Morphology and Surface States of Colloidal Probucol Nanoparticles Evaluated by Atomic Force Microscopy

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   Morphology and surface states of colloidal probucol nanoparticles after dispersion of probucol/polyvinylpyrrolidone (PVP)/sodium dodecyl sulphate (SDS) ternary ground mixture into water were investigated by atomic force microscopy (AFM). The observed particles had core-shell structure, *i.e.* drug nanocrystals were covered with PVP and SDS complex. The AFM phase image and the force curve analyses indicated that probucol nanoparticles with PVP K17 showed layer structure, compared to those with PVPK12. The structural difference was explainable in terms of the molecular states of PVP-SDS complex on the particle surface. These findings support not only the mechanism of drug nanoparticle formation but also the *in vivo* absorption results with the almost same particle size of *ca.* 40 nm.

   Key words   atomic force microscopy; colloidal nanoparticle; surface state; grinding; probucol

Co-grinding, where a drug is ground together with excipients, is a promising method for an effective reduction of particle size. A certain number of studies revealed that the co-ground mixture enhanced dissolution, resulting in the improvement of oral absorption and bioavailability.[1—3] Wet co-grinding method has been known as an effective method to produce stable nanosuspensions.[4—7] Liversidge and Cundy proposed a preparation method for crystalline nanoparticle by wet co-grinding of danazol with polyvinylpyrrolidone (PVP).[4] Oral absorption of poorly water-soluble drugs in nanocrystal form was remarkably improved.[4,5] Shi *et al.* reported the image of drug nanocrystals in the formulation by atomic force microscopy and complementary techniques.[8] They showed the morphology of nanocrystals, though the surface states of the nanocrystals were not well characterized.

   Compared with the wet co-grinding method, the dry co-grinding process has some advantages for solid pharmaceutical applications due to its simple preparation with free solvents. Our previous studies demonstrated that drug nanoparticle was successfully produced by the dry co-grinding of a poorly water-soluble drug with PVP and sodium dodecyl sulfate (SDS).[9—11] When probucol, which is mainly used as a cholesterol-lowering agent, was used as a model drug, solid-state ^{13}C-NMR studies revealed that the nanoparticle formation and stabilization were attributable to grinding-induced solid-state interactions among components of the drug/PVP/SDS ternary system.[12] The agglomeration of colloidal nanoparticles after dispersing the ground mixture (GM) into water was effectively inhibited in the presence of PVP and SDS. The results of particle size and zeta-potential measurements suggested that the adsorption of both PVP and SDS as the complex on the surface of crystalline particles stabilized the drug nanoparticles.[13] Because surface states of the probucol nanoparticles certainly affected the dissolution and the subsequent absorption, the direct observation of the nanoparticles in aqueous environment was an urgent requisite. We have observed the morphology of probucol nanoparticles in water by environmental scanning electron microscopy. However, it was difficult to evaluate the morphology of nanoparticles especially smaller than 100 nm. Structural changes of the nanoparticulate might also occur on the process of vacuum.

   Atomic force microscopy (AFM) has been used to evaluate the morphology nanoparticulate structures.[14,15] Ibuprofen-loaded nanoparticles were evaluated by AFM and transmission electron microscopy (TEM).[14] The core-shell structure was observed by TEM, but not by AFM because the observation was conducted using the dried sample. The AFM measurement in solution should be required to evaluate the structure. Recently, structural difference of micelles with or without cross-linking in solution was investigated by AFM.[15] The morphological changes were investigated but the physicochemical properties were not well characterized. The advantage of AFM measurement in solution was to evaluate not only the morphology but also the physicochemical properties of the sample. In this study, colloidal drug nanoparticles after dispersion of the probucol/PVP/SDS ternary GM in water were evaluated by AFM. We used silanized mica to immobilize the nanoparticles. The AFM topography and phase image observation as well as force curve analysis were conducted to evaluate the morphology and surface states of colloidal probucol nanoparticles.

