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# A novel method for measuring rigidity of submicron-size liposomes with atomic force microscopy

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**Abstract**

There are many useful colloidal drug delivery systems that use liposomes. The rigidity of the carrier particle is one of the most important properties affecting drug delivery effectiveness, assessed by particle stability, release profile of encapsulated drug, and blood circulation time. However, it is difficult to evaluate the rigidity of such fine particles; so far, no useful methods have been reported. We demonstrate a unique method to evaluate the rigidity of liposomes using atomic force microscopy (AFM) and dynamic light scattering (DLS) in this report. We showed that the combination of two types of particle-size measurements, tapping mode AFM in buffer solution with another conventional method such as DLS, is useful for evaluating the rigidity of submicron-size particles such as liposomes.


*Keywords:* Liposomes; Atomic force microscopy; Dynamic light scattering; Rigidity

## 1. Introduction

There are many useful colloidal drug delivery systems for intravenous injection; such systems use liposomes, lipid emulsions and polymeric micelles. Liposomes are a widely used drug carrier as well as being used to model the cell membrane to elucidate endocytosis mechanisms. Liposomes, which have a phospholipid bilayer membrane, are highly biocompatible and biodegradable, and their antigenicity and toxicity are low.

Carrier systems such as those for antitumor drugs must be retained in the bloodstream for a long time in order to achieve passive drug targeting with enhanced permeability and retention. We have demonstrated the feasibility of polymer coating the liposomal surface with modified hydrophilic polymers such as polyvinyl alcohol with a hydrophobic anchor at the end of its molecule (PVA-R). PVA-R coated liposomes showed an increase of circulation time with lower uptake by the reticuloendothelial system (Takeuchi et al., 2000).

Rigidity is one of the most important factors in improving the drug delivery efficiency of carrier particles. It affects their particulate stability and drug release profile as well as their blood circulation time. Several researchers have been reported that the rigid liposomes tend to be the most stable in the circulation and display the longest circulation lifetimes (Drummond et al., 1999) or liposomes in the bloodstream are picked up predominantly by macrophages (Kupffer cells) in the liver and also in the spleen and the rate or extent of their uptake depends on bilayer rigidity (Crommelin et al., 2003). Most of all studies for the rigidity are speculations corresponding to their results. Therefore in vitro evaluation of the rigidity of liposomes is one of the challenging aspects in pharmaceutical field.

It is thought that the rigidity of liposomes may be changed by altering the particle size or the type of phospholipid or amount of cholesterol in the formulation. The rigidity of liposomes can be roughly predicted from that information. However, it is difficult to directly evaluate the rigidity of such fine particles; no useful methods have been reported. To design novel-function, fine-particulate systems for drug delivery, an easier and quantitative method to characterize the rigidity of colloidal particles is required.

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In this paper, we try to capture and analyze images of liposomal particles using AFM, which has been reported as a useful microscopy to visualize not only submicron-size particles (Muhlen et al., 1996; Egawa and Furusawa, 1999; Tokumasu et al., 2003) but also DNA, protein or living cells (Moller et al., 1999; Rotsch et al., 2001; Umemura et al., 2001) at very high resolution without drying the samples. We propose a novel method to evaluate the rigidity of liposomes by combining these results with particle size data measured separately by dynamic light scattering (DLS).

## 2. Materials and methods

### 2.1. Materials

Egg phosphatidylcholine (EPC, COATSOME NC-50), L-α-dimyristoyl phosphatidylcholine (DMPC, COATSOME MC-4040) and L-α-distearoyl phosphatidylcholine (DSPC, COATSOME MC-8080) were purchased from Nippon oil and fats Co., Ltd., Japan. Taylor et al. reported that phase transition temperature of each phospholipid was −15 to −5, 23 or 55 °C, respectively (Taylor and Morris, 1995). Cholesterol (chol) and didodecyl dimethyl ammonium bromide (DDAB) were obtained from Sigma Chemical Co., Ltd. Stearyl amine (SA) was purchased from Tokyo Kasei Co., Ltd., Japan. Polystyrene particle was purchased from COREFRONT Co., Ltd., Japan (micromer®). Mica and the AFM probe (DNP-S20) were purchased from Veeco Co., Ltd., Japan. All other chemicals were commercial products of reagent grade.

