PHYSICOCHEMICAL CHARACTERIZATION OF SELF-ASSEMBLED NANOSTRUCTURES BY NMR SPECTROSCOPY

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ABSTRACT

Part I

α-Glucosylhesperidin (Hsp-G), a functional food additive, significantly enhances the solubility and bioavailability of poorly water-soluble drugs despite little surface activity. Herein we present investigations into the underlying mechanism by NMR techniques. A concentration dependence of the chemical shift of Hsp-G protons correlated well with a mass-action law model, indicating self-association of Hsp-G molecules. The critical micelle concentration was 5.0 mg/mL (6.5 mM) at 37 ºC. The aggregation number was calculated around 2-5 according to aggregation equilibrium and mass action law. The gradual rather than abrupt chemical shift variation upon Hsp-G aggregation would be different mode to conventional surfactants. Dynamic light scattering and 2D NOESY measurements demonstrated that Hsp-G molecules self-associated into particular small micelles, with the flavanone skeleton forming a hydrophobic core, and surrounding sugar groups working as a shell. The packing of the hydrophobic portion is not strictly oriented and the intermolecular arrangement of micelle shell is loose. Solubility enhancement was due to the incorporation of drugs into Hsp-G micelle, with naringenin being more soluble than flurbiprofen. This difference is possibly related to the structural similarities between the hydrophobic portion and the micelle core. Hsp-G micellization process with little loss of surface tension is a unique observation in surface and interface science.

Part II

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/polyvinylpyrrolidin
(PVP)/sodium dodecyl sulfate (SDS) in water. The 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.
INTRODUCTION

PART I

Oral administration is the most preferred and easiest route of drug delivery, but its use is limited when drug molecules have poor water-solubility and permeability. One approach to enhance the solubility and bioavailability of hydrophobic drugs taken orally is to use a particulate delivery system soluble or dispersible in water, e.g. using cyclodextrins\(^1\), solid dispersion systems\(^2-3\), nanoparticles\(^4\), and micelles\(^5\).

Since the late 1960s, micelles have drawn considerable attention as drug carriers owing to their ability to increase the solubility of sparingly soluble drugs in water, and their easily controlled properties for drug release and targeting\(^6-7\). Micelles are formed from unique hydrophilic-hydrophobic combinations when amphiphiles are placed in water. They consist of an inner core of assembled hydrophobic segments capable of solubilizing poorly water-soluble drugs and an outer hydrophilic shell serving as a stabilizing interface between the hydrophobic core and the external aqueous environment. However, low molecular weight surfactants, especially ionic surfactants such as sodium dodecyl sulfate (SDS), often have very high toxicity\(^8\). This delivery system is subject to severe dilution in the gastrointestinal fluid upon oral administration. Another amphiphiles assembly type is polymer micelles\(^9-11\), which usually consist of several hundred block copolymers and have diameters of approximately 10–100 nm. The molecular weight of the hydrophobic block is usually less than 2,000, while that of the hydrophilic part is about 1,000–12,000\(^9\). These polymeric micelle systems are currently a growing research field for drug delivery, with the small size and the high structural stability of polymeric micelles considered ideal for the attainment of stable, long-term circulation of the carrier system in the bloodstream\(^7\).
Recently, much attention has been paid to transglycosylated food additives as new pharmaceutical excipients in order to improve the dissolution and bioavailability of poorly water-soluble drugs. Hesperidin, an abundant flavonoid in citrus fruits, is well known as vitamin P. Glucosylhesperidin (Hsp-G), the transglycosylation product of hesperidin, is superior in water-solubility, as well as being substantially tasteless and odorless, free of toxicity, and readily hydrolyzable in vivo to hesperidin and D-glucose to exhibit the physiological activity inherent to hesperidin\textsuperscript{12}. Tozuka et al. reported that spray-dried powders of a water-insoluble drug and Hsp-G showed a pronounced enhancement of dissolution when compared to solid dispersions of the drugs with hydrophilic polymers\textsuperscript{13-14}. Moreover, in comparison with the untreated flurbiprofen (FP) crystals, the bioavailability of FP from the prepared spray-dried powders revealed 2.5- and 2.8-fold improvements in the Cmax and area under the curve (AUC) values, respectively\textsuperscript{13-14}. We confirmed that 10% (w/v) concentrations of Hsp-G solution did not have any toxic effect to Caco-2 cells, while a 0.1% (w/v) concentration of SDS solution had higher cytotoxicity\textsuperscript{15}. The surface tension of Hsp-G at 37 °C decreased from 72 mN/m of purified water to only 65.5 mN/m in concentrated Hsp-G solution\textsuperscript{15}, indicating that the surface activity of Hsp-G is remarkably weaker than that of conventional micelles such as SDS (35 mN/m). Thus, we postulated that Hsp-G formed a specific aggregation structure in concentrated solutions, which could create a micro-environment suitable for the entrapment and solubilization of hydrophobic drug molecules. However, the detailed structure of this aggregate and the solubilization mechanism of the encapsulated drugs in water has not been clearly understood.

