Information) was used to assign spectral lines from individual chemical components as well as nanoparticles. ^{1}H and ^{13}C spectra suggest that grinding of pure PBC-I results in PBC-II. The spectra of GM and PM samples were dominated by PBC and MMC components as shown in Figure 3a. ^{1}H and ^{13}C spectra reveal that PBC exists in the PBC-I form in PM while it converts to the PBC-II form in GM.

2D ^{1}H/^{1}H correlation experiments (similar to 2D NOESY) were performed under MAS using a pulse sequence shown in Figure S6 in the Supporting Information. A spin echo was applied before the signal acquisition to filter out peaks that have short T_2 values. 2D ^{1}H/^{1}H correlation spectra of PM and GM samples are given in Figure 4; 2D correlation spectra of pure PBC-I and PBC-II are also shown in the same figure for comparison with the spectra of PM and GM. All spectra exhibited relatively high-resolution in both frequency dimen-

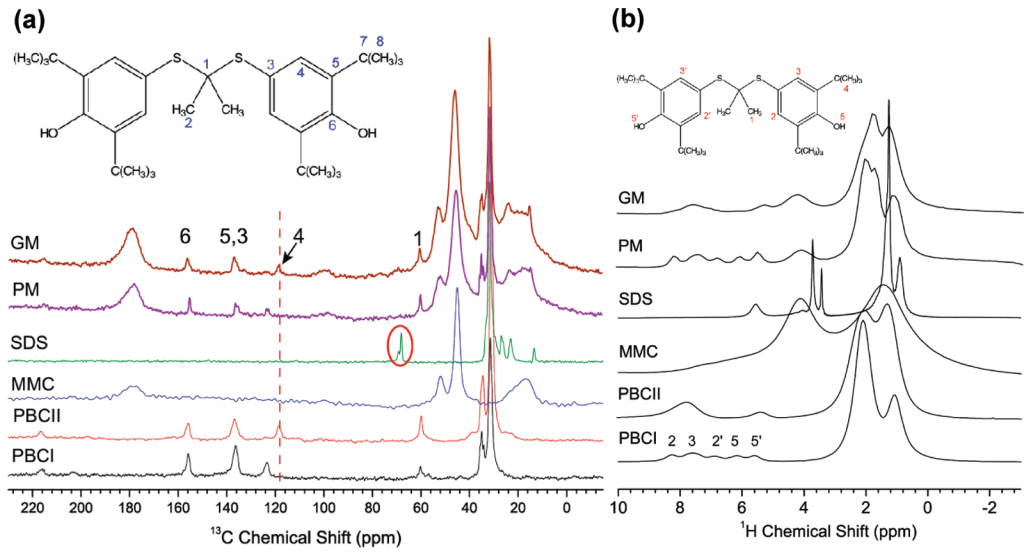

Figure 3. The ^{1}H and ^{13}C NMR spectra: (a) ^{13}C NMR spectra of SDS, MMC, PBC-II and PBC-I were recorded using a ramped-CP pulse sequence under 18 kHz MAS. (b) ^{1}H NMR spectra were recorded using a solid-echo pulse sequence under 18 kHz MAS at room temperature. Different refocusing times were used for different samples: 1 ms (SDS), 40 μs (MMC), 2 ms (PM), 2 ms (GM), 1 ms (PBC-I) and 1 ms (PBC-II).

sions. Peaks in 2D spectra were assigned based on the connectivity of proton resonances shown in the spectra (Figure 4). The presence of cross peaks between PBC and MMC in the 2D ^{1}H/^{1}H spectra of GM (indicated by a circle) and their absence in PM suggest that these two molecules are nearer in space in GM than in PM (see the experimental details in the Supporting Information). Specifically, CH_3 of PBC and CH_2 of MMC are within ~6 Å distance. This suggests that the hydrophobic interaction between the drug and the polymer plays an important role in the binding between MMC and PBC and thus in the formation of nanoparticles. An increase in the line width observed (particularly in ^{1}H spectra) in spectra of GM suggests that the cogrinding of the physical mixture changes the crystalline PBC to an amorphous state. This observation is in excellent agreement with our measurements using XRD and FTIR experiments (Figures S14 and S15 in the Supporting Information).

The reduction of particle size leads to a significant increase in the dissolution rate of the API (active pharmaceutical ingredient), which in turn can lead to a substantial increase in drug absorption.[16,17] There are several techniques to reduce the particle size to nano order, such as precipitation, supercritical fluid processing,[18] freeze-drying,[19] high pressure homogenization[20] and cogrinding.[21,22] The dry-cogrinding process has environmental and cost-effective advantages for production of solid pharmaceutical applications due to its simple procedure and freedom from the use of organic solvents. In addition, formulating a solid dosage form such as tablets or granules with or without capsules is quite important for the convenience of patients. Grinding has been shown to alter the structure of polypeptides[23] and pharmaceutical compounds.[24,25] In this study, we have applied MMC as a copolymer in the ternary cogrinding system.[26,27] MMC has been used as a coating material for oral pharmaceutical forms.[28,29] Its enteric property, which means the MMC dissolves in the neutral pH region but not in acidic conditions, is expected to protect nanoparticles as well as increase the solubility of hydrophobic drugs. To the best of our knowledge, this is the first study that successfully combines dry-cogrinding and film-coating to