### 2.2. Preparation of submicron-size liposomes

Submicron-size liposomes, which were composed of phospholipid, cholesterol and a positively charged additive, were prepared by the hydration method. These materials were dissolved in a small amount of chloroform, and the solution was placed in a rotary evaporator at 40 °C until a thin lipid film was obtained. This film was then dried in a vacuum dryer overnight to ensure complete removal of the residual chloroform, and hydrated with 10 mM phosphate buffer (pH 7.4) by vortexing, followed by incubation at 10 °C for 30 min. Submicron-size liposomes were prepared using an extruder (LiposoFast™-Pneumatic, Avestin Inc.) with a size-controlled polycarbonate membrane (pore sizes of membrane filter; 0.1, 0.4 or 0.8 μm, Whatman Inc.).

### 2.3. Physicochemical properties of the submicron-size particles

The particle size was measured with an aliquot of the particulate suspension diluted with a large amount of distilled water by DLS method (Zetasizer 3000HS$_A$, the system measures particles from 0.6 to 6000 nm, Malvern, UK). The zeta potential of a sample of colloidal particles was quantified using a Laser Doppler method (Zetamaster, Malvern, UK).

### 2.4. Atomic force microscopy

We used a commercial atomic force microscopy (AFM) apparatus (Nanoscope IIIa system controller, Digital Instruments Inc.) with an E-scanner possessing a maximum range of 10 μm × 10 μm × 2.5 μm. All images were captured in 10 mM phosphate buffer solution at room temperature with a silicon nitride probe (nominal spring constant: 0.32 N/m). The scanning speed was optimized between 1.0 and 2.5 Hz depending on the scan size. All images were recorded by both height and amplitude modes and their analyses were done on the height image mode. Freshly cleaved mica or surface-modified mica was used as the substrate for AFM observation. Mica was kept in the vacuum dryer before AFM observation or preparation of surface-modified mica. Surface-modified mica was prepared using 3-aminopropyltriethoxysilane (AP, M.W. 221.4) and *N*,*N*-diisopropylethylamine (DI, M.W. 129.2) as follows (Thomson et al., 2000). Positively charged mica was used only for negatively charged polystyrene particles in order to create an affinity between the mica surface and the polystyrene particles. Freshly cleaved mica was incubated in a plastic petri dish, first with AP (2 μl) for 2 h, then with DI (1 μl) containing the top layer from the microcentrifuge tubes for another 2 h.

### 2.5. Adsorption of submicron-size particles onto the substrate surface

The liposomal suspension was diluted with 10 mM phosphate buffer, and the lipid concentration of liposome was 2 mM. The polystyrene suspension was also diluted with same buffer to 2.5 μg/ml. Freshly cleaved or surface-modified mica was mounted and a quartz glass cell was set for the fluid tapping mode. Then, a suspension of submicron-size particles was incubated on the substrate surface for 30 min. After the submicron-size particles were adsorbed onto the substrate surface, the substrate surface was washed three times with 10 mM phosphate buffer solution to remove the non-adsorbed particles. AFM images were captured immediately after washing under suitable AFM conditions.

### 2.6. Evaluation of the rigidity of submicron-size particles

Particles were visualized by both height and amplitude modes at a 10 μm × 10 μm scale after adsorption onto the substrate. With respect to the height of liposome, the depth of all liposomes existed within an area of 10 μm × 10 μm mica surface was detected by AFM. The value of height represents the mode of histogram by using the software of this apparatus. Separately, the particle size ($P$) was measured using DLS method before the particles were adsorbed onto the substrate surface. These two parameters were introduced to describe the change in the height of particle image against the particle diameter as the rigidity of the submicron-size particles ($H/P$) according to Eq. (1):

$$H/P = \frac{\text{Mode of height of adsorbed particles } (H)}{\text{Particle size of particles } (P)} \quad (1)$$

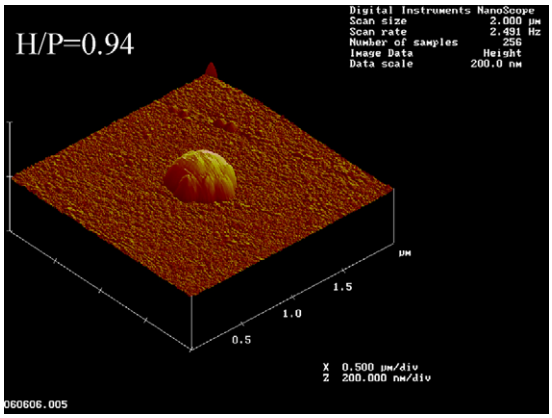


Fig. 1. An AFM image of polystyrene particle adsorbed onto surface-modified mica. Concentration of polystyrene particle: 2.5 μg/ml, scale: 4 $\mu m^2 \times 200$ nm.