In recent decades, NMR has become a powerful and reliable method for structural investigation and determination. Various one-dimensional (1D) and 2D NMR techniques have been successfully applied to study both the static (chemical environment, degree of association, size and shape) and the dynamic properties
(molecular mobility, kinetics of aggregation, solubilization) of micelles. The 1D $^1$H chemical shift appears very sensitive to subtle changes in the local environment of the proton and proves to be a useful tool for the rapid detection of molecular association. 2D $^1$H-$^1$H nuclear Overhauser effect spectroscopy (NOESY), based on the dipole-dipole interactions between nuclei in spacial proximity, is an effective method of studying the relative arrangement of the aggregates and their related structure.

The aim of the present study was to clarify the specific aggregation behavior of Hsp-G in aqueous solution showing the least surface activity, and to understand the solubilization mechanism of Hsp-G for poorly water-soluble drugs by NMR spectroscopy. In this study, two model hydrophobic drugs, flurbiprofen and naringenin, were used. 1D $^1$H NMR and 2D $^1$H-$^1$H NOESY spectra were employed to evaluate the aggregation of Hsp-G in water and the spatial localization of drugs.
INTRODUCTION

PART II

Drug nanoparticle formation has emerged as a promising strategy for enhancing the bioavailability of hydrophobic drugs \(^{20-22}\). Information on the size or morphology of drug nanoparticles can be obtained by analytical techniques such as light scattering or by microscopic measurements \(^{23}\). However, only a little research has been done on the molecular state of drug nanoparticles in a suspension \(^{24-26}\); it is considered that a large amount of liquid water or solvent molecules obstruct the direct evaluation of the solid molecules \(^{24-26}\). 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 \(^{27}\), 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 \(^{28}\) technique that reduces the dipole-dipole interactions and chemical shift anisotropy of the solid components dispersed in water, thus enabling the measurement of an NMR spectrum of the suspension \(^{29-30}\). Recently, *in situ* solid-state NMR experiments in various fields have revealed unknown aspects of suspensions \(^{28,31-32}\). 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 \(^{24-26}\). For the abovementioned reason, the application of this approach has been restricted to the field of pharmaceutics. In this research, we successfully prepared a stable and highly concentrated nanosuspension for \textit{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 \(^{33-36}\). Probucol nanosuspensions showed high stability and their bioavailability was significantly enhanced \(^{22}\). Solid-state \(^{13}\)C-NMR studies revealed that when a drug/PVP/SDS ternary ground system was dispersed in water, grinding-induced solid-state interactions among the components of the ternary system played an important role in nanoparticle formation \(^{33,35}\). Atomic force microscopy proved that colloidal probucol nanoparticles were coated with the PVP-SDS complex \(^{37}\). 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 \textit{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 (Figure 2). \textit{In situ} solid-state and liquid-state NMR measurements were performed for evaluating the molecular state of the nanosuspension. A freeze-dried sample of the nanosuspension was also evaluated by powder X-ray diffraction (PXRD) and \(^{13}\)C solid-state NMR measurements, and the results were compared with those of \textit{in situ} solid-state and liquid-state NMR measurements.
EXPERIMENTAL

PART I NMR characterization of self-aggregation behavior of α-glucosylhesperidin in water

Materials

Flurbiprofen (FP) and naringenin (NRG) were purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan) and used without further purification. α-Glucosylhesperidin was a gift from Toyo Sugar Refining Co., Ltd. (Tokyo, Japan). Commercial 99.9% deuterium oxide (D₂O) (Aldrich, St. Louis, USA) was used as received. All other chemicals and solvents were of reagent grade quality and used without further purification. The chemical structures and proton numbering of Hsp-G, FP, and NRG are depicted in Figure 1.

Preparation of spray-dried powders

Spray-dried powders of FP/Hsp-G or NRG/Hsp-G were prepared using a spray-drying method. To prepare samples by the spray-drying method, 500 mg of FP or NRG and defined ratios of Hsp-G were dissolved in an ethanol/water solution (8:2 v/v). The loading ratio of drug/Hsp-G was varied from 1:10 to 1:30 (w/w). This solution was fed to a spray dryer (GS31; Yamato, Tokyo, Japan) at a rate of 10 mL/min and sprayed into the chamber from a nozzle with a diameter of 406 μm at a pressure of 0.13 MPa. The inlet and outlet temperatures of the drying chamber were maintained at 120 and 70 °C, respectively. All spray-dried powders were dried in a desiccator with silica gel under reduced pressure for 1 day before their physicochemical properties were tested.

