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Molecular-level characterization of probucol nanocrystal in water by *in situ* solid-state NMR spectroscopy

Junying Zhang, Kenjirou Higashi, Waree Limwikrant, Kunikazu Moribe, Keiji Yamamoto*

Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8675, Japan

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ABSTRACT

The molecular state of colloidal probucol nanoparticles with additives was evaluated by ^{13}C *in situ* solid-state NMR spectroscopy. The nanoparticles were obtained by dispersing a ternary co-ground mixture of probucol/polyvinylpyrrolidon (PVP)/sodium dodecyl sulfate (SDS) in water. Their mean particle size was found to be approximately 150 nm by dynamic light scattering and cryogenic-scanning electron microscopy measurements. The results of the ^{13}C *in situ* solid-state NMR spectroscopy showed that probucol existed in the crystalline state (form I) in water. ^{13}C liquid-state NMR results indicated that PVP and SDS interacted with probucol in water. Their broad signals suggested that the surface interaction of the probucol nanocrystal with PVP and SDS stabilized the suspension. In addition, a freeze-dried sample of the suspension was studied by ^{13}C solid-state NMR and powder X-ray diffraction experiments, which confirmed the presence of the probucol nanocrystals. The combination of the *in situ* solid-state, solid-state, and liquid-state NMR measurement results provided molecular-level insights about the role of intermolecular interactions in the design of nanoformulations.

1. Introduction

Drug nanoparticle formation has emerged as a promising strategy for enhancing the bioavailability of hydrophobic drugs (Ruenraroengsak et al., 2010; Shudo et al., 2008; Zhang et al., 2010). Information on the size or morphology of drug nanoparticles can be obtained by analytical techniques such as light scattering or by microscopic measurements (Sharma et al., 2011). However, only a little research has been done on the molecular state of drug nanoparticles in a suspension (Mayer, 2002, 2005a); it is considered that a large amount of liquid water or solvent molecules obstruct the direct evaluation of the solid molecules (Mayer, 2002). It is important to understand how drugs and additives coexist in a suspension because the molecular state of the drugs and additives and the interaction between them determine the stability and solubility of drug nanoparticles in the suspension. The stability and solubility significantly affect the bioavailability and development of the drug. Although it is possible to characterize freeze-dried samples of a nanosuspension by various solid-state analytical techniques (Abdelwahed et al., 2006), the molecular state of dissolved or colloidal components in the suspension cannot always be evaluated accurately because the freeze-drying process converts the liquid sample into solid and sometimes causes aggregation of the colloidal particles.

In situ solid-state NMR spectroscopy involves the magic angle spinning (MAS) technique that reduces the dipole–dipole and chemical shift anisotropy interactions of the solid components dispersed in water, thus enabling the measurement of an NMR spectrum of the suspension (Crisp et al., 2010; Friebolin et al., 2010). Recently, *in situ* solid-state NMR experiments in various fields have revealed previously unknown aspects of suspensions (Blasco et al., 2010; Skogsberg et al., 2007; Xu et al., 2009). The *in situ* solid-state NMR spectroscopy is a non-invasive, non-destructive tool for direct observation of solid materials in suspensions, which may provide us useful information on the crystallinity, type of crystal, mobility, and molecular interaction of drugs, additives, etc. However, the inherent insensitivity of NMR necessitates a highly concentrated suspension and a longer duration to perform the measurement. Moreover, in MAS, which exerts a strong centrifugation force, the suspension is required to retain its physical and chemical properties for a long time (Mayer, 2005a,b). For the abovementioned reason, the application of this approach has been restricted to the field of pharmacy. In this research, we successfully prepared a stable and highly concentrated nanosuspension for *in situ* solid-state NMR evaluation.

We have reported that the co-grinding of poorly water-soluble drugs in the presence of polyvinylpyrrolidon (PVP) and sodium dodecyl sulfate (SDS) could induce drug nanoparticle formation (Wanawongthai et al., 2009; Itoh et al., 2003; Pongpeerapat et al.,

* Corresponding author. Tel.: +81 43 226 2865; fax: +81 43 226 2867.
E-mail address: k-yamamoto@faculty.chiba-u.jp (K. Yamamoto).

