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Homogeneous Nanoparticles To Enhance the Efficiency of a Hydrophobic Drug, Antihyperlipidemic Probucol, Characterized by Solid-State NMR

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Abstract: A low absorption in the gastrointestinal tract of hydrophobic pharmaceutical compounds in use today considerably limits their bioavailability, and therefore they are taken in large doses in order to reach the therapeutic plasma concentration, which inevitably results in undesired side effects. In this study, we demonstrate a new nanoparticle approach to overcome this problem, and our experimental results show that this approach has a high efficiency of drug loading and is easily adaptable to industrial scale. Characterization of nanoparticles containing a cholesterol-lowering hydrophobic drug, probucol, using a variety of biophysical techniques revealed higher homogeneity of these particles compared to those prepared using other approaches. Intermolecular interactions of these nanoparticles are probed at high resolution by magic angle spinning solid-state NMR experiments.

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

Introduction

Poor solubility and therefore a low absorption in the gastrointestinal tract of >40% of the pharmaceutical compounds in use today considerably limit their bioavailability after being administered.[1,2] As a result, these hydrophobic drugs must be taken in large doses in order to reach the therapeutic plasma concentration, which inevitably results in undesired side effects.[3] Therefore, it is essential to develop novel techniques to enhance the effectiveness of hydrophobic drugs. Recent studies have shown that nanoparticle formulation by wet-grinding[4−6] or cogrinding[7−10] is one of the

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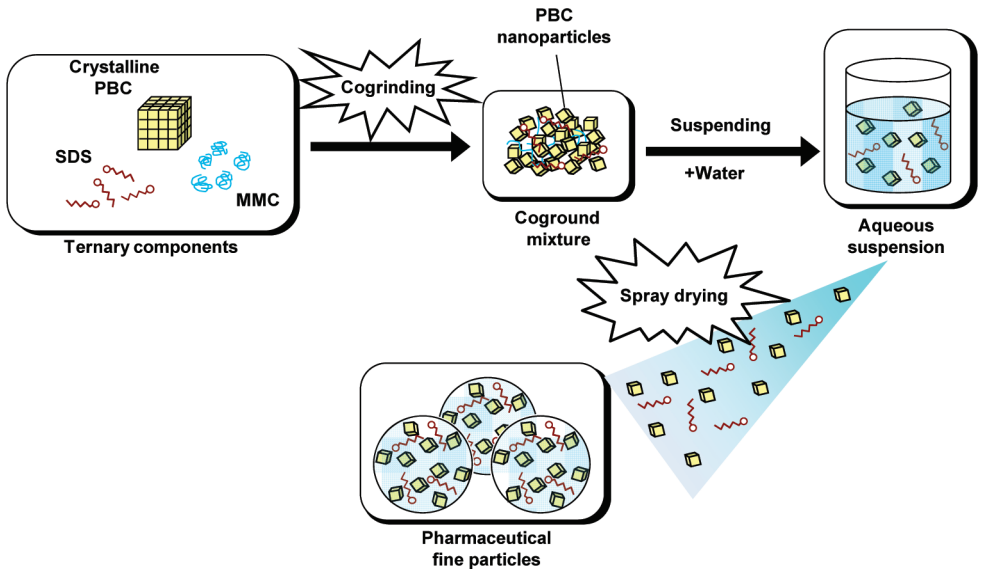

Figure 1. A schematic representation of the preparation of pharmaceutical fine particles containing probucol nanoparticles.

promising methods to increase the solubility of hydrophobic drugs. However, the wet-grinding approach typically results in submicrometer size drug particles which aggregate when dried for the preparation of solid dosage forms.[11,12] While the cogrinding approach resulted in nanoparticles (<100 nm), they are unfortunately heterogeneous in size and therefore less effective. In this paper, we demonstrate a new approach by integrating cogrinding and spray-drying to enable hydrophobic pharmaceutical compounds to maintain a fully soluble state and at the same time increase the rate of absorption to reach the therapeutic plasma concentration. Our results on the nanoparticle formation of a hydrophobic cholesterol-lowering drug, probucol (PBC), demonstrate that this one-step continuous process of nanoparticle formation approach has a high efficiency of drug loading and is easily adaptable to industrial scale.[13−15] This concept is schematically shown in Figure 1.