Particle size analysis:

The volumetric particle size distribution was determined by a dynamic light scattering method using Microtrac UPA® (Nikkiso, Japan). The detection range of UPA is 0.0008–6.5 μm.
**NMR measurements**

All NMR spectra were recorded on a JEOL Lambda 400 MHz spectrometer (Tokyo, Japan). The samples were dissolved in D$_2$O and measured at 37 °C. Chemical shifts are referenced to the internal signal of 0.05% 3-(trimethylsilyl) propionic-2,2,3,3-$d_4$ acid, sodium salt (TSP) at 0.0 ppm. Resonance assignments for Hsp-G were made by comparison with referenced data$^{38-40}$ and by 2D $^1$H-$^1$H correlation spectroscopy (COSY) NMR. The peaks of FP and NRG were assigned by comparison with the literature$^{38-40}$. The chemical shifts of various protons in the Hsp-G units were monitored as a function of concentration (1, 2, 3, 4, 5, 6, 7, 10, 20, 30, and 40 mg/mL). $^1$H-NMR spectra of drug-loaded Hsp-G solutions (concentration equivalent to 20 mg/mL of Hsp-G) with different loading ratios were also recorded.

The 2D $^1$H-$^1$H NOESY spectra for 1.0 and 20 mg/mL Hsp-G solutions were measured to verify the solution structure of Hsp-G. The spectra were recorded with 512 data points in the $t_2$ time domain, 256 $t_1$ increments and 64 scans. The mixing time was 1.0 s. A relaxation delay of 6.3 s was used between the scans. A sine apodization function was applied in both dimensions before Fourier transformation. The spectra were zero-filled in the $f_1$ dimension to give a 512 × 512 data matrix in the frequency domain. Under similar conditions, the NOESY spectra of 20 mg/mL FP-loaded Hsp-G and NRG-loaded Hsp-G solutions were also recorded to validate the interaction positions between drug and Hsp-G molecules.
Figure 1. Chemical structures and atom numbering of (A) α-glucosylhesperidin (Hsp-G), (B) flurbiprofen (FP), and (C) naringenin (NRG).
EXPERIMENTAL

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

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.

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

It is reported that the suitable weight ratio for drug/PVP/SDS is 1:3:1 as it shows the highest recovery of drug nanoparticles $^{33-36}$. 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.

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).

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).

**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.

**Powder X-ray diffraction (PXRD) measurement**

PXRD measurements were performed using a Rigaku Miniflex diffractometer (Rigaku, Japan) with a CuKα radiation source at a voltage of 30 kV, a current of 15 mA, and a scanning speed of 4° min⁻¹.

**13C solid-state and in situ solid-state nuclear magnetic resonance (NMR) spectroscopy**

13C 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 1H 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 signal to noise (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.

$^{13}$C liquid-state NMR spectroscopy

$^{13}$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$_2$O. 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 8,000 accumulations, and rotational speed of 15 Hz were used.
Figure 2. Chemical structures and carbon atom numbering of (A) probucol, (B) PVP (K12, $M_w \approx 2,500$), and (C) SDS.
RESULTS AND DISCUSSION

PART I NMR characterization of self-aggregation behavior of α-glucosylhesperidin in water

Aggregation behavior of Hsp-G in aqueous solution

The $^1$H chemical shift is often sensitive to subtle changes in the local environment of the molecule and is usually adopted to study molecular association. $^1$H-NMR was performed on gradual concentrations (1, 2, 3, 4, 5, 6, 7, 10, 20, 30, and 40 mg/mL) of Hsp-G as shown in Figure 3. The results revealed that the chemical shifts (ppm) of all protons decreased with increased concentration of Hsp-G, suggesting that some aggregation structure was formed as the Hsp-G concentration was increased. The flavanone skeleton protons ($H-2'$, $H-5'$, $H-6'$, $H-2$, $H-6$ and $H-8$) were considerably more shielded. Meanwhile the sugar protons ($H-1$$''$, $H-1$$''''$ and $H-6$$''''$) were less shielded, except $H-1$$''$ which is close to the flavanone skeleton. The significant upshift of flavanone skeleton protons could be caused by the formation of intermolecular interactions, such as π-π interaction during the process of Hsp-G aggregation. Consequently, the flavanone skeleton of the Hsp-G molecule should play an important role in its aggregation. The chemical shifts of sugar protons were further influenced by the effects of ring currents and thus exhibited small change in chemical shift during the aggregation process.