## 3. Results and discussion

### 3.1. Evaluation of the rigidity of polystyrene particle

Size-controlled polystyrene particles are often used as a standard in the evaluation of particulate drug delivery carriers. Burchell et al. reported that polystyrene latex can be readily synthesized over a wide size range with a well defined spherical morphology and narrow size distributions. In addition, polystyrene has a high glass transition temperature, which means that the latex is rigid and non-deformable at room temperature (Burchell et al., 1999). Therefore, particles of size-controlled polystyrene may be suitable for use as a benchmark of the rigidity. Fig. 1 shows an AFM image of polystyrene particle adsorbed onto surface-modified mica. The mode of height of polystyrene particles is estimated as $94.8 \pm 1.30$ nm by the depth analysis software of the AFM apparatus, while the particle size of the same particle is $100.8 \pm 0.32$ nm by DLS. The rigidity of polystyrene particle was calculated to be $0.94 \pm 0.001$. The polystyrene particle was not significantly distorted during the AFM measurements and therefore makes an ideal particle for evaluating or comparing with the liposomal rigidity.

Several researchers have reported that the height was nearly identical with the actual size of particles (Ramirez-Aguilar and Rowlen, 1998; Rasa and Philipse, 2002). Since there is a good correlation between the mode of height and particle size, it can be assumed that the mode of height (obtained from the amplitude scan of the tapping mode) represents the length of the liposome perpendicular to the mica surface.

### 3.2. Effect of the type of phospholipids on the rigidity of liposome

It has been reported that the type of phospholipids in a given lipid composition may affect the resultant drug carrier functions of the liposomes such as its particulate stability, the efficacy of the encapsulated drug and the blood circulation time (Storm et al., 1987; Mayer et al., 1989). Three types of liposomes composed of the three different phospholipids (phospholipid:chol:SA = 7:3:1, EPC731, DMPC731 or DSPC731) were prepared. The particle size of the three types of liposomes, measured with DLS, was approximately $103.6 \pm 0.85$, $115.8 \pm 0.36$ or $139.1 \pm 0.85$ nm, respectively. The AFM images of EPC or DMPC liposomes showed a flattened shape. On the