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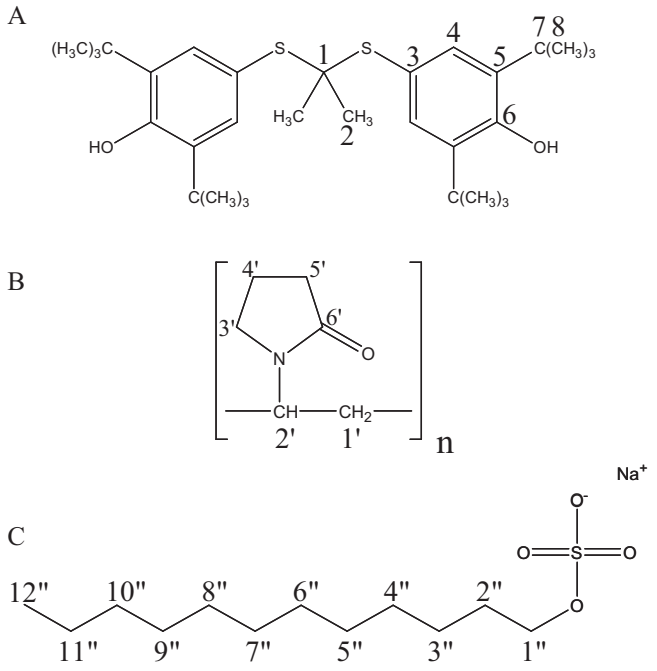

Fig. 1. Chemical structures and carbon atom numbering of (A) probucol, (B) PVP (K12, $M_w \approx 2500$), and (C) SDS.

2006, 2008). Nanosuspensions thus prepared show high stability and their bioavailability is significantly enhanced (Shudo et al., 2008). Solid-state ^{13}C NMR studies have revealed that when a drug/PVP/SDS ternary ground system is dispersed in water, grinding-induced solid-state interactions among the components of the ternary system play an important role in nanoparticle formation (Wanawongthai et al., 2009; Pongpeerapat et al., 2006). Atomic force microscopy has shown that colloidal probucol nanoparticles are coated with the PVP-SDS complex (Moribe et al., 2008). However, the molecular details of nanoparticles dispersed in water are still unknown.

The aim of this research was to obtain molecular-level information on probucol and additives in a nanosuspension by *in situ* solid-state and liquid-state NMR spectroscopies. In this study, probucol was used as a poorly water-soluble drug. PVP (PVP K12, $M_w \approx 2500$) and SDS were employed as the water-soluble polymer and ionic surfactant, respectively (Fig. 1). *In situ* solid-state and liquid-state NMR measurements were performed for evaluating the molecular state of the nanosuspension. Then, a freeze-dried sample of the nanosuspension was evaluated by powder X-ray diffraction (PXRD) and ^{13}C solid-state NMR measurements, and these were compared with those of *in situ* solid-state and liquid-state NMR measurements.

2. Materials and methods

2.1. Materials

Probucol was supplied by Daiichi-Sankyo (Japan). PVP (Kollidon® 12 PF, $M_w \approx 2500$) was obtained from BASF (Japan). SDS was purchased from Wako Pure Chemical Industries (Japan). All other chemicals used were of reagent grade.

2.2. Preparation of physical mixture (PM) and ground mixture (GM)

It is reported that the suitable mixing ratio for drug/PVP/SDS is at a weight ratio of 1:3:1 as it showed the highest recovery of drug nanoparticles (Wanawongthai et al., 2009; Itoh et al., 2003). The PM was prepared by physically mixing probucol, PVP, and SDS at a weight ratio of 1:3:1 in a glass vial using a vortex mixer. For the preparation of the ternary GM, the PM was ground in a TI-500ET vibrational rod mill (CMT, Japan) for 30 min. The temperature during the grinding process was controlled and maintained at $0 \pm 5\,^{\circ}C$ using a nitrogen-gas flow cooling system. The grinding cell and rod were made of stainless steel.