Figure 4 shows concentration-dependent changes of $^1$H chemical shifts for some key Hsp-G signals that were typically affected by the aggregation. NMR chemical shift values corresponding to different nuclei in molecular units can be used to detect self-aggregation behavior, e.g. micellization, has been previously documented. This method could also apply to the investigation of large molecular weight polymer micelles. Here, the value of critical micelle concentration (cmc) was used as the index.
parameter of the self-assembly. Below the cmc, the average environment of various protons in the molecule was surrounded by solvent molecules and the observed chemical shift, \( \delta_{\text{obs}} \), was the chemical shift of the monomer, \( \delta_{\text{mon}} \). Above the cmc, \( \delta_{\text{obs}} \) was the weighted average of the monomer and micelle chemical shifts, based on the assumption that the exchange of monomers and micelles in the solution is comparatively fast on the NMR time scale. If the monomer concentration was constant above the cmc, the observed chemical shift could be described as follows using the mass-action law model (Eq. (1)) \(^{42,45-46} \):

\[
\delta_{\text{obs}} = \delta_{\text{mic}} - (\text{cmc}/C)(\delta_{\text{mic}} - \delta_{\text{mon}}) \quad \text{(Eq. (1))}
\]

A plot of \( \delta_{\text{obs}} \) as a function of 1/C should consist of two straight lines—one above and the other below the cmc. The intersection of the two lines is the point corresponding to 1/C = 1/cmc. Figures 4A and 4B represent plots of \( \delta_{\text{obs}} \) (H) at H-2` and H-1` as a function of the reciprocal of the total Hsp-G concentration, respectively. The lines of the experimental data fitted to the theoretical mode. The chemical shift variation broke abruptly at a specific concentration. The cmc value determined from these chemical shift dependences was about 5.0 mg/mL (6.5mM) at 37 ºC. This value was in accordance with one previously obtained by surface tension measurements, although the decrease in surface tension is very small\(^{15} \). The critical aggregation of conventional surfactants, e.g., dodecyldimethylbenzylammonium chloride, provides an abrupt chemical shift variation corresponding to the critical micelle concentration\(^{42,45-46} \). The gradual chemical shift upon Hsp-G aggregation, on the contrary, would be different mode of aggregation to conventional surfactants. The proposed Hsp-G aggregation may not be categorized as a strict definition of “critical micelle concentration”. It has been reported that a self-aggregation of bile salts surfactants provides a gradual change in
NMR chemical shift\textsuperscript{47}. This was explained by their specific structures, which are rather different from those of the conventional surfactants\textsuperscript{47}. Similarly to bile acids, there exists a range of concentration where Hsp-G aggregation took place. A negligible shift was observed for the terminal methyl group in hydrophilic sugar groups of Hsp-G as shown in Figure 4C. It confirmed that this proton does not change its chemical environment through aggregate formation of Hsp-G molecules.

The Hsp-G aggregation number was further calculated by the chemical shift variation according to aggregation equilibrium and mass action law (Eq. (2))\textsuperscript{48}, where \( n \) and \( K \) are the aggregation number and aggregation equilibrium constant, respectively. The plot of \( \log (C(\delta_{\text{obs}}-\delta_{\text{mic}})) \) versus \( \log(C(\delta_{\text{mon}}-\delta_{\text{obs}})) \) should give a straight line. The slope yields the value of the aggregation number \( n \).

\[
\log(C(\delta_{\text{mon}}-\delta_{\text{obs}})) = n \log(C(\delta_{\text{obs}}-\delta_{\text{mic}})) + \log(nK) + (1-n)\log(\delta_{\text{mon}}-\delta_{\text{obs}}) \quad (\text{Eq. (2)})
\]

At the Hsp-G concentration below 5 mg/mL, the aggregation number was calculated as 2. It could show a dimer formation (Figure 5). At the Hsp-G concentration above 5 mg/mL, the aggregation number was increased to 4-5. It suggested that Hsp-G molecules self-associated into particular small micelles with low aggregation number.