Experimental Section

Materials. Probucol (MW 516.84, crystalline powder of PBC-I form), 4,4′-{(1-methylethylidene)bis(thio)}-bis{2,6-bis(1,1-dimethylethyl)}phenol, was supplied by Dai-ichi Sankyo Co., Ltd. (Tokyo, Japan). Methacrylic acid-methyl methacrylate 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, 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) and PBC-II Form. In the ternary system, a blend of PBC, MML and SDS (weight ratio of 1:5:1) was physically mixed in a glass vial using a vortex mixer for 10 s. A physical mixture 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 condition. PBC was

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alone ground under the same conditions mentioned above to obtain the crystalline powder of PBC-II form and used for solid-state NMR experiments.

Preparation of Spray-Dried (SD) Particles. *Preparation of Suspension.* The formulation of spray-drying suspension was summarized in Table S1 in the Supporting Information. GM was dispersed in water, and then 1.7% ammonium hydroxide was gradually added and equilibrated with slow agitation via magnetic stirrer for 60 min. Polysorbate 80, triethyl citrate and glycerol monostearate were dispersed in hot water (80 °C) and left until the ambient temperature had decreased with stirring. This glidant suspension was poured into GM suspension for a further agitation overnight.

Spray-Drying Process. A desktop spray-dryer (SD-1, Tokyo Rika Corp., Tokyo, Japan, Figure S2 in the Supporting Information) was used to prepare the spray-dried particles. The processing parameters were as follows: inlet air temperature, 85−120 °C; outlet air temperature, 75 °C (SD75), 90 °C (SD90) and 105 °C (SD105); blow flow, 0.6 m^3/min; spray rate, 2.0 g/min; and atomizing air pressure, 0.8 kg/cm^3 (see Table S2 in the Supporting Information).

Particle Morphology. Spray-dried particles were viewed by scanning electron microscope (SEM) measurements, JSM-6360LV (JEOL, Tokyo, Japan), and samples were adhered to a sample stage using an adhesive tape. A sputter coater, JEC-1600 (JEOL Ltd., Tokyo, Japan), was used to coat the samples with gold/platinum at 20 mA for 30 s. E800 POL polarized microscope (Nikon, Tokyo, Japan) was used to observe the crystalline components in a polymeric film. Optical images of samples were captured using a digital sight camera system DS-5M-L1 (Nikon Co., Tokyo, Japan).

Caco-2 Permeability Experiments.[16,17] *Cell Culture.* The Caco-2 cells (ATCC, Rockville, MD) were seeded at 3 × 10^5cells onto polycarbonate filter membranes (pore size 3.0 μm, growth area 4.67 cm^2) in clusters of 6 wells (Transwell, Corning Costar Corp., Cambridge, MA). The cells were grown in a medium consisting of DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The cultures were maintained at 37 °C (CO_2 incubator, SANYO, Japan) in an atmosphere of 5% CO_2 and 95% air, at 95% relative humidity. The growth medium was changed three times a week until time of use. Cells from passage numbers 18−21 were used in the experiments at ages ranging from 14 to 21 days.

Permeability Experiments. The permeability of presuspended PBC in SD particles 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. Before the permeability experiments, the cell monolayers were rinsed twice with HBSS, pH 7.4, and equilibrated in the transport buffers under experimental conditions for 15 min. Transepithelial electrical resistance (TEER) was measured using a Millicell ERS Voltohmmeter (Millipore Corp., Bedford, MA) and monolayers with TEER values below 250 Ω cm^2 were discarded. Particle samples (0.1 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 at 15, 30, 45, 60, 75, 90, 105, and 120 min and immediately replaced by an equivalent volume of fresh HBSS. All of the transport experiments were conducted at least in triplicate ($n = 3$).