2.3. Preparation of freeze-dried mixture (FD)

In this research, to achieve enough the signal to noise (S/N) ratio and avoid precipitation during long *in situ* solid-state NMR measurement, a stable and highly concentrated nanosuspension was prepared for NMR evaluation and other measurements. To prepare the suspension, the GM was dissolved in 20 mL of distilled water with a probucol concentration of 5 mg/mL and the mixture was then sonicated for 2 min. The suspension was stored for 24 h at 25 °C and then freeze-dried under 15 Pa and a trap temperature of −30 °C for 72 h using the EYELA freeze dryer FD-1000 (Tokyo Rikakikai, Japan).

2.4. Analysis of particle size

The PM, GM, and FD were dispersed in distilled water and then sonicated for 2 min to form the suspension. The drug concentration in the suspension was fixed at 5 mg/mL. The particle size was determined by the dynamic light scattering method using Microtrac UPA® (Nikkiso, Japan; measurement range: 0.0008–6.5 μm) and by the light scattering method using Microtrac HRA® (Nikkiso, Japan; measurement range: 0.1–700 μm).

2.5. Morphology observation by cryogenic-scanning electron microscopy (cryo-SEM)

The morphology of the suspension was investigated using a scanning electron microscope (JSM-6510A) equipped with a cryo-SEM unit (JEOL, Japan). The GM with 5 mg/mL probucol was dispersed in water. The sample was loaded onto the cryo-specimen holder, cryo-fixed in slush nitrogen, and then quickly transferred to the cryo-SEM unit in a frozen state. The frozen sample was then fractured by striking them with a pre-cooled razor blade, at the point on the sample surface where the fracture plane was required. The specimen was then sputter-coated with gold, and a cryo-SEM image was obtained at an acceleration voltage of 1.5 kV.

2.6. Powder X-ray diffraction (PXRD) measurement

PXRD measurements were performed using a Rigaku Miniflex diffractometer (Rigaku, Japan) with a Cu Kα radiation source at a voltage of 30 kV, a current of 15 mA, and a scanning speed of $4^{\circ}\,min^{-1}$.

2.7. ^{13}C solid-state and in situ solid-state nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR spectra were measured on a JNM-ECX400 NMR spectrometer (JEOL Resonance, Japan). The solid-state and *in situ* solid-state NMR spectra were recorded with a 6-mm HX probe. The spectra were obtained by cross-polarization/magic angle spinning/total spinning sideband suppression (CP/MAS/TOSS) techniques. MAS was carried out at a rotational speed of 4 kHz. The pertinent parameters included a ^{1}H 90° pulse of 6 μs and relaxation delay of 2 s. The CP contact time was 10 ms for *in situ* solid-state NMR and 2 ms for solid-state NMR. For solid-state NMR spectroscopy, powder samples weighing *ca.* 150 mg

were placed in a 6-mm zirconia rotor. For the *in situ* solid-state NMR measurement, a GM sample with 5 mg/mL probucol was dispersed in water such that the S/N ratio was sufficient. The measurements were performed by filling the rotor with the suspension (160 μL). To achieve a suitable S/N ratio, the numbers of accumulations were 20,000 for *in situ* solid-state NMR and 12,000 for solid-state NMR. All spectra were externally referenced by setting the methine peak of hexamethylbenzene at 17.3 ppm.

2.8. Liquid-state NMR spectroscopy

Next, ^{13}C liquid-state NMR spectra were acquired on a JNM-ECX400 NMR spectrometer (JEOL Resonance, Japan) by a conventional single pulse decoupling method as follows: the samples with 5 mg/mL probucol were dissolved in D_2O. The measurements were performed at 37 °C. Chemical shifts were referenced to an internal signal of 0.05% 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP) at 0.0 ppm. A relaxation delay of 2.0 s, scan of 8000 accumulations, and rotational speed of 15 Hz were used.