It should be noted that the surface tensions at 37 °C decreased slightly from 72 mN/m to 65.5 mN/m of purified water when the concentration of Hsp-G was increased. Hsp-G had surface activity although the surface activity was considerably weaker than that of conventional micelles such as SDS (35 mN/m) or fluorinated random copolymers (20~30 mN/m)\textsuperscript{49}. It is currently accepted that common surfactants or amphiphiles are first adsorbed at the water surface to form a Gibbs monolayer and then form micelles in the solution above cmc. However, Matsuoka \textit{et al.}, reported that a new class of amphiphiles, which consisted of hydrophobic and ionic polymer chains, showed
“micelle formation without Gibbs monolayer formation”. Its origin was suggested to be image charge repulsion at the air/water interface. Owing to its hydrophobicity, the block copolymer would be adsorbed at the air/water interface. However, near the interface a strong electrostatic repulsion from the interface was induced by the image charge effect. As the hydrophilic chain consisted of polyions, this electrostatic repulsion was so strong that the polymers could not be adsorbed but formed micelles in bulk solution. This was explained as a totally new observation in the field of physical chemistry of surfaces and interfaces\textsuperscript{50-54}. Since Hsp-G is non-ionic material, the surface activity could not be explained by an image charge effect. It could be mentioned that the Hsp-G is energetically more favorable in an aggregation, compared with in a surface monolayer. The modest decrease in surface tension probably corresponds to a distribution of “gaseous” like Hsp-G molecules at the surface. For an appreciable reduction in surface tension, a higher surface concentration of Hsp-G at the surface is required. However, it seems that the large polar head groups of Hsp-G geometrically preclude the possibility of a significant association of the non-polar rings at the planar air/water interface. Thus, a high surface concentration can not be achieved, since it would be unfavorable to have sugar moieties at the surface. In contrast, a small aggregation would provide such an environment to decrease the unfavorable hydrophobic interaction between water and the rings. This assumption is supported by the results from dynamic light scattering (DLS) method that the particle size in Hsp-G solution increases from ca. 1 nm to 3 nm as a function of the concentration (Figure 6), although it is not easy to determine the accurate size due to the principle of the measurement.

To further probe the conformation of Hsp-G molecules in either non-aggregated or aggregated states, 2D \textsuperscript{1}H-\textsuperscript{1}H NOESY experiments were performed at Hsp-G concentrations of 1 and 20 mg/mL, which are below and above the cmc value (= around 5.0 mg/mL (6.5 mM)) (Figure 7). The correlation peaks observed in the 2D \textsuperscript{1}H-\textsuperscript{1}H
NOESY spectra are the result of cross-relaxation between neighboring protons that are spatially close to each other (roughly below 5 Å). The NOESY spectrum of a 1 mg/mL Hsp-G solution shown in Figure 7A gave a few weak cross-peaks only among sugar proton pairs, which could be due to intramolecular interaction. In addition, we did not observe any NOE interactions between flavanone skeleton protons of the Hsp-G molecule. These results showed that in a non-aggregated or monomolecular state, the Hsp-G molecules are probably in an extended rather than folded conformation. By contrast, when the concentration of Hsp-G was increased from 1 to 20 mg/mL, many more additional correlation peaks were observed, implying much denser packing of the molecules in the aggregates. The peaks were ascribed to the formation of the hydrophobic core because most of new cross-peaks appeared in the hydrophobic region, confirming the hydrophobic interaction of the flavanone skeleton. At the hydrophilic portion, the intensity of all cross-peaks among sugar protons was also increased in comparison with the non-aggregated state. Additionally, we did not observe any NOE interaction between flavanone skeleton and the hydrophilic portion of the Hsp-G molecule. These results offer insight into the spatial proximity between the respective molecular fragments and give information about the preferred conformation of Hsp-G.

In many articles, the self-aggregations of amphiphilic materials are generally described as micelles even when the aggregation number is lower than $10^{55-57}$. For instance, bile salts such as sodium cholate (NaC) and sodium taurocholate (NaTC) form dimmer and trimer aggregations, which are widely accepted as micelles or small micelles$^{47,56}$. In this study, it is most probable that due to steric hindrance, amphiphilic Hsp-G molecules self-associate into small micelles. We speculate the Hsp-G micelle structure to be core-shell like where the flavanone skeleton is segregated from the aqueous exterior to form an inner core encased by a shell of sugars. The conventional core-forming segments, such as alkyl or aryl chains, could form the core through a
combination of intermolecular forces. Interestingly, flavanone skeleton moieties show appreciable self-associating behavior, driven by hydrophobic interactions, which are substantial for the core formation. The micropolarity of such a hydrophobic core comprising flavanone skeletons may provide a better solubilizing microenvironment for the desired encapsulation of a specific drug with a similar flavanone skeleton or aromatic structure, since the loading capability was mainly dependent upon the compatibility of the drug and the hydrophobic segment.