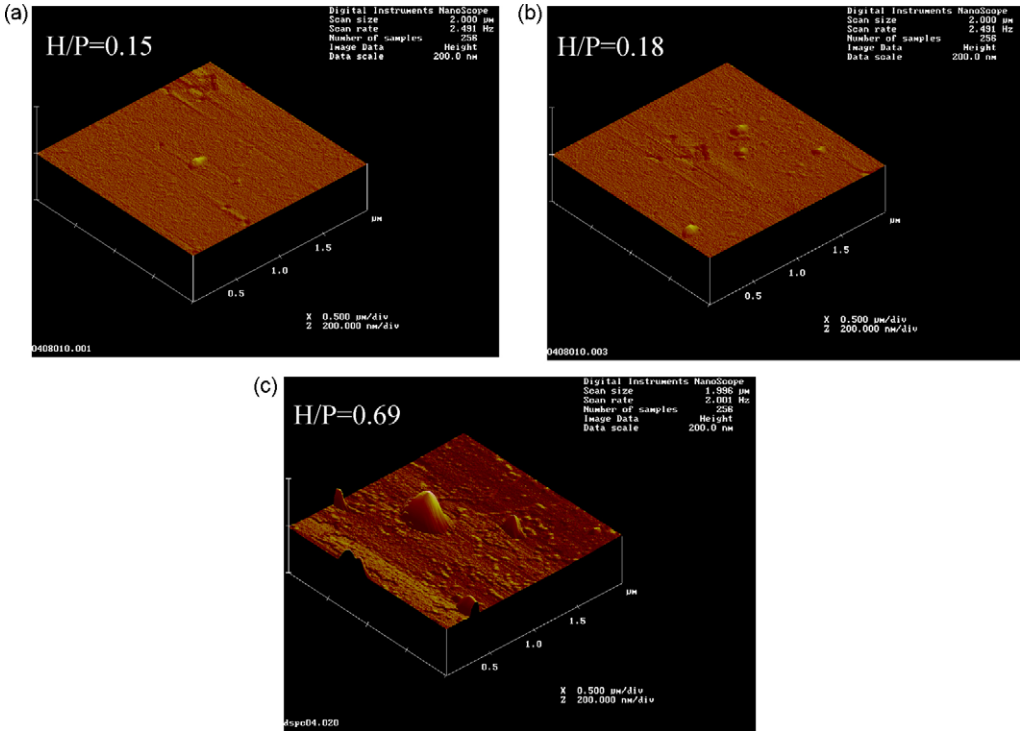


Fig. 2. The effect phospholipid on the liposomal rigidity. (a) EPC731 (EPC:chol:SA = 7:3:1), (b) DMPC731 (DMPC:chol:SA = 7:3:1) and (c) DSPC731 (DSPC:chol:SA = 7:3:1), scale: 4 $\mu m^2 \times 200$ nm.

Table 1
AFM and DLS data of polystyrene particle or liposomes ($n = 3$)

| Lipid composition | Mode of height (nm ± S.D.) | Particle size (nm ± S.D.) | *H*/*P* ratio |
|---|---|---|---|
| Polystyrene particle | 94.8 ± 1.30 | 100.8 ± 0.32 | 0.94 ± 0.01 |
| EPC731 | 15.3 ± 1.17 | 103.6 ± 0.85 | 0.15 ± 0.011 |
| DMPC731 | 21.0 ± 1.40 | 115.8 ± 0.36 | 0.18 ± 0.012$^{*}$ |
| DSPC731 | 96.3 ± 1.12 | 139.1 ± 0.85 | 0.69 ± 0.009$^{**}$ |
| DSPC551 | 22.4 ± 1.10 | 118.2 ± 0.69 | 0.19 ± 0.008 |
| DSPC911 | 133.1 ± 1.30 | 148.8 ± 0.66 | 0.89 ± 0.005$^{\dagger\dagger}$ |
| DSPC731 (0.1 μm) | 96.3 ± 1.12 | 139.1 ± 0.85 | 0.69 ± 0.009 |
| DSPC731 (0.4 μm) | 202.8 ± 1.21 | 470.0 ± 0.44 | 0.43 ± 0.002$^{\#\#}$ |
| DSPC731 (0.8 μm) | 237.9 ± 1.71 | 691.2 ± 0.61 | 0.34 ± 0.003$^{\#\#}$ |
| DSPC731 (DDAB) | 34.3 ± 1.00 | 113.3 ± 0.60 | 0.30 ± 0.009 |

Lipid composition: EPC731 (EPC:chol:SA = 7:3:1), DMPC731 (DMPC:chol:SA = 7:3:1) and DSPC731 (DSPC:chol:SA = 7:3:1), DSPC551 (DSPC:chol:SA = 5:5:1), DSPC911 (DSPC:chol:SA = 9:1:1) and DSPC731 (DDAB) (DSPC:chol:DDAB = 7:3:1), 0.1, 0.4 and 0.8 μm: pore size of membrane filter on extruding, $^{*}p < 0.05$, $^{**}p < 0.01$: significantly different from the *H*/*P* ratio of EPC731, $^{\dagger\dagger}p < 0.01$: significantly different from the *H*/*P* ratio of DSPC551, $^{\#\#}p < 0.01$: significantly different from the *H*/*P* ratio of DSPC731 (0.1 μm).

other hand, DSPC liposomes kept a spherical shape under the conditions in the AFM measurement as shown in Fig. 2. The *H*/*P* values were calculated using Eq. (1) after analyzing the mode of height within the 10 μm square of the captured image. All *H*/*P* values determined in the present experiment are listed in Table 1. The liposomes composed with the phospholipid having the highest phase transition temperature (DSPC) showed the highest *H*/*P* value in the three types of liposomes. The phase transition temperature of EPC and DMPC are $-10 \pm 5$ and 23 °C, respectively, and they exist as the liquid crystalline state at the room temperature, indicating the high fluidity of these liposomal membranes. A schematic model of liposomes adsorbed on the mica surface is depicted in Fig. 3. The mode of height after analyzing liposomes composed of EPC showed 15.3 ± 1.17 nm. Jass et al. reported that the single bilayer showed 6.0–7.5 nm of apparent height (Jass et al., 2000). In this case, the possibility that the top bilayer and bottom bilayer was stacked on the mica cannot be excluded, arising from the collapse of liposomes by interaction with mica surface. The collapsed liposomes may indicate two-fold of single bilayer (ca. 15 nm). Although liposomes composed of EPC or DMPC were distorted on AFM images, the histogram did not represent the existence of the single bilayer on the surface.

