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AFM Phase Imaging of Soft-Hydrated Samples: A Versatile Tool to Complete the Chemical-Physical Study of Liposomes

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Despite of the several approaches applied to the physicochemical characterization of liposomes, few techniques are really useful to obtain information about the surface properties of these colloidal drug-delivery systems. In this paper, we demonstrate a possible new application of tapping mode atomic force microscopy (AFM) to discriminate between conventional and pegylated liposomes. We showed that the differences on liposomal surface properties revealed by the phase images AFM approach well correlate with the data obtained using classical methods, such as light scattering, hydrodynamic, and nuclear magnetic resonance analysis. 15 20

Keywords liposomes, atomic force microscopy, phase tapping mode images, photon correlation spectroscopy, ¹H HR-MAS NMR

Introduction

Liposomes are micro- or nanoparticulate vesicles formed by self-assembly of lipids in an aqueous environment (Chatterjee and Banerjee, 2002). First described by Bangham et al. (1965) roughly 40 years ago, liposomes have moved a long way toward becoming a widely used pharmaceutical carrier for numerous clinical applications (Juliano and Layton, 1980; El Aneed, 2004; Torchilin, 2006). Different kinds of lipidic vesicles have been proposed as drug and gene carriers (i.e., long circulating 25 30

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liposomes with modified surfaces), pH-sensitive liposomes formulated by using pH sensitive components, cationic liposomes for gene therapy, immunoliposomes, and “specialized liposomes,” such as transferosome and niosome (Ceh et al., 1997; Maruyama et al., 1999; Cevc, 1996). Generally, the physical properties can be easily modified by changing the lipidic composition, the preparation procedure, or the surface by conjugation with antibodies, peptides, polymers, and other selective ligands (Madelmont et al., 2003; Forssen and Willis, 1998; Allen et al., 1998; Drummond et al., 1999).

In order to evaluate the physical properties (e.g., morphology, size, polydispersity index, number of lamellae, charge, bilayer fluidity, lipidic composition, and encapsulation efficiency), several analytical techniques have been applied (Edwards and Baeumner, 2006): dynamic light scattering (DLS, also known as photon correlation spectroscopy; PCS), nuclear magnetic resonance (NMR), X-ray photoelectron spectroscopy (XPS), electron paramagnetic resonance (EPR), and transmission electron microscopy (TEM). Moreover, in recent years, atomic force microscopy (AFM), one member of the family of scanning probe microscopes, was successfully applied to evaluate the morphological and technological properties of liposomes (Ruozi et al., 2007). To investigate soft samples such as liposomes, two types of operating dynamical modes are currently used. In the “non contact-mode” AFM, the oscillation amplitude is fixed and the output signal is related to the resonance frequency. In the “intermittent contact-mode” (dynamic force mode or tapping-mode AFM), the oscillation frequency of the tip is maintained near its frequency recording two type of images (Albrecht et al., 1991; Zhong et al. 1993): 1) the “height image” coming from the record of changes in z-axis of the piezo necessary to maintain the fixed oscillation amplitude through a feedback loop and 2) the “phase image” generated on the phase-delay oscillator with respect to the excitation signal. The image contrast in the phase imaging describes the variation of the phase during the cantilever oscillation (Koop-Marsaudon et al., 2000). The phase lag is affected by the tip-sample adhesion, the elasticity, and the viscosity, which is related to the composition and other different properties, such as the hydrophilicity and hydrophobicity of the sample surface. Thus, the tapping mode AFM is an extremely versatile probe for local surface studies (Magonov et al., 1997; Tamayo and Garcia, 1996; Whangbo et al., 1998; Noy et al., 1998).