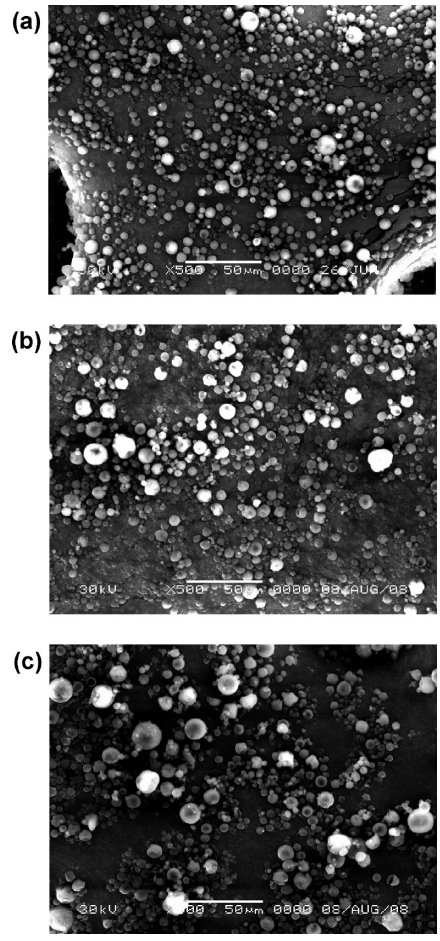

Figure 2. SEM photomicrographs of powders spray-dried at SD75 (a), SD90 (b) and SD105 (c).

Particle Size Analysis of PBC Nanoparticles. The SD particles were dispersed into pH 6.8 HBSS and then sonicated for 10 min to make the suspension. The volumetric particle size distribution for each suspension was determined at 37 °C by the dynamic light scattering method using NICOMP 380ZLS (Agilent Technologies Inc.).

Solid-State NMR Experiments. Solid-state NMR experiments were performed on a 400 MHz Varian/Chemagnetics spectrometer (9.4 T) at the University of Michigan, and final spectra presented in this paper were acquired using a 900 MHz Bruker spectrometer (21.1 T) at biomolecular NMR facility, East Lansing, MI, using a 4 mm triple-resonance

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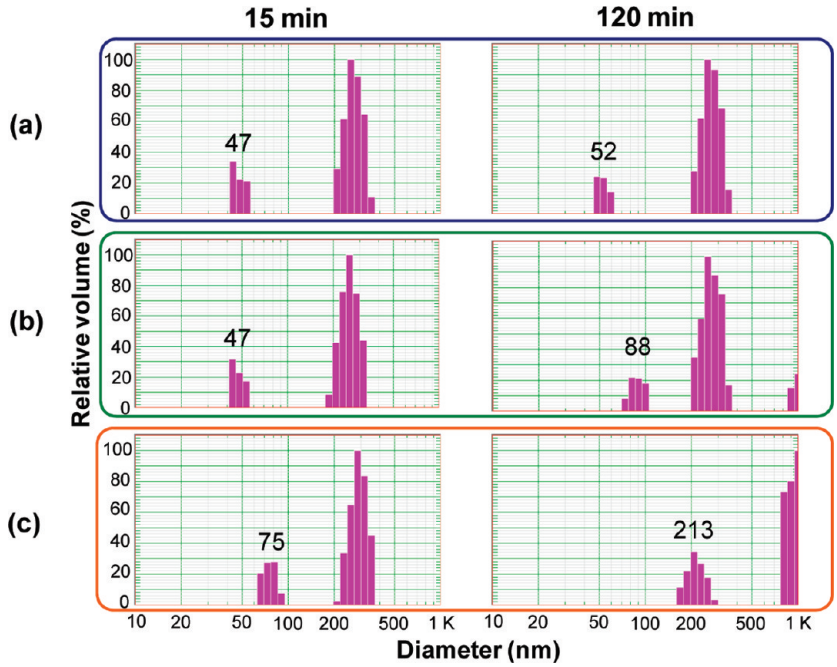

Figure 3. Particle size distributions of PBC nanoparticles after SD in pH 6.8 HBSS solution. SD75 (a), SD90 (b) and SD105 (c).

MAS probe. All spectra were obtained under 18,000 ± 5 Hz MAS at 27 °C. Experimental parameters, sample conditions and other details are given in figure captions. All data were processed using the NMR Pipe software.