To further understand the Hsp-G micelle, the key cross sections from the 2D NOESY spectra were taken and are shown in Figure 7C. Figures 7C (a, c, e) display cross-sections along the $f_2$ dimension from the NOESY spectrum of Figure 7A taken at 7.05 ppm, 5.11 ppm, and 1.09 ppm, which are the chemical shifts of H-2`, H-1`` and H-6```, respectively. Correspondingly, Figures 7C (b, d, f) show cross-sections from Figure 7B taken at 6.88 ppm, 4.88 ppm, and 1.08 ppm representing H-2`, H-1`` and H-6``` resonance. These three proton environments represent an aromatic proton, anomeric proton, and terminal sugar proton, respectively. At low Hsp-G concentration, no NOE interactions can be detected for these protons. At the higher Hsp-G concentration of 20 mg/mL, numerous intra- and intermolecular NOE interactions can be seen in this trace. For H-2` (Figure 7C (b)), several intra- and intermolecular NOESY cross-peaks were detected involving H2`-H8,6, H2`-H2, H2`-H4`(OCH$_3$), H2`-H3 pairs. These groups participate in the formation of the hydrophobic core and are thus in close proximity with each other. In the hydrophobic portion of the Hsp-G molecules, the distance between H-2` and H-8,6 is too large for intramolecular interactions to be significant. The observed cross-peak could be explained as the result of intermolecular interactions between different Hsp-G molecules. Emin et al., and Yuan et al. reported the surfactant structures of Hyamine-M and Triton X-100, respectively, by NMR spectroscopy. The packing of the hydrophobic chains was not strictly oriented during aggregation, this

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being confirmed by NOE contacts\textsuperscript{16,60}. Hence, we speculated that the packing of the hydrophobic part in Hsp-G micelles is similarly not strictly oriented, i.e. H-2\textsuperscript{`} group of one molecule might be packed near the H-8,6 groups of another molecule. The additional cross-peak between H-2\textsuperscript{`} and H-4'(OCH\textsubscript{3}) can similarly be attributed to this disorder. For the anomeric signal H-1```(Figure 7C (d)), sugar proton accumulation could also be observed. For terminal sugar signal H-6``` (Figure 7C (f)), no NOE correlation peak can be detected in the observed range. In the micellization of alkyl or alyl surfactants with low molecular weight, aggregation of the hydrophilic portion can occur in accordance with the concentration changes\textsuperscript{42}. The least dense cross-peaks indicate less intermolecular interaction at the surface of Hsp-G micelles even at high concentration. Thus, we assumed that in Hsp-G micelles, the sugar protons are loosely packed. Although the correlation of surface activity and micelle structure has not been extensively discussed in the literature, the loose packing of the sugar shell appears to be related to little surface activity of Hsp-G.

**Solubilization mechanism of poorly water-soluble drugs**

Figure 8 shows the \textsuperscript{1}H-NMR spectra of Hsp-G in D\textsubscript{2}O at 37 °C before (8A) and after (8B, or 8C) being spray-dried with FP or NRG, together with assignment of the most prominent resonance signals. The peaks of FP and NRG were clearly detected. In general, solution NMR cannot be used to analyze molecules with low mobility e.g. crystals due to the broad signals. The solution NMR properties suggest that these drugs in Hsp-G solution are in molecular-dispersed state rather than crystal state. The solubilities of FP and NRG were calculated at 37 °C from the integration values of \textsuperscript{1}H peaks using 0.05% TSP as a reference. The concentration of NRG was calculated as 1.42 mg/mL (untreated NRG: 0.05 mg/mL), indicating almost 30 times greater solubility when compared to NRG crystals. This result was in accordance with our previously published data, which indicated the enhancement effect of Hsp-G on the
solubility of NRG by HPLC\textsuperscript{13}. The solubility of FP prepared as spray-dried powders of FP/Hsp-G also showed a remarkable increase in water, with the concentration of FP calculated as 0.49 mg/mL (untreated FP: 0.04 mg/mL). The enhancement of the drug solubility is due to the incorporation of the drug into the Hsp-G micelle via intermolecular interaction, as Hsp-G micelles have an inner core of assembled flavanone skeleton segments capable of solubilizing poorly water-soluble substances. They also have an outer hydrophilic shell serving as a stabilizing interface between the hydrophobic core and the external aqueous environment. Since both FP and NRG molecules possess a hydrophobic character, they are incorporated in the Hsp-G hydrophobic core. The intermolecular interactions in the spray-drying process facilitate the drug-solubilized Hsp-G micelle formation in water. The solubility enhancement of NRG was significantly higher than that of FP. Many studies indicate that the most important factor related to drug solubilization capacity of a micelle is the compatibility between the drug and the core-forming segment\textsuperscript{59}. For this reason, the choice of core-forming segment is very important; the more similar the polarity of the hydrophobic micelle core to the solubility characteristics of the drug, the more effective the drug incorporation should be\textsuperscript{61}. It can be rationalized that NRG could more easily gain ingress to the hydrophobic part of micelles compared with FP, primarily due to similarity in the polarity of NRG with Hsp-G micelle cores.