Therefore, we propose the application of the phase image obtained by using tapping mode AFM to characterize the local properties of liposomal surface apart from the topographic data. In this study, conventional and polyethylene glycol-grafted liposomes (PEG-grafted liposomes) were compared. PEG-grafted liposomes have been proposed for clinical applications to solve several problems associated with *in vivo* liposomal stability, such as the fast clearance from the circulation and the rapid accumulation in the cells of the mononuclear phagocyte system (MPS). The *in vitro* characteristics of pegylated liposomes and their *in vivo* behavior have been deeply examined, applying few techniques in order to confirm the liposome surface coverage with PEG but, to our best knowledge, without reporting any microscopical investigation. Thus, the present study dealt with the use of PCS and NMR studies as classical approaches to evaluate the presence of PEG coverage on the liposome surface and the stability of our preparations. The data were then implemented with the AFM studies; on the basis of the phase images, we demonstrated that this microscopical approach is a useful tool to discriminate between liposomes with different surface characteristics.

Materials and Methods

Materials

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Cholesterol (Chol) was obtained from the Sigma-Aldrich Company (Milan, Italy). Egg-yolk phosphatidylcholine (PC) was purchased from Fluka (Büchs, Switzerland). Monocholesteryl PEG amine (Chol-PEG-NH₂) (Figure 1) was synthesised according to Pan et al. (2007).

A MilliQ water system (Millipore, Bedford, Massachusetts, USA), supplied with distilled water, provided high-purity water (18 MΩ) for these experiments. All other chemicals were obtained commercially as reagent-grade products and used without purification.

Liposome Preparation

Liposome formulations, composed of PC:Chol 1:0.1 mol:mol (sample 1) and PC:Chol-PEG-NH₂ 1:0.1 mol:mol (sample 2), were prepared by the thin-layer evaporation method (Table 1). Practically, the lipidic mixture was dissolved in a chloroformic solution and vacuum dried (10 mbar), using a rotary evaporation device (B-480; Büchi, Büchs, Switzerland) at 20°C. The dried lipidic film was vacuum dried over 3 hours (0.15 mbar) and then hydrated at room temperature with 4 mL of MilliQ water. The preparation was vortexed for 3 minutes (Zx³; Velp Scientifica, Usmate, Italy) and warmed in a water bath at room temperature for 3 minutes. The cycle was repeated for three times. Sample 1 was also extruded through the polycarbonate filter (Liposofast Basic; Avestin, Ottawa, Canada) of a 200-nm pore size (19 cycles) to obtain liposomes with size comparable with that of sample 2. This sample is reported in Table 1 as sample 1a. All formulations were stored at 4°C, protected from light, and analyzed within 15 days.

Particle Size and Zeta Potential

Liposomes were analyzed for particle size and zeta potential by PCS and laser Doppler anemometry using a Zetasizer Nano ZS (Malvern, UK; Laser 4 mW He-Ne, 633 nm, Laser attenuator Automatic, transmission 100% to 0.0003%, Detector Avalanche photodiode, Q.E. > 50% at 633 nm, T = 25°C) without any dilution of the samples.

To evaluate the stability of lipidic samples, the software was also used in trend mode, which allows multiple measurements in a range of temperature to be recorded. The parameters selected were: initial and final temperatures of the trend 4 and 40°C; temperature interval 4°C; number of steps 10; number of measurements to be made at each step after

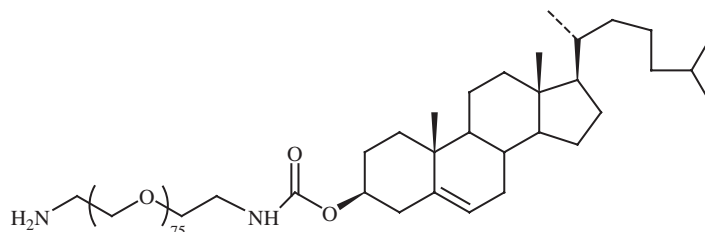


Figure 1. Monocholesteryl PEG amine (Chol-PEG-NH₂).