3D Structural Modeling. Materials Studio (Accelrys Software Inc., San Diego, CA) was used for the construction of the 3D structural model based on solid-state NMR experimental constraints.

Results and Discussion

Scanning electron microscope (SEM) photographs of spray-dried powders prepared at 75 °C (SD75), 90 °C (SD90) and 105 °C (SD105) are shown in Figure 2. These powder samples are spherical shaped particles with a smooth surface as seen in Figure 2 that are composed of nanoparticles. Although the mean diameters of these spherical particles were similar (~3 μm, see Table S3 in the Supporting Information) among SD75, SD90 and SD105 samples, smaller sized particles were found abundantly in SD75 and less in SD105 as seen in Figure 2 (see Figure S3 and Table S3 in the Supporting Information). This means suggest that inner water phase gradually evaporated at lower temperatures providing smaller particles, whereas a crust was formed on the outer surface of the spray droplets at higher temperatures, resulting in larger particle size[18] (see Figure S4 in the Supporting Information). The dynamic-light scattering measurement of the size distribution of drug nanoparticles present in the spherical spray-dried powder revealed the presence of ~47 nm sized nanoparticles in SD75 and SD90 samples (Figure 3). On the other hand, the size of relatively large (~75 nm diameter) nanoparticles observed from SD105 sample increased with time as shown in Figure 3.

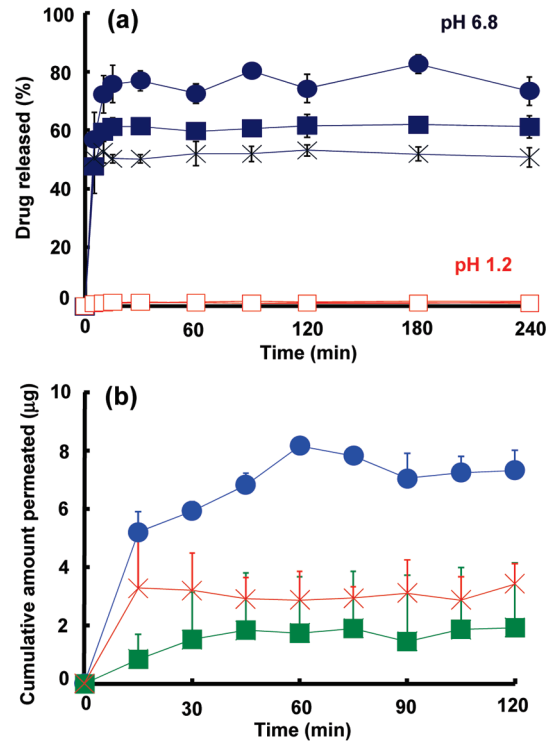

Figure 4. (a) Drug release profiles of PBC from SD75 (●), SD90 (■), and SD105 (×) nanoparticles at pH 1.2 (red) and pH 6.8 (blue). (b) Permeation of PBC drug across the living Caco-2 cell monolayer at 37 °C and pH 6.8. Each error bar indicates the mean + SD (n = 3).