Experimental

   Materials   Probucol was supplied by Daiichi-Sankyo Pharmaceutical Co., Ltd. (Japan). PVP K12 (Kollidon® 12 PF, Mw 2500) and PVP K17 (Plasdone® C15, Mw 10000) were obtained from BASF Japan Ltd. and ISP Technologies, Inc. (U.S.A.), respectively. Sodium dodecyl sulphate was purchased from Wako Pure Chemical Industries Ltd. (Japan). All other chemicals used were of reagent grade.

   Preparation of Ground Mixture (GM) Suspension   Probucol (0.500 g), PVP (1.500 g) and SDS (0.500 g) (weight ratio of 1:3:1) were physically mixed in a glass vial using a vortex mixer (physical mixture, PM). For the preparation of probucol/PVPK12/SDS ternary GM, the physical mixture was ground in a vibrational rod mill (TI-200, Heiko Seisakusho, Japan) for 30 min. The GM powder was dispersed in water and then sonicated for 2 min to prepare the colloidal nanosuspension. The particle size was measured by the dynamic light scattering method using Microtrac UPA® (Nikkiso, Japan) and estimated as 30—40 nm. To prepare the GM suspensions with the particle size of 30—40 nm, probucol/PVPK17/SDS was cryoground in a vibrational rod mill (TI-200, Heiko Seisakusho, Japan) under liquid nitrogen flow for 90 min.

   Atomic Force Microscopy   Atomic force microscope (MFP-3D, Asylum Research, U.S.A.) was used to observe morphology and surface states of colloidal probucol nanoparticles. A silanized mica was prepared by dropping 0.1% of 3-aminopropyltriethoxysilane (APTES) solution onto a mica surface. After storing it for 30 min at room temperature, excess amount of APTES was washed with water. The positively charged mica surface can easily immobilize the negatively charged drug nanoparticles. The probucol/PVP/SDS ternary GM powder (0.5—0.6 mg) was dispersed in 1 ml of purified water and sonicated for 2 min. The colloidal probucol nanoparticles were dropped onto the silanized mica to immobilize them onto the surface. Alternative contact mode atomic force microscopy was performed in the liquid environment. A silicon cantilever (BL-RC150VB-C1, OLYMPUS, Japan) was used for the observation. The AFM topography image, which reflects topographic features of the surface, was obtained from the amplitude change of the cantilever oscillation. The AFM phase image, which reflects

the probe motion, was obtained from the phase shift relative to a driving oscillator. The phase difference of the sample surface was expressed in a contrast between light and shade.

Force *versus* distance measurements in the liquid environment were performed using silicon nitride cantilever (OMCL-RC800PSA-W, OLYMPUS, Japan). Deflection of the cantilever was plotted as a function of z height of the sample. When the sample was not touching on the tip (sample height $<$0), the deflection was constant. Samples were measured at the center of the particles. Stiffness and layer thickness of the particle surface were evaluated by comparison with the reference where no samples were added on silanized mica.

In Vivo Absorption Studies *In vivo* experiment was performed by the similar method reported previously.[16] Ground mixture suspensions of probucol/PVP/SDS at the weight ratio of 1 : 3 : 0.5 were used to reduce the effect of surfactant on the drug absorption. The particle size was adjusted within the range of 30—40 nm. All *in vivo* studies were conducted according to the guide for the care and use of laboratory animals stood by association for assessment and accreditation of laboratory animal care international (AAALAC). Probucol formulations were administered by oral gavages to male Sprague Dawley rats. All rats were made to fast prior to the dose administration and remained fasting until 6 h after the dose administration. Drug concentration of the suspensions were adjusted at 25 mg/ml before the administered to rats at 8 ml/kg by oral gavage. Blood samples (*ca.* 0.3 ml) were collected at 0, 1, 2, 3, 4, 7, 10, 24 and 48 h after drug administration ($n=3$). The plasma concentration was measured by HPLC with the same condition previously reported.[16] The area under the blood concentration–time curve (*AUC*) value (0—48 h) of the plasma profiles was calculated using the logarithmic and linear trapezoidal rules.