Table 1
Atomic force microscopy (AFM) and photon correlation spectroscopy (PCS)
data of liposomes

Samples	Lipidic composition (molar ratio)	Mean diameter [nm] (\pm SD)		
		AFM measurements	PCS measurements (z-average diameter)	Zeta potential [mV] (\pm SD)
1	PC:Chol 1:0.1	$\text{\O} = 350 \pm 85$ $h = 38 \pm 9$	280 ± 61 (PDI 0.77)	-16 ± 5
1a (extruded liposomes)	PC:Chol 1:0.1	$\text{\O} = 213 \pm 75$ $h = 18 \pm 6$	172 ± 11 (PDI 0.24)	-11 ± 5
2	PC:Chol-PEG-NH ₂ 1:0.1	$\text{\O} = 266 \pm 63$ $h = 21 \pm 7$	183 ± 14 (PDI 0.30)	31 ± 7

The experiments were carried out at $25^\circ\text{C} \pm 1$; samples were analyzed without any dilution. Data are means \pm SD for three different experiments.

equilibration time 2, and equilibration time at each temperature 2 minutes. The equilibrium time of 2 minutes was capable of ensuring that the sample viscosity was equilibrated before each determination. All the data were collected as “mean count rate versus temperature versus average diameter.” 120

NMR Analysis

¹H HR-MAS NMR spectra were recorded on an Avance-400 spectrometer (Bruker BioSpin Corp, Billerica, Massachusetts, USA) operating at 400.13 MHz and equipped with a ¹H/¹³C dual HR-MAS probe with gradients at lock channel. Typically, 50 μL of samples prepared by using 99.96% D₂O were loaded into a 4-mm ZrO₂ cylindrical rotor equipped with a PTFE spherical insert. A zgpcpr standard pulse sequence was employed (Bruker software) for residual HDO signal presaturation and spinning rate was maintained at 12,000 Hz. Spectral parameters were: SW 20 ppm, 2 second presaturation, 32-k points, 64 scans. 125 130

Atomic Force Microscopy: Tapping Mode Measurements

The AFM experiments were performed with a Nanoscope IIIa (Digital Instruments, Santa Barbara, California, USA) operating in tapping mode at room temperature. Using a commercial silicon tip-cantilever (tip diameter \cong 5–10 nm) with a stiffness of about 40 Nm^{-1} and a resonance frequency of around 150 KHz, both the height- and the phase-imaging data were acquired simultaneously. All the AFM images were obtained, without any dilution of the samples, with a scan rate of 0.7 or 1 Hz over a selected area in the dimension of $1.2 \times 1.2 \mu\text{m}$ or $0.5 \times 0.5 \mu\text{m}$. Freshly cleaved mica was used as the substrate for AFM observations. 135 140

The force applied to the surface is roughly adjusted by the ratio of the engaged or set-point amplitude A_{sp} to the free-air amplitude A_0 . The set point amplitude was adjusted to

10–25% of the free-air amplitude for “high force” and to 40–70% or 75–90% of the amplitude for the moderate- and low-force imaging, respectively (McLean and Sauer, 1997). All data presented in this paper were obtained in moderate force mode (set point adjusted to 50–60%).

Images were processed and analyzed by using a program obtained from Digital Instruments software, version V5-31 (Veeco Group, Santa Barbara, California, USA). The height and diameter of liposomes were measured from the profile section of AFM line scans analyzing the height images.

Results and Discussion

Characterization of Liposomes: PCS and NMR Studies

Table 1 summarizes the mean diameter obtained elaborating the AFM images, the z-average diameter (the mean diameter based on the intensity of scattered light), and the polydispersity index (PDI; an estimate of the width of distribution) measured by using the PCS of the conventional liposomes (sample 1) and PEG-grafted liposomes (sample 2). The average diameter of PEG-grafted liposomes (sample 2) was lower than that of conventional liposomes (sample 1). Moreover, the presence of PEG significantly reduces the size distribution and the PDI. The presence of PEG coverage on the liposomal surface of sample 2 was suggested also by the higher positive surface charge due to the protonation in the aqueous environment of the amino groups located at the end of the PEG molecules.