DSPC has the highest phase transition temperature (55 °C) of the three phospholipids considered and exists as a gel state at room temperature, leading to the low fluidity of DSPC–liposomal membranes.

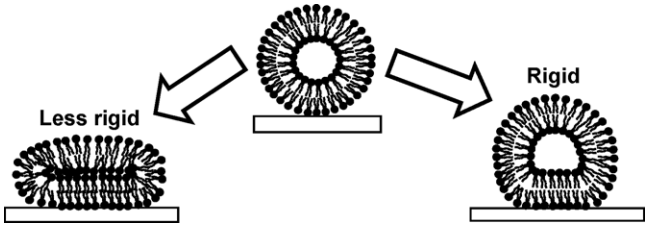


Fig. 3. A schematic model of liposomes adsorbed on the mica surface.

### 3.3. Effect of the cholesterol amount on the rigidity of liposome

Generally, cholesterol molecules interact with the acyl groups of phospholipids and restrict the activity of the liposomal membrane. They affect not only the stabilization of the liposomal membrane but also the content or condition of the incorporated drug (Takeuchi et al., 2000). Liposomes with different molar concentrations of cholesterol, DSPC:chol:SA = 5:5:1 (DSPC551), 7:3:1 (DSPC731) and 9:1:1 (DSPC991) were prepared. AFM measurements of DSPC551 liposomes showed a flattened shape, while DSPC731 or DSPC911 maintained their spherical shape as shown in Fig. 4. The *H*/*P* value of DSPC551 liposomes was lower than those of the other two formulations. DSPC731 and 911 liposomes showed almost the same *H*/*P* value (Table 1). Ladbrooke et al. reported that addition of cholesterol up to maximum of 50 mol% causes a reduction in the lipid transition temperature and this is consistent with the cholesterol causing a reduction in the cohesive forces between the adjacent hydrocarbon chains of the lecithin (Ladbrooke et al., 1968). Utsumi et al. also reported that addition of cholesterol to lecithin is regarded to promote the permeability in dipalmitoyl and dimyristoyl lecithin–cholesterol–water system (Utsumi et al., 1976). The AFM observation and the rigidity represented by the *H*/*P* values in the present experiment reflect these findings.

### 3.4. Effect of the particle size on the rigidity of liposome

The particle size of liposomes is also one of the most important factors for determining the behavior of liposomes in the body. It was reported that the particles smaller than 100 nm easily penetrate tumor tissues because they have discontinuous capillaries and the size of fenestra is approximately 100 nm (Mayer et al., 1989; Burchell et al., 1999). Furthermore, small particles can avoid recognition or incorporation into mononuclear phagocyte systems such as macrophages. Various size-controlled liposomes were prepared by varying the pore size of the membrane filter. Particles of DSPC:chol:SA (7:3:1) were prepared with filter sizes of 0.1, 0.4 and 0.8 μm. Fig. 5 shows the AFM images of the various liposomes and their *H*/*P* values. Each image shows the spherical shape determined from the AFM observation. The calculation of their *H*/*P* reveals that the rigidity of liposomes decreases with an increase in the particle size (Table 1). This tendency may be attributed to the different curvatures of liposomes depending on the particle size. The molecular interactions between phospholipids and cholesterol in the smaller liposomes particles such as those extruded through the 0.1 μm filter are more restricted than those for the larger particles which were extruded through 0.8 μm filter. In the case of the larger particles, partially flattened liposomes were also observed and the height of the flattened region was almost 8–10 nm (data not shown). It has been reported that some larger liposomes tend to flatten from a sphere to a lipid bilayer (Jass et al., 2000). Thus, the smallest particles will maintain their spherical shape and rigidity.