Results and Discussion

Atomic force microscopy measurement in water was performed using silanized mica. Because of the negative charges of the probucol GM particles, they were fixed on the mica surface through electrostatic interaction. The obtained images of a colloidal probucol nanoparticle with PVP K17 are shown in Fig. 1. The prepared nanoparticles were well dispersed on the mica surface. The AFM topography image (Fig. 1A) represented that the particle was covered with foggy substance. The AFM phase image gives us the information about the physicochemical properties, such as stiffness. As shown in Fig. 1B, the phase image was quite different between the core and the surface of the particle. From our previous study using NMR and particle size measurements, we speculated that probucol was existed as nanocrystals covered with PVP and SDS complex.[12,13] The AFM phase image clearly showed that the observed particle had core-shell structure and that the stiff core originated from probucol nanocrystals was covered with soft substance, *i.e.* PVP K17 and SDS complex. It is speculated that grinding-induced intermolecular interactions, probucol-PVP K17 and PVP K17-SDS, were reflected in the morphology of the nanoparticles in aqueous solution.

Comparison of AFM phase images of colloidal probucol nanoparticles with PVP K12 and those with PVP K17 are shown in Fig. 2. In probucol nanoparticles with PVP K17, the surface of the nanoparticles was covered with PVP K17 and SDS complex as demonstrated in Fig. 1. However, the surface coverage was hardly observed on those with PVP K12. These results indicated that surface coverage states of PVP-SDS complex were apparently different depending on the molecular weight of PVP. Force-distance curves of the probucol nanoparticles are shown in Fig. 3. The cantilever deflection was proportional to the sample height when the tip was touching the reference silanized mica. The linearity is characteristic for stiff materials. In the case of colloidal probucol nanoparticle with PVP K12, threshold where the
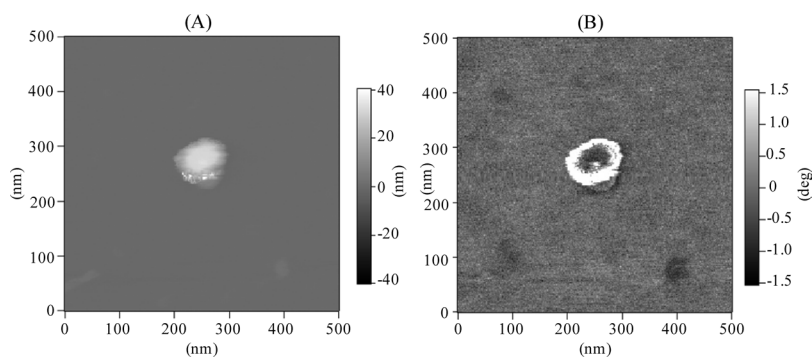

Fig. 1. Atomic Force Microscopy (AFM) Images of Colloidal Probucol Nanoparticles with PVP K17

(A) AFM topography image. (B) AFM phase image. Probucol nanoparticles prepared by the co-grinding with PVP K17 and SDS were dispersed in water before measurement.
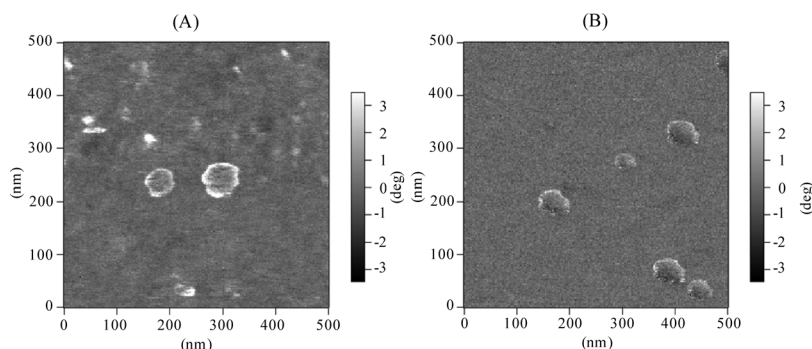

Fig. 2. Comparison of AFM Phase Images of Colloidal Probucol Nanoparticles with PVP K12 and Those with PVP K17