These data agree with those reported in the literature for the PEG-grafted liposomes. According to the literature (Ueno et al., 2003; Garbuzenko et al., 2005), the presence of a low amount of PEG molecules on the surface of liposomes ($\leq 10\%$ mol PEG-grafted lipid) easily prevents the vesicles' aggregation (reducing the attractive forces) owing to steric hindrance due to the great hydration of polyethylene oxide. This limited aggregation results in a gradual reduction in the liposome size and size distribution. Moreover, the curvature of the liposomal surface increases to balance the lateral repulsive force in the lipidic bilayers produced by the extensive hydration around the polar head groups of PEG incorporated into vesicles. These phenomena lead to the decrease of the mean diameters of vesicles (Ueno and Sriwongsitanont, 2005).

Since liposomes of the same size were required in order to allow a comparison with sample 2 and to correctly evaluate the physicochemical parameters, sample 1 liposomes were extruded. Both the z-average and the polydispersity index of this extruded sample, called sample 1a, indicate the presence of homogeneous population of large unilamellar vesicles (Table 1).

In order to study the relationship between pegylation and the liposome stability in terms of aggregation, solubilization and changes in molecular conformation, the size distribution, and scattering-intensity variations were evaluated *versus* temperature. PCS can easily monitor temperature-dependent changes in the conformation of polymer and lipidic particles. Figure 2 shows the effect on the mean count rate and z-average diameter of a lipidic vesicle produced by the temperature increase. As reported by Michel et al. (2006), the measured count rate could vary, since it is affected by the alteration in the size and/or in the structure of the particle membrane and in the concentration of the sample. In the present study, the concentration of the samples was 5 mg/mL without changes during the measurements; therefore, when the temperature changes, the variation in the measured scattering intensity, which reveals the discontinuity in the mean count rate, can really reflect differences in the optical properties of the liposomes or in size distribution.