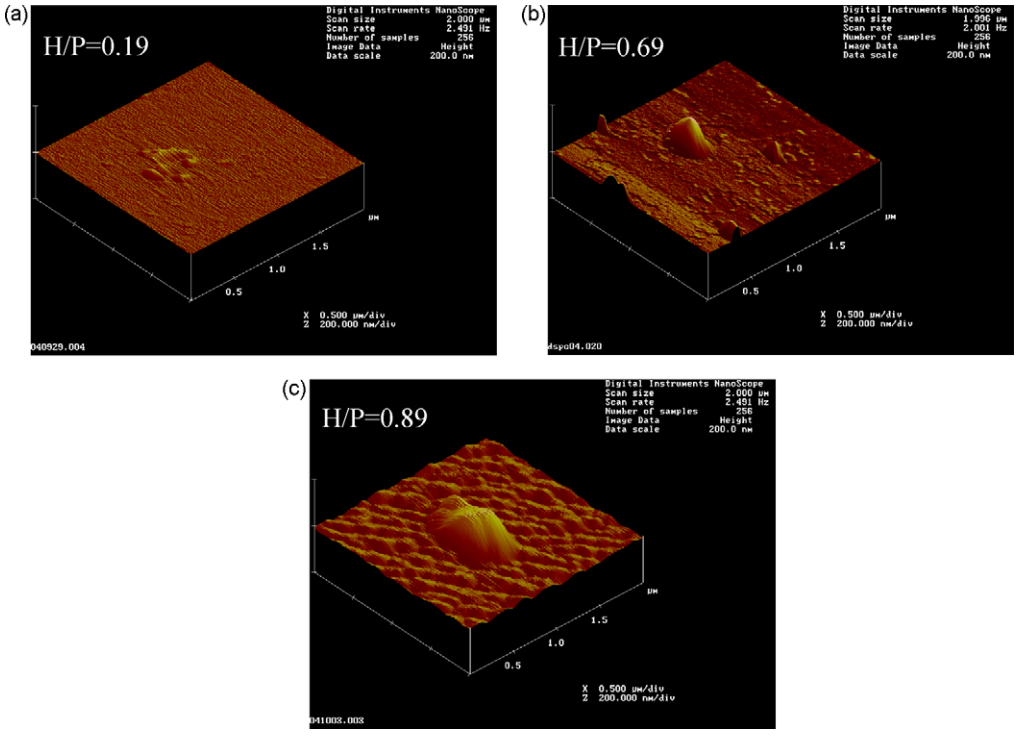


Fig. 4. The effect of cholesterol concentration on the liposomal rigidity. (a) DSPC551 (DSPC:chol:SA = 5:5:1), (b) DSPC731 (DSPC:chol:SA = 7:3:1) and (c) DSPC911 (DSPC:chol:SA = 9:1:1), scale: 4 $\mu m^2 \times 200$ nm.

### *3.5. Effect of the types of positively charged additive on the rigidity of liposome*

Recently, the technique of gene delivery has developed in which the cationic material plays important role as a vector of the nucleotide. The development of safe vectors with excellent transfection efficiency and low cytotoxicity is required for the human application of gene therapy. Cationic liposomes are one of the most effective vectors for gene targeting because these liposomes form complexes with target nucleotide for stable gene