3. Results and discussion

3.1. Size measurement

Fig. 2 shows the mean particle size of the ternary probucol/PVP/SDS system. The mean particle size of probucol in the PM, measured by the laser diffraction method, was 53.7 μm (Fig. 2A). The mean particle size of the GM was approximately 40 nm after it was dispersed into water (primary nanoparticles) (Fig. 2B) and 150 nm after it was stored at 25 °C for 24 h (Fig. 2C). These particle sizes were different from previously reported data (Wanawongthai et al., 2009; Pongpeerapat et al., 2006). This difference was attributed to the difference in the concentration of probucol; the concentration of probucol in this study, i.e., 5 mg/mL, was 10 times higher than that in previous studies. Fig. 2C suggests that secondary nanoparticles were formed when the suspension was stored for 24 h. The secondary nanoparticle suspension showed superior stability; its particle size was maintained at approximately 150 nm even after one month of storage (data not shown). For *in situ* measurement, this concentrated suspension was spun at 4 kHz in an NMR sample tube for 96 h. The mean particle size of the suspension was 154 nm after spinning (Fig. 2D). It should be noted that centrifugation for a long duration also had no effect on the particle size of the suspension. From these results, we inferred that this concentrated nanosuspension from ternary GM could be successfully evaluated by *in situ* solid-state NMR spectroscopy. The FD suspension showed a mean particle size of *ca.* 178 nm (Fig. 2E), indicating that the size of the particles only slightly increased after the freeze-drying process. Hence, the probucol in the FD powder was expected to exhibit the same physiochemical properties as that in the GM suspension.

3.2. Morphology observation by cryo-SEM measurement

Cryo-SEM images of the GM suspension after storage at 25 °C for 24 h are shown in Fig. 3. Cryo-SEM is used for direct viewing of hydrated (wet) samples, thus providing information about the solid or semisolid components in the suspension. The images show that the nanoparticles in water were spherical with a size of approximately 150–200 nm, which was in agreement with the DLS results. The particles appeared to be an agglomeration of primary nanoparticles.

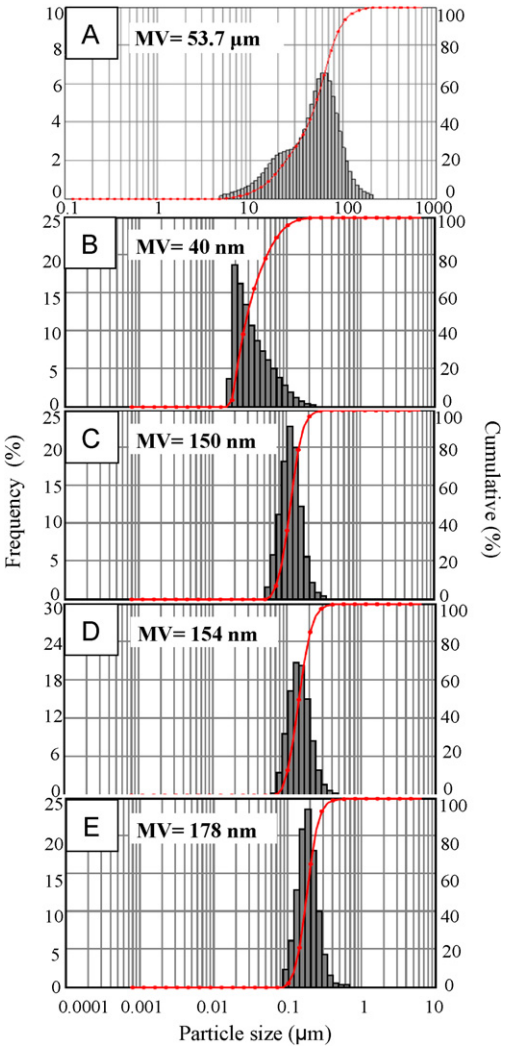

Fig. 2. Particle size distribution of probucol/PVP/SDS systems in water: (A) PM suspension (measured by laser diffraction method), (B) GM suspension after dispersion, (C) sample B storage at 25 °C for 24 h, (D) sample C after spinning at 4 kHz for 96 h, and (E) FD suspension (B–E measured by dynamic light scattering method).