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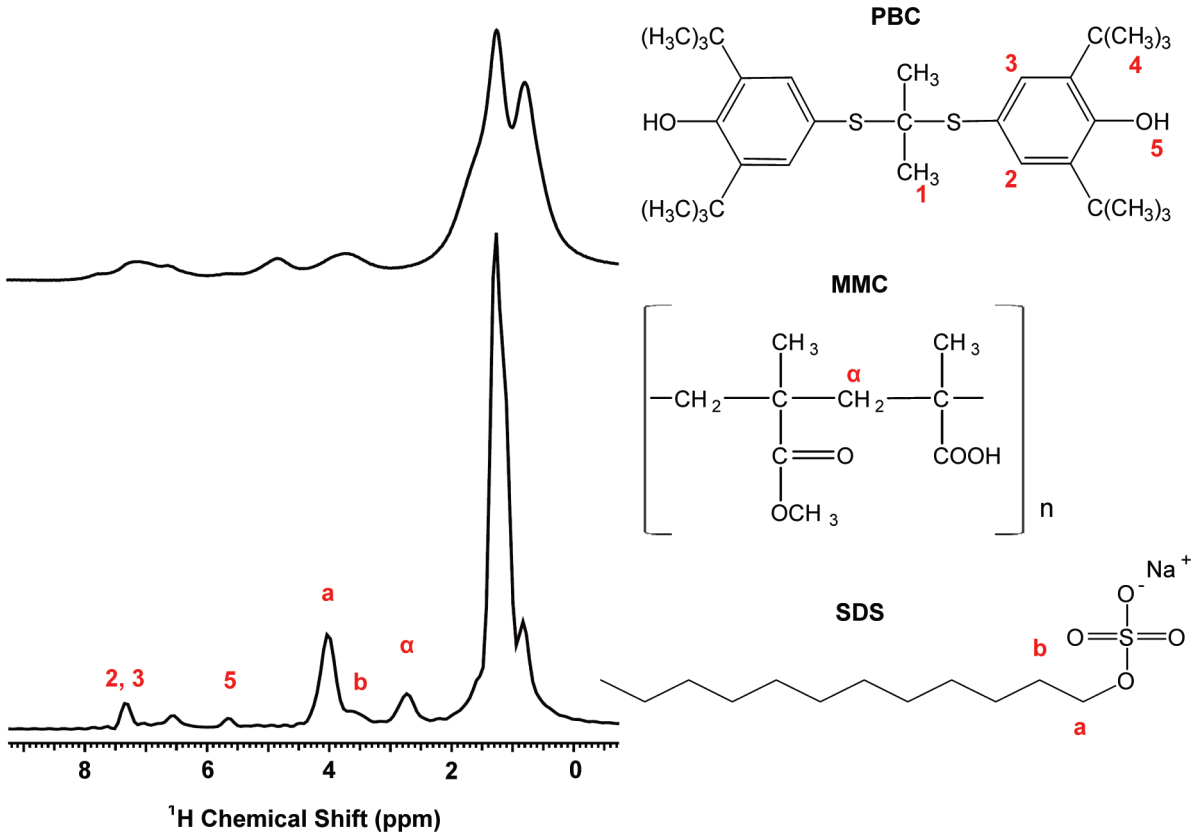

Figure 5. ¹H chemical shift spectra of a ground mixture of MMC, SDS and PBC prepared with (bottom) and without (top) spray-drying. The 1D spectra were obtained using a solid-echo pulse sequence with a 1 ms refocusing time under 18 kHz MAS at 27 °C in a Bruker 900 MHz NMR spectrometer and 16 scans.

The percentages of the probucol drug released from SD75, SD90 and SD105 powders at pH 1.2 and pH 6.8 buffers, mimicking the stomach and small intestine pH environments respectively, are given in Figure 4a. The PBC releasing percentages from all three samples were below 2% at pH 1.2 even after 240 min. On the other hand, SD75, SD90 and SD105 respectively released 80, 60 and 50% of PBC immediately at pH 6.8. These data infer that SD75 had the best enteric property and released most of the drug nanoparticles within 15 min. These results further suggest that (a) the agglomeration of drug nanoparticles occurred during the spray-drying process at a higher temperature as shown in Figure S4 in the Supporting Information and (b) these nanoparticles exhibit pH-dependent functional properties and therefore the hydrophobic drugs can be effective in the alkaline environment of the small intestine.

The Caco-2 cell monolayer from human intestines was used to examine the cell membrane permeation of the nanoparticles. Experimentally measured permeation rates of PBC from nanoparticles are given in Figure 4b. Our results showed the best permeation for SD75, that is ~8 μg of PBC was transported from apical to basal side of monolayer over 120 min. The 2-fold faster permeation speed of SD75 nanoparticles compared to that of nanoparticles prepared without the spray-drying process[7] suggests that the spray-drying process can drastically improve the efficiency of hydrophobic drugs. This Caco-2 cell permeation test is consistent with the dissolution study (shown in Figure 4a) and particle size analysis (shown in Figure 3) of cogrinding−spray-drying processed drug nanoparticles. The solubility, fraction and size of nanoparticles correlate with the dissolution rate according to the Noyes−Whitney equation;[19] therefore smaller-size nanoparticles would be efficient for the cell membrane permeation.