We applied this same approach using the chemical shifts of some key signals from drug/Hsp-G spray-dried powders with different drug-loaded ratios. Figures 9A-B show chemical shift variation of the \textsuperscript{1}H chemical shifts in the Hsp-G unit on the different drug-loaded ratios (1:10, 1:20, and 1:30) of the same Hsp-G concentration. Compared with untreated Hsp-G, drug-loaded Hsp-G exhibited upshift variation in the hydrophobic part (H-4′, H-5′6′, H8 and H-3), whereas no significant change was observed in the hydrophilic part (H-6″″). This upshift is probably due to interaction of
the flavanone skeleton of Hsp-G with the FP or NRG molecules. Additionally, the chemical shift variation of the Hsp-G signals showed a good relationship with the amount of drug loaded. It is clear that drug inclusion influenced the structure of Hsp-G micelle. Furthermore, the overall effect of the drug on the chemical shift of Hsp-G was in agreement with the above-mentioned solubilization enhancement.

2D NOESY spectroscopy was used to confirm the localization of FP and NRG. Figure 10 show NOESY spectra of FP-loaded Hsp-G and NRG-loaded Hsp-G. The key cross-peaks to deduce the possible interaction sites between the Hsp-G molecules and FP or NRG molecules are highlighted. A significant number of NOE interactions connecting drugs and Hsp-G were found. These indicated that the FP and NRG were bound to the Hsp-G micelles. These new cross-peaks presented were not observed in the NOESY spectrum of the pure micelle without the drugs (Figure 7). Figure 10A shows that the aromatic protons of FP exhibited NOE contacts with the flavanone skeleton as well as with methoxy group (H-4′OCH₃) of Hsp-G. NOE interactions between the aromatic proton (H-c, H-f, H-b′, and H-f′,) of FP and Hsp-G (H-2′,5′,6′) were observed. Additionally, the methyl group of FP (H-h), and the methoxy group of Hsp-G (H-4′OCH₃) exhibited cross-peak, whereas no NOE interactions connecting FP to the hydrophilic moiety of Hsp-G could be found. It was confirmed that the intermolecular interactions between FP and Hsp-G were between the flavanone skeleton and the FP molecules. The same NOESY experiment was carried out with NRG-loaded Hsp-G as shown in Figure 10B. Since there is a high degree of similarity between the flavanone skeleton of NRG and the Hsp-G structure, a number of signals showed significant overlap, making the assignment of the NOE cross-peaks uncertain. However, a well-defined NOE cross-peak indicates the proximity of two flavanone skeletons between Hsp-G (H-2′) and NRG (H-B′, H-F′). The observed NOE interaction can be interpreted as a result of the insertion of NRG into the core interior, and the flavanone skeletons of
Hsp-G and NRG were relatively close to each other. All NMR observations undoubtedly confirmed our assumption that the solubilized FP and NRG mainly accumulated and was stabilized in the Hsp-G hydrophobic core by hydrophobic interaction.

The micellization of Hsp-G indicated by NMR measurements is somewhat similar to that of bile salts surfactants. The bile salts surfactants, with bulky molecules and large molecular weight, have a gradual rather than abrupt cmc, and aggregate with low numbers around 2-5, which are different from other alkyl surfactant 47, 55-57. The particle size of ca. 1-3 nm from DLS measurements supported that Hsp-G self-associates into small micelles, in which the aggregate number of Hsp-G molecules was around 2-5. A simplified schematic representation of the Hsp-G micelles is given in Scheme 1. Interestingly, Hsp-G molecules which show less surface activity are shown to undergo self-assembly to form small micelles with a core-shell like architecture in aqueous solution. Flavanone skeletons are segregated from the aqueous exterior to form a drug-loading core through hydrophobic driving forces. The core is stabilized by shell-forming segments and the packing of these hydrophobic parts is not strictly oriented with the intermolecular arrangement of micelle surface being loose. Furthermore, poorly soluble drugs could be effectively incorporated into the Hsp-G hydrophobic core through hydrophobic interactions.
Figure 3. Partial $^1$H NMR spectra of Hsp-G proton signals at different concentrations ranging from 1 to 40 mg/mL recorded in D$_2$O at 37 ºC. The concentrations are given on the right hand side of the spectra.
Figure 4. Variation of chemical shifts versus reciprocal Hsp-G concentrations ranging from 1 to 40 mg/mL: (A): H-2`, (B): H-1``, and (C): H-6``. The chemical shift data used in this graph were indicated in Figure 3 at the concentration of 1 and 40 mg/mL.
Figure 5. The plot of $\log(C(\delta_{\text{obs}} - \delta_{\text{mic}}))$ versus $\log(C(\delta_{\text{mon}} - \delta_{\text{obs}}))$ of the H-2' signal of Hsp-G in D$_2$O at 37 °C.
Figure 6. Particle size distribution of Hsp-G at different concentrations ranging from 1 to 50 mg/mL measured by DLS.
Figure 7. 2D $^1$H/$^1$H NOESY spectra and cross sections of Hsp-G (A) NOESY spectrum of 1 mg/mL (below cmc) and (B) NOESY spectrum of 20 mg/mL (above cmc). (C) cross sections taken at proton resonance of (a): H-2' (1 mg/mL, 7.05ppm), (b): H-2' (20 mg/mL, 6.88ppm), (c): H-1`` (1 mg/mL, 5.11ppm), (d): H-1`` (20 mg/mL, 4.88ppm), (e): H-6''' (1 mg/mL, 1.09ppm), and (f): H-6''' (20 mg/mL, 1.08ppm).
Figure 8. $^1$H NMR spectra of (A) Hsp-G, 20 mg/mL; (B) FP-loaded Hsp-G, 22 mg/mL; and (C) NRG-loaded Hsp-G, 22 mg/mL. Stars indicated peak-overlap between Hsp-G and drug signals.
Figure 9. Chemical shift variation at the different chemical moieties of Hsp-G after spray-drying with different weight ratios (drug:Hsp-G) of (A): FP and (B): NRG.
Figure 10. 2D $^1$H/$^1$H NOESY spectra of (A) FP-loaded Hsp-G and (B) NRG-loaded Hsp-G. New cross peaks are highlighted.
**SCHEME 1.** Schematic representation of the micelle structure of Hsp-G based on NMR constraints.
RESULTS AND DISCUSSION