3.3. PXRD characterization

Fig. 4 shows PXRD patterns of the probucol/PVP/SDS system. The PXRD pattern of the PM showed the superimposition of the diffraction peaks of probucol and SDS crystals (Fig. 4A). For the GM, the diffraction peaks disappeared and a halo pattern was observed (Fig. 4B). This result suggests that crystalline probucol may have been transformed into amorphous or very small crystals with some disorder by co-grinding. Diffraction peaks of both crystalline probucol and SDS were observed in the PXRD pattern of the FD samples, as shown in Fig. 4C. It is supposed that the crystallization of probucol occurred after the GM was dispersed in water, and SDS was crystallized from its dissolved state during the freeze-drying process.

3.4. NMR characterization

Figs. 5–7 show the ^{13}C solid-state, *in situ* solid-state, and liquid-state NMR spectra of the probucol/PVP/SDS system. The solid-state spectrum of the ternary PM (Fig. 5A) showed superimposition of the spectrum of each component in the PM (data not shown), indicating little interaction among probucol, PVP, and SDS. The solid-state

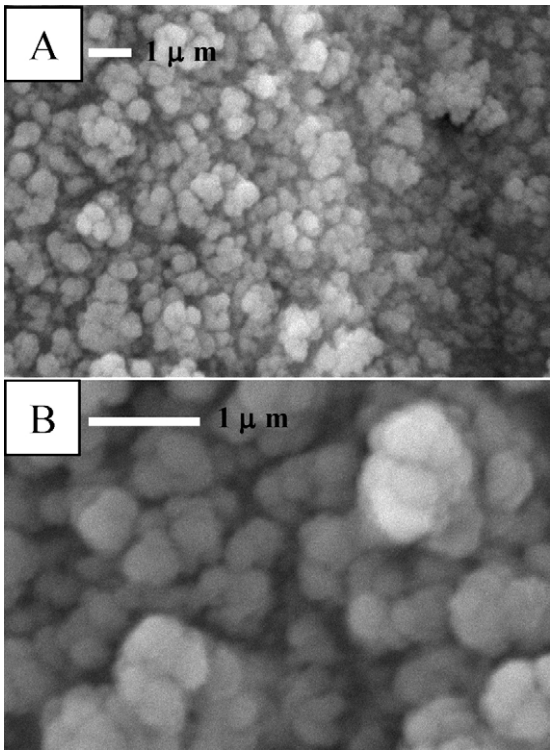

Fig. 3. Cryo-SEM images of probucol/PVP/SDS GM suspension after 24 h storage at 25 °C. A and B indicate a varying scale bar.

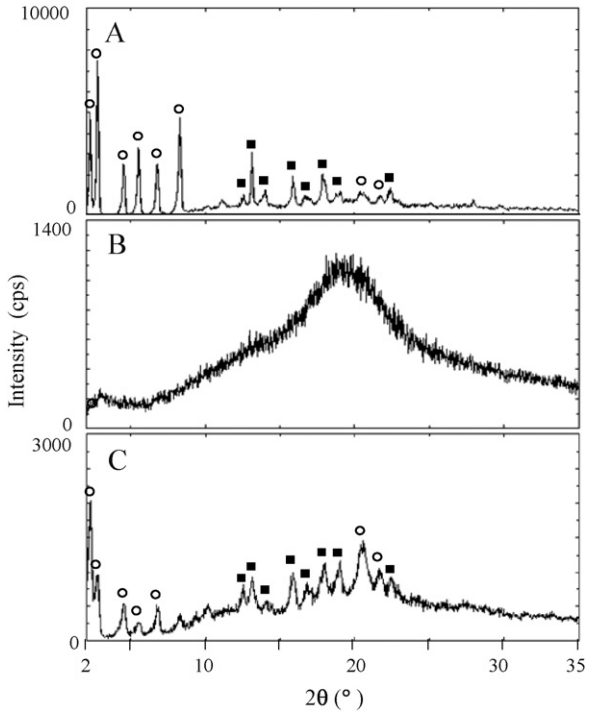

Fig. 4. PXRD patterns of probucol/PVP/SDS system: (A) PM, (B) GM, and (C) FD. Filled squares and unfilled circles represent the characteristic peaks of probucol and SDS, respectively.