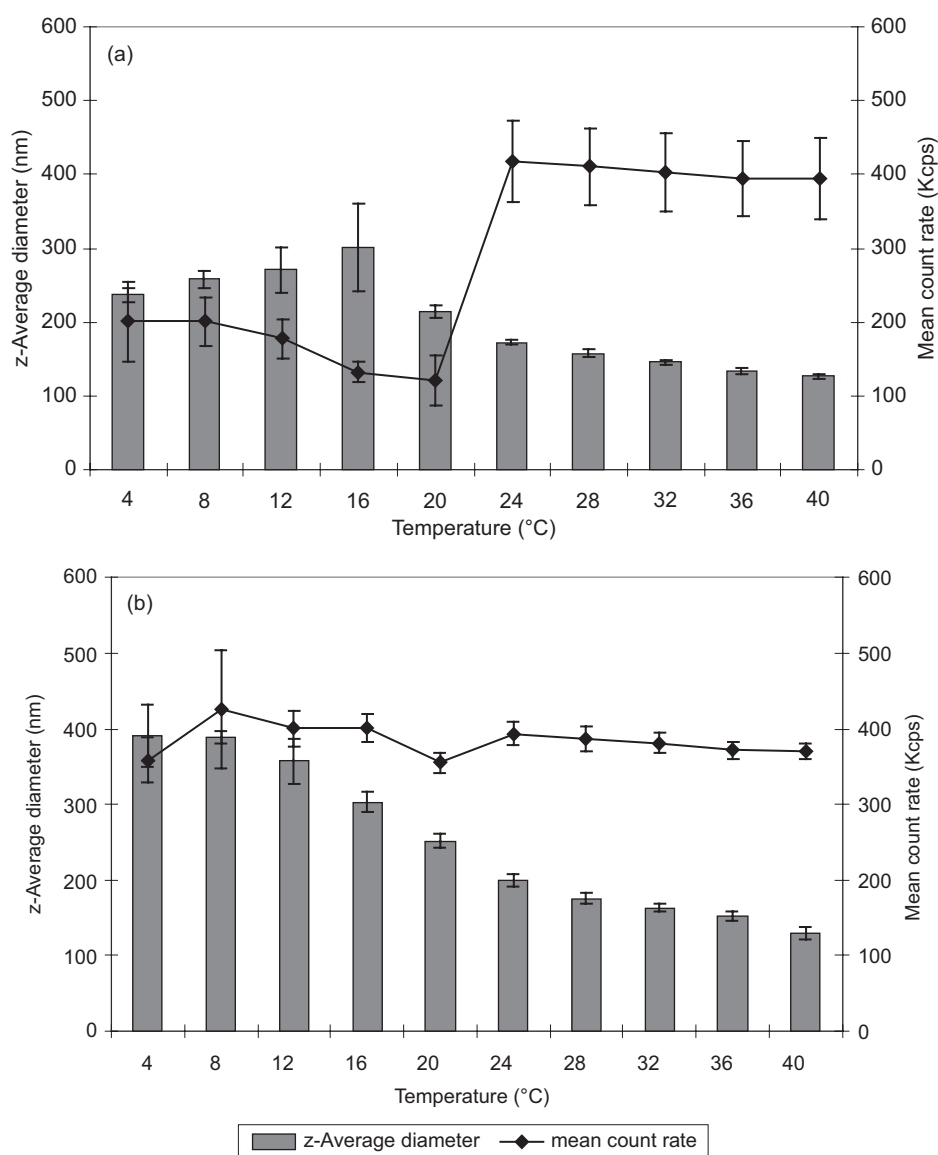


Figure 2. The z-average diameter (nanometers) and the mean count rate (kilo counts per second) obtained as a function of temperature (°C): A: sample 1a and B: sample 2.

The extruded liposomes (sample 1a) decreased their size with the increase of the temperature, although it can be emphasized that an anomalous swelling behaviour until about 20°C and variation in the mean count rate were observed. The thermally reduction of the bending rigidity of the bilayers provided the size reduction of liposomes. As the change in z-average was less dramatic than the change in mean count rate, it can be also hypothesized as an alteration in the structure of liposomes likely due to the polymorphic phase transition of the lipidic mixture (Epand et al., 2005). When the liposomes were prepared using 10% mol of Chol-PEG-NH₂ (sample 2), the z-average diameter decreased as the

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temperature increased. On the contrary, the mean count rates kept constant upon heating, indicating a good stability of this system. The decrease in the mean diameter with increasing temperature in the pegylated sample was already described by Garbuzenko et al. (2005). This behavior could be explained considering that the high temperature increases the mobility of molecules, reducing bilayer thickness. On the other hand, the grafted PEGs strengthen the repulsive forces on the liposome surface (Kenworthy et al., 1995).

Samples were analyzed through ^1H -NMR with the HR-MAS technique, which allows the broadening due to the dipolar interactions and chemical shift anisotropy to be reduced. This feature is typical of solid and semisolid materials, such as liposomes, cells, and tissue specimens. This goal is achieved by spinning the samples (at rates in the range 3–15 kHz) along an axis held at 54.7 degrees with respect to the static magnetic field used in the magnetic resonance instrumentation. Signals from molecules that possess high mobility are narrow, while those deriving from species possessing an intermediate mobility are broader. Signals from structural species, such as those involved in cell membranes, give prevalently background contributions.

Figure 3 shows the ^1H HR-MAS (12,000 Hz) NMR spectra of conventional extruded liposomes (sample 1a) and PEG-grafted liposomes (sample 2).