Our results suggest that the combination of cogrinding and spray-drying process has significantly improved the solubility and bioavailability of the hydrophobic PBC drug. MAS solid-state NMR experiments were performed to characterize the structural integration of nanoparticles at high resolution. ¹H as well as ¹³C resonances of the coground mixture were assigned using 2D ¹H/¹H NOESY and 2D ¹H/¹³C chemical shift correlation experiments under MAS[7] (Figure S7 in the Supporting Information). The two types of PBC crystal forms were distinguished based on ¹H and ¹³C spectra (Figures S8 and S9 in the Supporting Information). NMR spectra of nanoparticles prepared using the combined cogrinding and spray-drying process revealed the existence of the drug in PBC-II form as shown in Figure 5 and Figure 6. ¹H spectral lines of nanoparticles prepared with this approach are significantly narrower than that of nanoparticles prepared

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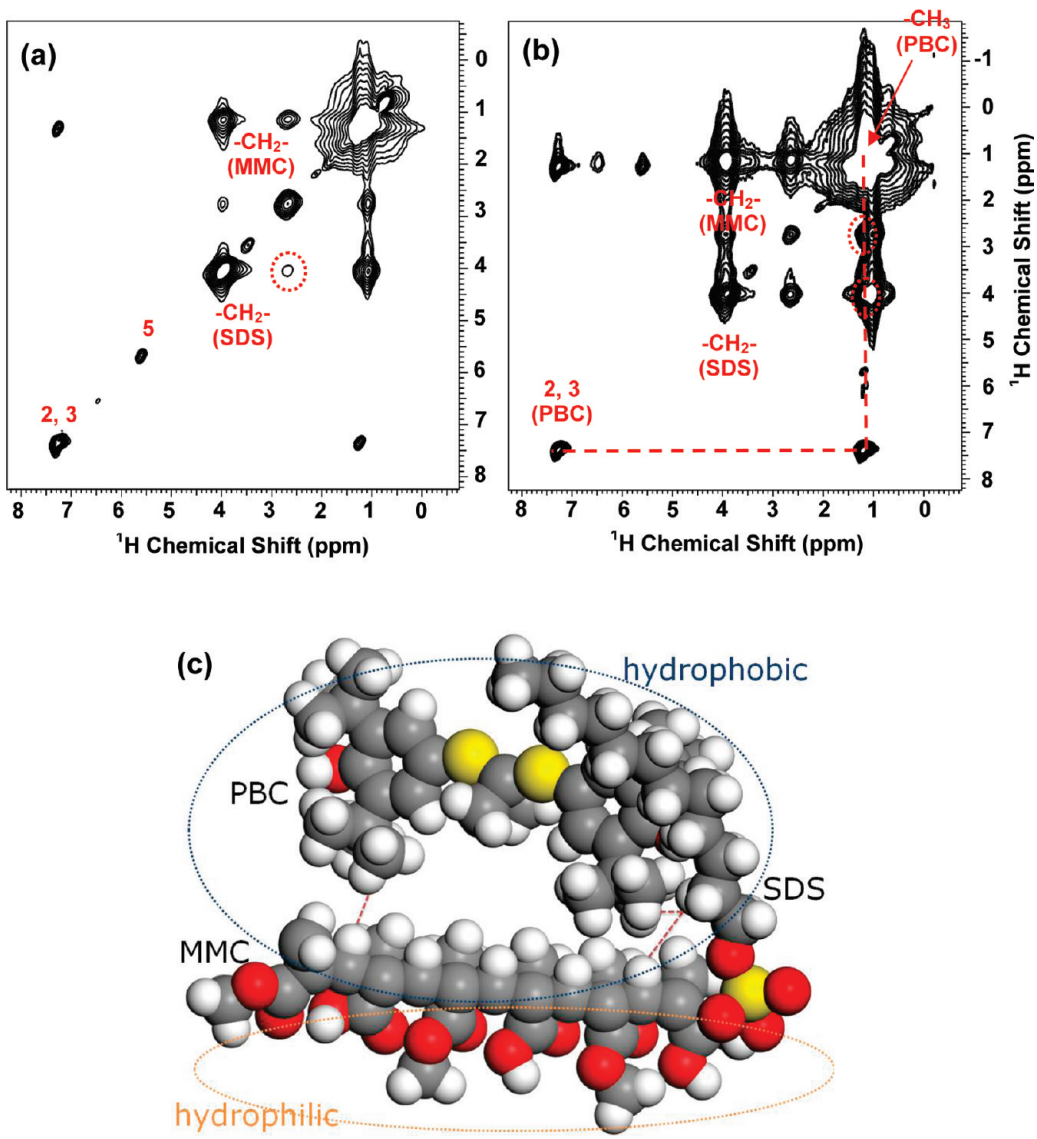