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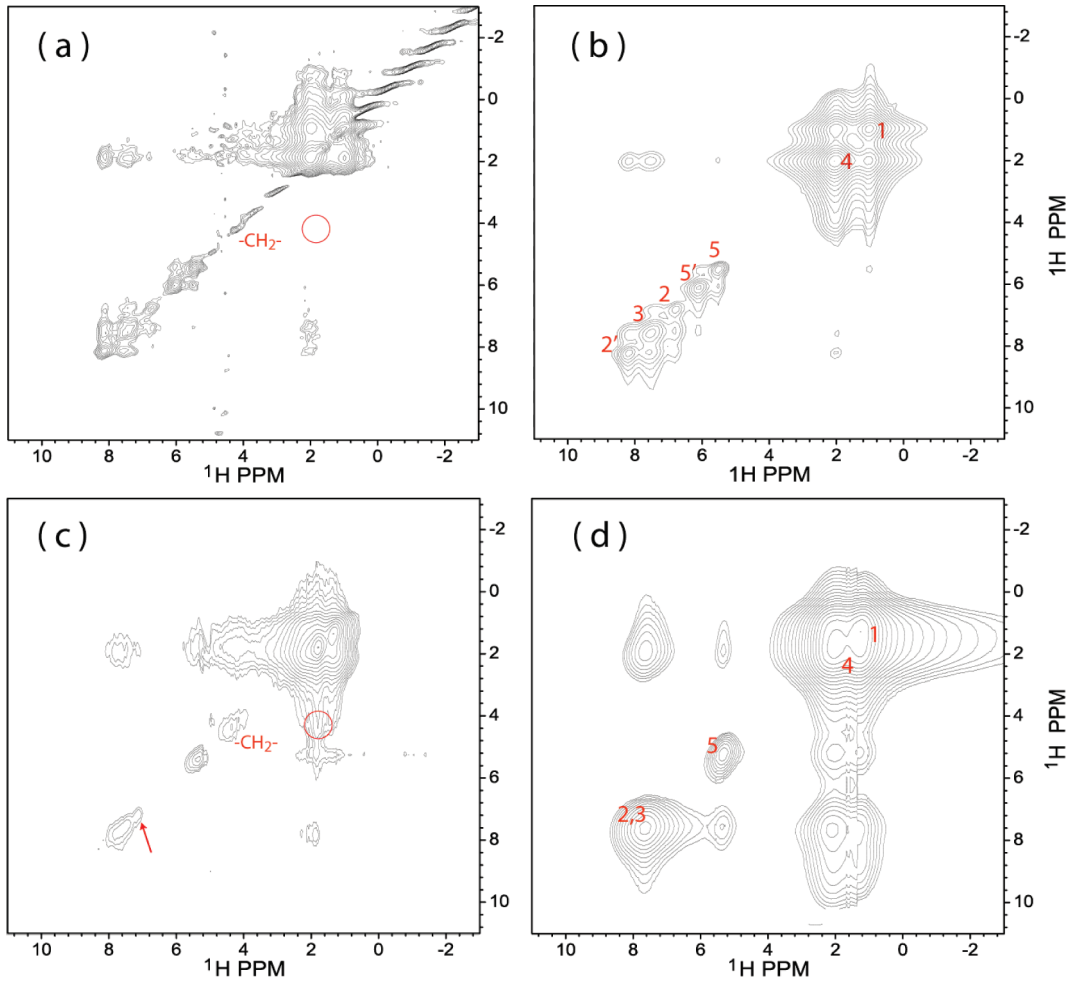

Figure 4. 2D H/H correlation spectra of PM (a), PBC-I (b), GM (c) and PBC-II (d) obtained using the pulse sequence given in Figure S6 in the Supporting Information. $\tau = 1$ ms, an 11.11 ms mixing time, 16 scans and 62 t1 increments were used. The spinning frequency of the sample was synchronized with the mixing time of the pulse sequence. States et al. method was applied to achieve quadrature detection in the indirect frequency dimension.

prepare the polymeric dosage of drug nanoparticle. In this study, we have successfully accomplished the following tasks: (i) preparation of PBC nanoparticle by ternary cogrinding, (ii) formulation of pharmaceutical fine granule for easy handling by patients, (iii) increase in the solubility of PBC, (iv) increasing the permeation of PBC-nanoparticle across a model membrane that mimics the human intestinal cell membrane, and (v) elucidation of intermolecular interactions in ternary component that lead to the formation of nanoparticles.

Conclusions

Recent research in this field is also focused on the diversity of pharmacological action of PBC, such as antioxidation and inflammatory effects.[9,10] These effects are expected to be important in treating myocardial infarction by inhibiting oxidation of LDL and its accumulation in coronary artery. Since this is an important requirement of cholesterol-lowering drugs, our approach presented in this paper will enable PBC to efficiently function *via* multiple routes in the human body. In addition, ongoing investigations include exploring the possibility that our approach could be useful for more general applications to formulate other hydrophobic drugs or organic compounds which are poorly soluble in water. Therefore, this study has broad implications in pharmaceutical research.

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Supporting Information Available: Details of the experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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