In the spectrum of PEG-grafted liposomes, the lipidic signals near 1.5–1 ppm have a wide line, suggesting an increased rigidity of the bilayer when compared to the conventional liposomes. On the contrary, a PEG signal near 3.7 ppm is much more narrow, thus indicating a good mobility of the PEG chain. As reported in the literature (Hashizaki et al., 2005), the addition of PEG-lipid to liposomes produced a lateral phase separation both in the gel and liquid crystalline states. Moreover, the fluidity in the interfacial region of liposomal bilayer membranes was markedly increased by the addition of PEG-lipids.

Characterization of Liposomes: Height and Phase Tapping Mode Images

As demonstrated using PCS and the NMR approach, conventional liposomes and PEG-grafted liposomes showed several differences in physical-chemical parameters. To evaluate the surface properties and to complete the characterization, the tapping mode AFM approach was used.

Figure 4 shows the height, three-dimensional height (3D), and phase images of conventional extruded liposomes (sample 1a; panel A) and PEG-grafted liposomes (sample 2;

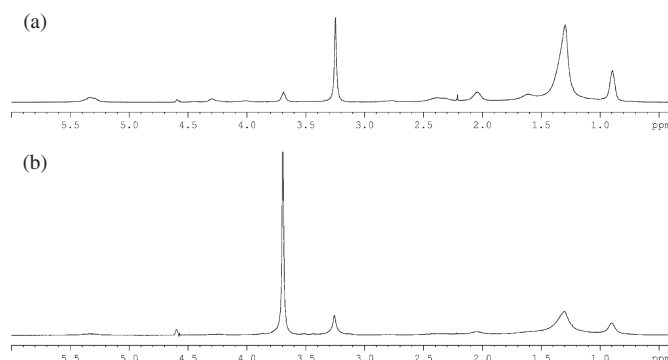


Figure 3. ^1H HR-MAS NMR spectra of A: sample 1a and B: sample 2.