Figure 6. (a and b) 2D ^{1}H/^{1}H NOESY spectra of a ground mixture of MMC, SDS and PBC prepared with spray-drying were obtained using a pulse sequence given in Figure S7 (in the Supporting Information) with $t = 1$ ms and a mixing time of 11.11 ms (a) and 55.55 ms (b) under 18 kHz MAS at 27 °C in a Bruker 900 MHz NMR spectrometer; 16 scans and 62 t_1 increments were used with the synchronization of the MAS spinning speed and the mixing time. (c) A structural model based on NMR constraints depicts molecular interactions between MMC, SDS and PBC and gives an insight for the water-soluble mechanism of PBC, a hydrophobic molecule.

without spray-drying as shown in Figure 5. This observation suggests that the spray-drying process increased the homogeneity of not only the physical size of nanoparticles but also their chemical composition. It also infers that the intermolecular interactions that enable the nanoparticle formation are effectively utilized in the spray-drying process.

To further probe these intermolecular interactions, NOESY spectra of nanoparticles prepared with (Figures 6a and 6b) and without (Figure S10 in the Supporting Information) spray-drying were recorded. The increase in the spectral resolution due to spray-drying further confirmed the higher homogeneity of the intermolecular interactions in nanoparticles prepared with spray-drying than that of the nanoparticles prepared without spray-drying.[7]

The intensity of cross peaks in the 2D ^{1}H/^{1}H NOESY spectra can be used to determine the through space distance between protons and possibly the structural assembly of molecules that constitute the nanoparticle. The presence of cross peaks between MMC and SDS (indicated by a circle in Figures 6a and 6b) suggests that these two molecules are near in space within the nanoparticle. Specifically, CH_2 of MMC and CH_2 of SDS are within ~9 Å distance. Also, CH_2 of MMC and CH_3 of PBC are within ~3 Å distance and CH_2 of SDS and CH_3 of PBC are within ~3 Å distance. These results suggest that the methyl groups of the PBC drug are stabilized by hydrophobic interactions with CH_2 of MMC and SDS. Therefore, it is highly likely that the optimization of the

hydrophobic interaction among drug, SDS and MMC could further enhance the homogeneity and the functional efficiency of nanoparticles. Our results also demonstrate that solid-state NMR experiments can be used to examine the quality of nanoparticles and to provide atomic-level resolution insights on the role of intermolecular interactions toward the design of nanoparticles. While these nanoparticles are not amenable for studies using most commonly used high-resolution physical techniques like X-ray crystallography and solution NMR spectroscopy, NMR data presented in this paper suggest that the use of a variety of MAS techniques and recently developed solid-state NMR approaches[20−25] on nanoparticles containing molecules labeled with specific isotopes such as ^{13}C and ^{2}H could provide additional high-resolution insights on the structure, intermolecular assembly, and dynamics of individual molecules. Such information may be utilized in the design of more effective nanoparticles.

Conclusions

In summary, our results suggest that the nanoparticles of PBC prepared with spray-drying maintain the pharmaceutical property, exhibit enhanced oral bioavailability under the physiological alkaline condition (pH 6.8), and effectively absorbed through the mucous membranes in the human small intestine. We believe that this novel approach of cogrinding and spray-drying could be of significant advantage for effective use of probucol to patients who require a higher and faster rate of mucosal absorption when taken orally. Our method could be generally applicable for other poorly soluble compounds on an industrial scale production. In addition this technique may find applications in material science, particularly in the design of multifunctional nanoparticles consisting of hydrophobic compounds.

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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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