spectrum of the GM (Fig. 5B) showed broadening of the peaks of probucol, and a new peak was observed at around 143 ppm, shown by a diamond. The peak of SDS (C-1″) at around 66.7 ppm was upshifted. It has been reported that the new peak and upshift variation occur because of probucol-PVP and PVP-SDS interactions during co-grinding (Pongpeerapat et al., 2006). The solid-state NMR spectrum of the FD sample (Fig. 5C) was quite similar to that of the PM in Fig. 5A, thus confirming the results of the PXRD measurements that probucol and SDS existed in a crystalline state. However, a little broadening of the probucol peak (i.e., C-3, 5) for the FD sample was observed compared with the peak shape for the PM sample. This difference could be attributed to the difference between the

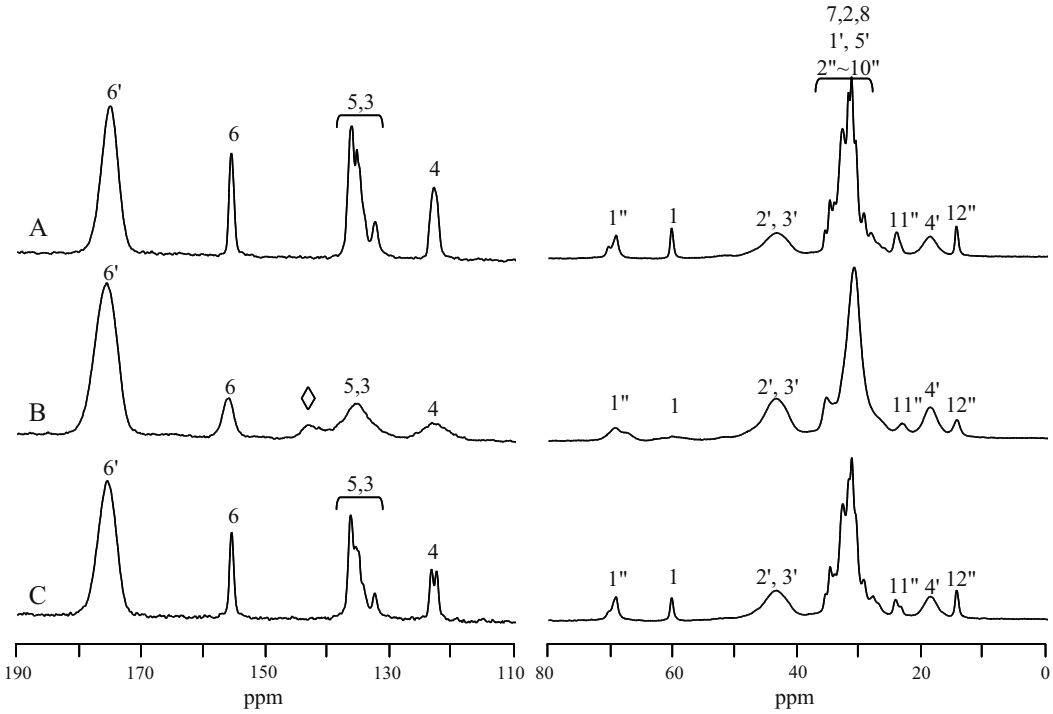

Fig. 5. ^{13}C solid-state CP/MAS/TOSS NMR spectra of probucol/PVP/SDS systems between 0–80 ppm (right) and 110–190 ppm (left): (A) PM, (B) GM, and (C) FD. The diamond denotes the new peak.