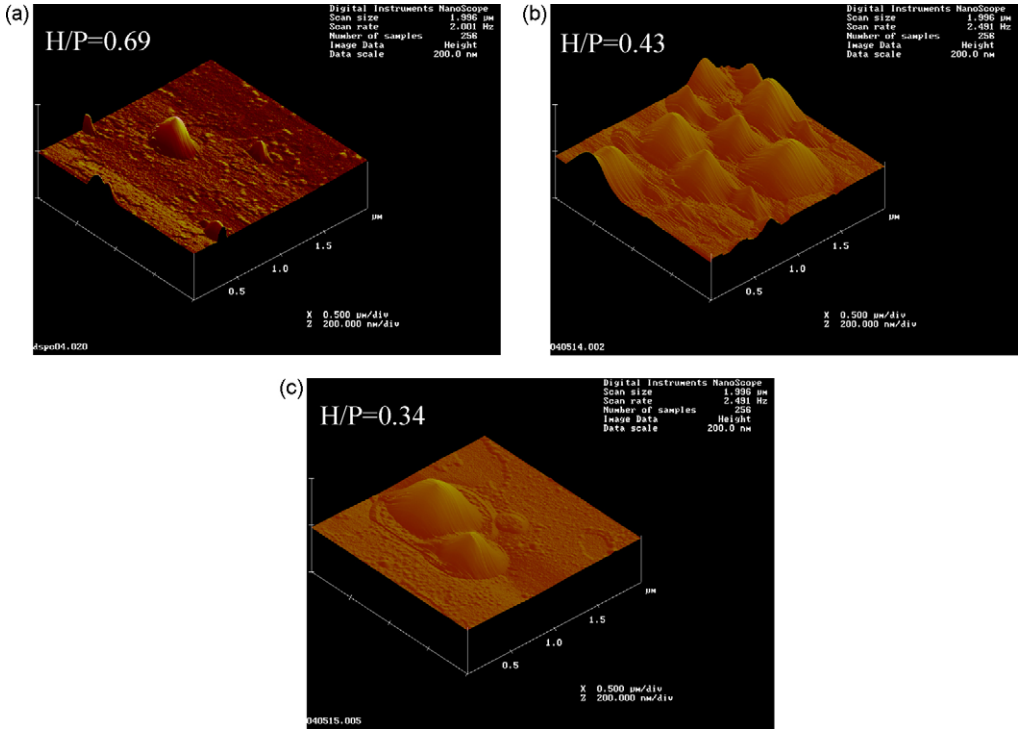


Fig. 5. The effect of particle size on the liposomal rigidity. Lipid composition: DSPC731 (DSPC:chol:SA = 7:3:1), pore size of membrane filter on extruding: (a) 0.1, (b) 0.4 and (c) 0.8 μm, scale: 4 $\mu m^2 \times 200$ nm.

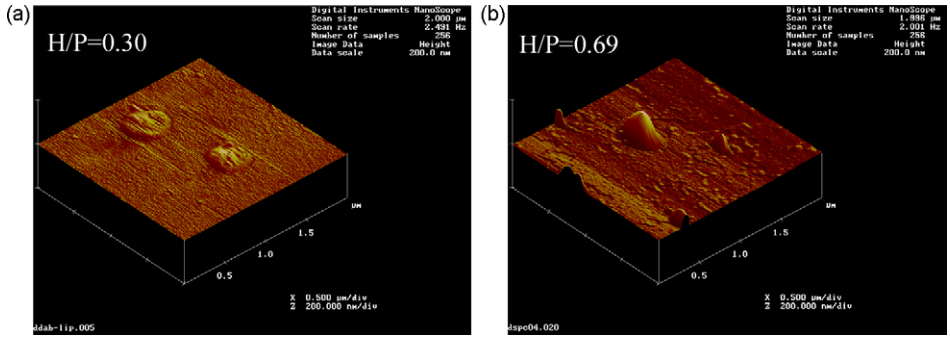


Fig. 6. The effect of cationic additive on the liposomal rigidity. Lipid composition: (a) DDAB–liposomes (DSPC:chol:DDAB = 7:3:1) and (b) SA–liposomes (DSPC:chol:SA = 7:3:1), scale: 4 $\mu m^2 \times 200$ nm.

delivery using the electrostatic interaction between the positively charged liposomes and negatively charged DNA. The complexes show a surface charge that promotes a fast and efficacious association with the cellular barrier and are then incorporated into cytoplasm by the optimization of their lipid composition. The selection of cationic materials is important for the appropriate gene therapy. DDAB has been reported as a potential positively charged additive especially for gene delivery carriers in the recent study (Ruozi et al., 2003). AFM imaging and rigidity calculations were carried out for both SA, which is often used for preparing positively charged liposomes, and DDAB containing liposomes in this study. The images of DDAB liposomes showed a flattened shape compared with SA containing ones as shown in Fig. 6. The *H*/*P* value of DDAB containing liposomes was lower than that of SA-containing ones (Table 1). In other words, SA-containing liposomes keep their shape better than DDAB containing ones. The different orientation of DDAB and SA in the lipid membrane might be responsible for the different rigidity of the resultant liposomes. Our present work provided that the rigidity of liposomes might be related to the value of *H*/*P* index which is calculated from AFM and DLS data. However, it is clear that much more work, e.g., force extension curves using AFM, should be required to obtain a more complete understanding of the quantitative correlation between the particle size from DLS measurement and elasticity of liposomes.

## 4. Conclusions

The present studies reveal that the rigidity of submicron-size particles can be successfully evaluated using AFM. *H*/*P* values calculated with the particle size data using the fluid tapping mode of AFM and DLS provide a parameter of the rigidity of the submicron-size particles. The *H*/*P* values clearly demonstrate that the rigidity of liposomes can be altered by changing the type of phospholipid, the cholesterol content, particle size and the type of the positively charged additive.

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