(A) Colloidal probucol nanoparticles with PVP K17. (B) Colloidal probucol nanoparticles with PVP K12. Probucol nanoparticles prepared by the co-grinding with PVP K12 or PVP K17 and SDS were dispersed in water before measurement.
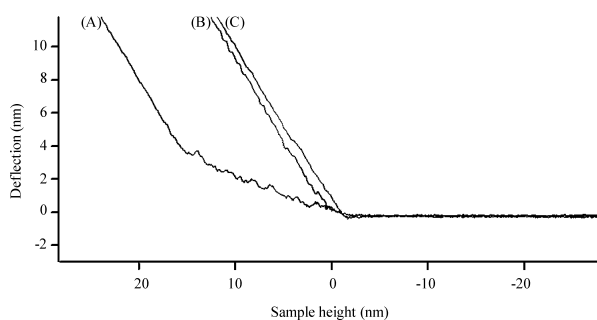
Fig. 3. Force Curve Analysis of Colloidal Probucol Nanoparticles with PVP K12 and Those with PVP K17

(A) Colloidal probucol nanoparticles with PVP K17. (B) Colloidal probucol nanoparticles with PVP K12. (C) Silanized mica surface. Probucol nanoparticles prepared by the co-grinding with PVP K12 or PVP K17 and SDS were dispersed in water before measurement.

linearity began was slightly shifted to higher sample height (*ca.* 1 nm or less). The linear increase of the deflection after touching on the sample indicated that the tip was touching stiff materials, *i.e.* probucol nanocrystals. The surface should be covered with PVP K12 and SDS, but the coated layer structure was extremely thin. On the nanoparticle with PVP K17, however, the deflection increased very slowly and the linearity appeared when the sample height reached *ca.* 15 nm. The gradual increase of the force curve of the sample with PVP K17 was characteristic for soft materials. Furthermore, the subsequent linear increase of the deflection indicated the core-shell structure of the particle. From the results, we confirmed that probucol nanocrystal was covered with soft layer formed by PVP K17 and SDS complex.

The effect of surface states of probucol nanoparticles on the pharmacokinetic parameters was evaluated. We prepared the ternary GMs with PVP K12 and PVP K17, the mean particle size of which were 40 and 36 nm, respectively. As shown in Table 1, the *AUC* values of the GM suspension with PVP K12 were apparently higher than that with PVP K17. The results suggested that the difference of the layer structure formed by PVP and SDS, not the particle size, mainly influenced the *in vivo* absorption of probucol.

Our previous study indicated that ternary GM with PVP K17 was stable in aqueous media, compared with that with PVP K12.[13,16] Since PVP K17 interacted with SDS as a necklace structure in aqueous solution, the soft layer structure seemed to be observed by the AFM measurement. The layer structure stabilized the probucol nanoparticles in aqueous solutions. On the contrary, PVP K12 interacted with SDS but the complex could not form the necklace structure because of the short chain length of PVP K12. The coated layer was very thin as shown in the AFM phase image. Thus, probucol nanoparticles with PVP K12 could not be stabilized well by the complex and gradually agglomerated compared with those with PVP K17. However, in terms of the dissolution of drug from the particle surface, it was speculated that probucol molecules could dissolve more easily from the surface of the nanoparticles with PVP K12.

In conclusion, AFM measurement revealed morphology and surface states of colloidal GM nanosuspensions. The *in vivo* results indicated that surface state of probucol nanopar-

Table 1. Effect of Molecular Weight of PVP on Pharmacokinetic Parameters of Probucol Following Oral Administration of the Colloidal Nanoparticles

	Probucol/PVP K12/SDS GM (40 nm)	Probucol/PVP K17/SDS GM (36 nm)
AUC (μg h/ml)	23.6±7.2	13.4±1.1
C_{max} (μg/ml)	1.61±0.48	1.29±0.07
T_{max} (h)	2.33±0.33	2.00±0.0

Mean particle size was shown in the parenthesis. Results are expressed as mean±S.E. ($n=3$).

ticles covered with PVP-SDS complex influenced the absorption properties when the particle sizes were almost same. The prepared GM nanosuspensions are transparent and the probucol molecules are usually regarded as "dissolved". However, the solution is composed of probucol nanoparticles with core-shell structures and the surface properties are reflected on dissolution of probucol molecules. Observation of colloidal drug nanoparticles by AFM is a promising method to evaluate the molecular states of drug and the subsequent dissolution and absorption behavior.

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