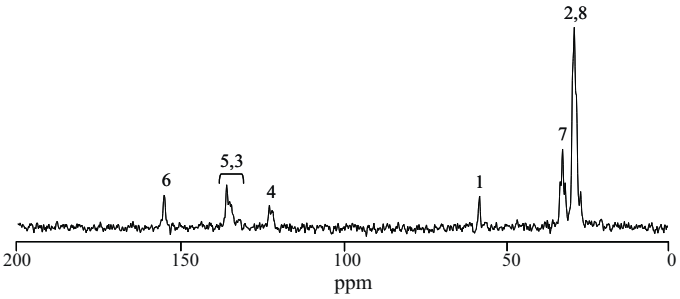

Fig. 6. ^{13}C *in situ* solid-state CP/MAS/TOSS NMR spectrum of probucol/PVP/SDS ternary GM suspension after storage at 25 °C for 24 h.

particle size of probucol crystals in the FD sample (nanocrystals) and that in the PM sample (several μm size crystals). Fig. 6 shows an *in situ* solid-state NMR spectrum of the GM dispersed in water and stored at 25 °C for 24 h. *In situ* solid-state NMR techniques have expanded the application scope of NMR spectroscopy from liquid- or solid-state materials to suspended-state materials. During CP, the transfer of nuclear magnetization from ^{1}H to ^{13}C occurs mainly via the ^{1}H–^{13}C through space dipolar interactions, which are present in both solids and liquids. In isotropic liquids, these dipolar interactions are averaged to zero, and therefore, the liquid component cannot be detected (Crisp et al., 2010). In this study, the ^{13}C peaks of samples in a relatively rigid solid state were prominent in the spectrum. The probucol signals could be detected in the spectrum of the GM suspension, possibly indicating the presence of a probucol nanocrystal core in water. These results coincided with the solid-state NMR results of the FD sample. Probucol has two polymorphs, named form I and form II (Gerber et al., 1993). The chemical shift of the characteristic peak at 122 ppm coincided with probucol form I, while the characteristic peak of probucol form II at 117 ppm was not shown (Pongpeerapat et al., 2006). Further, no peaks were recorded from the PVP and SDS molecules. These results indicated that most of the PVP and SDS molecules were either freely mobile or soluble in water. The broad amorphous peaks of probucol and a new peak at 143 ppm in the solid-state GM spectrum (Fig. 5B) disappeared in the NMR spectrum of the GM suspension. It was speculated that the spectral differences for probucol were induced by the transformation of amorphous/or very small crystals with some disorder into a nanocrystalline state after dispersion in water. Probucol nanocrystals in water could be stabilized by having PVP and SDS on the surface, although the strength of interaction on the surface was too low to be detected.

The suspensions were further investigated by ^{13}C liquid-state NMR spectroscopy to study the interactions at the surface of the nanoparticles (Fig. 7). No peaks were observed for the drug in both the PM and GM spectra despite the high drug concentration. The results suggested almost all probucol existed in a crystalline state with low mobility, which could not be detected in the liquid-state NMR spectra. The peaks of PVP and SDS were clearly observed in the spectra. In addition, the spectrum of the GM (Fig. 7B) was slightly different from that of the PM (Fig. 7A). The broader peaks of PVP (C-2′, C-3′) and SDS (i.e. C-1″, C-4″ ∼ 9″) were observed for the GM suspension. In the PM suspension, a PVP/SDS complex was expected to exist where some SDS micelles were bound to the PVP chain. In the GM suspension, solid-liquid interaction between probucol crystals and the PVP/SDS complex were expected at the surface. The PVP/SDS complex was found to alternate between its bound and unbound forms. This exchange in the solution was fast, as inferred from the NMR time scale, and thus, the NMR signals of PVP and SDS were broadened (Stockman and Dalvit, 2002). Such interaction on the probucol crystal surface stabilizes the suspension (Moribe et al., 2008).