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

Size measurement

Figure 11 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 (Figure 11A). The mean particle size of the GM was approximately 40 nm after it was dispersed into water (primary nanoparticles) (Figure 11B) and 150 nm after it was stored at 25 °C for 24 h (Figure 11C). These particle sizes were different from previously reported data. 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. Figure 11C 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 (Figure 11D). 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 (Figure 11E), 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.

Morphology observation by cryo-SEM measurement
Cryo-SEM images of the GM suspension after storage at 25 °C for 24 h are shown in Figure 12. 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.

**PXRD characterization**

Figure 13 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 (Figure 13A). For the GM, the diffraction peaks disappeared and a halo pattern was observed (Figure 13B). 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 Figure 13C. 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.

**NMR characterization**

Figures 14~16 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 (Figure 14A) 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 spectrum of the GM (Figure 14B) 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 $^{33}$. The solid-state NMR spectrum of the FD sample (Figure 14C)
was quite similar to that of the PM in Figure 14A, 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 particle size of probucol crystals in the FD sample (nanocrystals) and that in the PM sample (several μm size crystals). Figure 15 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 \(^{29}\). 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 \(^{62}\). 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 \(^{33}\). 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 (Figure 14B) 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 (Figure 16). 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 (Figure 16B) was slightly different from that of the PM (Figure 16A). 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. Such interaction on the probucol crystal surface stabilizes the suspension.

Thus, this study confirmed the proposed nanoparticle structure in the ternary probucol/PVP/SDS GM suspension 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.
Figure 11. 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.).
Figure 12. Cryo-SEM images of probucol/PVP/SDS GM suspension after 24 h storage at 25 °C. A and B indicate a varying scale bar.
Figure 13. 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.
**Figure 14.** $^{13}$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.
Figure 15. $^{13}$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.
Figure 16. $^{13}$C liquid-state single pulse with decoupling NMR spectra of probucol/PVP/SDS systems between 0-80 ppm (right) and 110-190 ppm (left): (A) PM suspension and (B) GM suspension after storage at 25 ºC for 24 h.
CONCLUSIONS

Part I

Concentration-dependent variation of proton chemical shift in amphilic Hsp-G revealed the self-association of these molecules in solution. The gradual chemical shift variation indicated the specific Hsp-G aggregation, which was different from that of conventional alkyl surfactants. The cmc for Hsp-G was determined to be around 5.0 mg/mL (6.5 mM). The aggregation number was calculated around 2-5 according to aggregation equilibrium and mass action law. Further 2D NOESY and DLS measurements confirmed that Hsp-G molecules self-associated into particular small micelles with a core-shell like architecture, in which the flavanone skeleton is segregated from the aqueous exterior to form a novel drug-loading core surrounded by a hydrophilic shell of sugar groups. As Hsp-G has little surface activity, Hsp-G micellization should be a unique phenomenon. Moreover, we found that two model drugs, FP and NRG, were effectively trapped into the core, as the structure dependent manner. The chemical shift variation of the Hsp-G signals showed a good relationship with the quantity of drug loaded, indicating that drug loading affects the Hsp-G micelle. Hsp-G, a food additive, could prove attractive as non-toxic and less expensive excipient for drug delivery in pharmaceutical industry.

Part II

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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LIST OF PUBLICATIONS

This thesis is based on the following publications:


LIST OF COMMITTEE

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