Thus, this study confirmed the proposed nanoparticle structure in the ternary probucol/PVP/SDS GM suspension (Pongpeerapat et al., 2006, 2008; Moribe et al., 2008) and that the probucol nanocrystals were covered by PVP/SDS complexes. Moreover, the direct observation of the nanosuspension at a molecular level not only elucidated the crystal structure of probucol but also indicated that the PVP–SDS complex achieved equilibrium by alternating between its bound and unbound forms on the surface of the probucol nanocrystal. For *in situ* solid-state NMR spectroscopy, the suspension is required to maintain its stability at high concentration or during the long MAS process. The knowledge acquired by *in situ* solid state NMR spectroscopy was useful for understanding the nanoparticle structure in water. Furthermore, a combination and comparison of the results obtained by solid-state and liquid-state NMR spectroscopies can provide insight about the molecular states of drug and additives in a suspension.

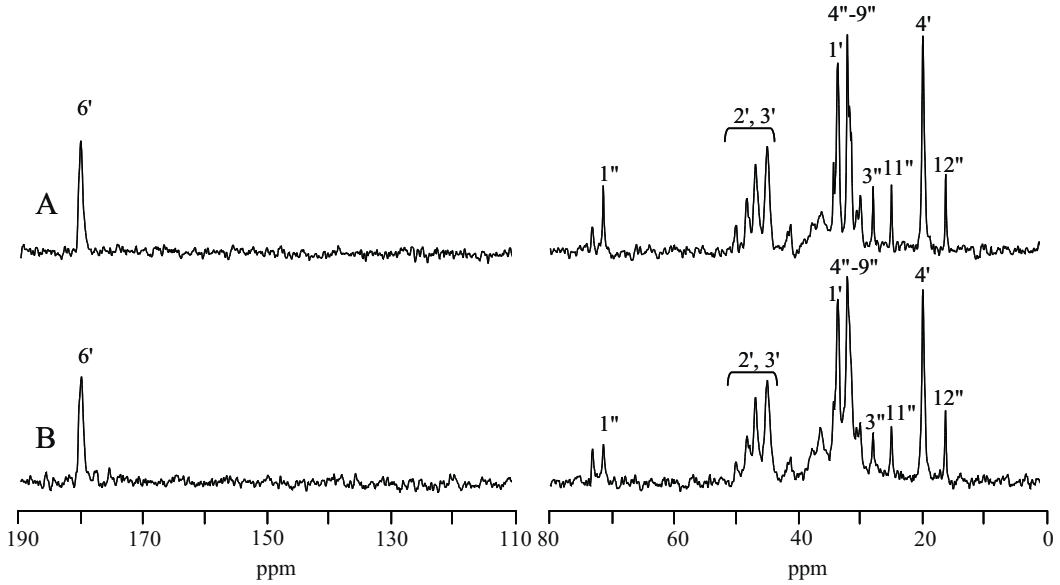

Fig. 7. ^{13}C liquid-state single pulse with decoupling NMR spectra of probucol/PVP/SDS systems between 0 ppm and 80 ppm (right) and 110–190 ppm (left): (A) PM suspension and (B) GM suspension after storage at 25 °C for 24 h.

4. Conclusions

Nanoparticles were obtained by dispersing the probucol/PVP/SDS ternary co-ground mixture in water. The nanoparticles with a mean particle size of approximately 150 nm were directly evaluated by ^{13}C *in situ* solid-state and liquid-state NMR spectroscopies. The ^{13}C *in situ* solid-state NMR spectra showed that probucol existed as a nanocrystal (form I) in water. The ^{13}C solid-state NMR and powder X-ray diffraction characterization of the FD sample confirmed the presence of the probucol nanocrystal. PVP and SDS observed by liquid-state NMR spectroscopy suggested that they interacted on the surface of the probucol nanocrystals to provide stability to the nanoparticles. The advantage of *in situ* solid-state and liquid-state NMR spectroscopies is that the drug and additives in a nanosuspension can be observed at a molecular level. We will perform further *in situ* solid-state NMR measurements for monitoring the dynamic interactions that occur at the surface of nanoparticles.

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