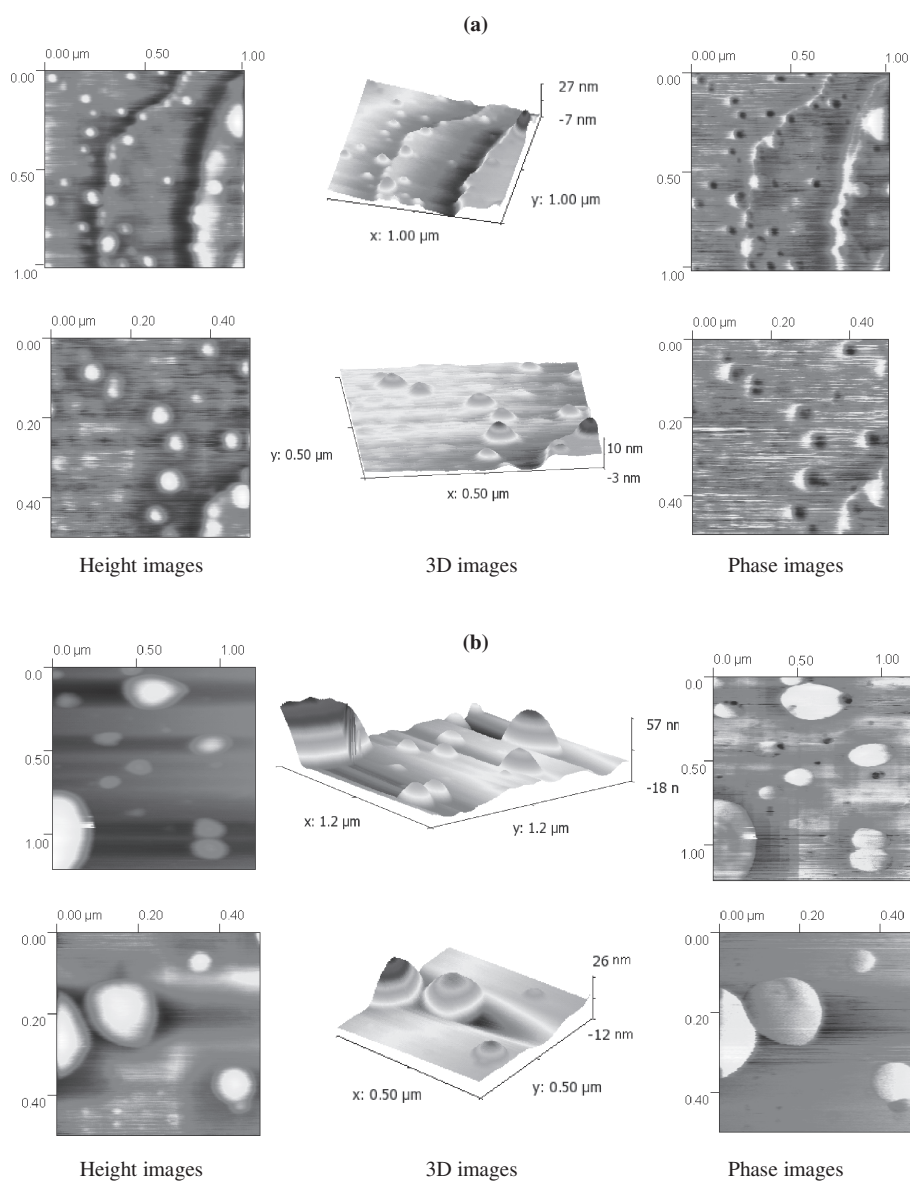


Figure 4. Tapping mode height images, 3D reconstructions, and phase images for A: sample 1a and B: sample 2. Area of scansion, $1.2 \times 1.2 \mu\text{m}$ and $0.5 \times 0.5 \mu\text{m}$.

panel B) acquired at two different selected areas of scansion ($1.2 \times 1.2 \mu\text{m}$; $0.5 \times 0.5 \mu\text{m}$), using the same force applied to the surface (set point amplitude adjusted to 50–60% of the free-air amplitude). 230

The two types of images revealed a corresponding phase shift for the protrusions relative to the surface of the liposomes. The height images of the samples showed separated, and more defined, vesicles with a height of 20 nm and a diameter of approximately 200–250 nm. 235

As expected, these data poorly correlate with the particle size obtained by PCS, and it suggests that our vesicles were distorted during the AFM measurements. As described in our previous work (Ruozi et al., 2005) and confirmed by Nakano et al. (2008), liposomes composed with the phospholipids having the lowest phase-transition temperature (e.g., PC) showed a high fluidity of liposomal membrane and tended to collapse by interaction with the mica surface. Also, pegylated liposomes were flattened into the mica surface, showing their high fluidity. Considering the phase images, the investigations suggest the presence of remarkable differences in the surface properties between the conventional and the PEG-grafted liposomes. Conventional liposomes (sample 1a) comprised a dark frame as a consequence of a negative phase shift, while PEG-grafted liposomes appeared with a well-defined bright phase contrast (positive phase shift).

The force applied to the surface can dramatically change the phase data, making difficult the interpretation of phase contrast results. To more rationally interpret the AFM phase images, several researchers carried out the AFM experiments by using different forces (Wabnig et al., 2007; Bar et al., 1997; Raghavan et al., 2000), relating the force to the driving amplitude (A_0) and the set-point amplitude (A_{sp}) ratio. Owing to this choice, the *set point* is the key factor capable of affecting both the relative phase and the relative height signal on a heterogeneous sample. In fact, an inversion of the phase and height contrast can occur as the *set point* is changed under certain conditions (Bar et al., 1998; Garcia et al., 1998). Setting the force applied to the surface of the liposomes, it was possible to correlate the change in energy dissipation during the scanning with the change in material properties (when the morphology of samples did not affect the analysis).

Several theoretical and mathematical models have been developed to describe the nature of the phase contrast. Some researchers described the surface properties of stiff samples by using Young's modulus and ascribing a larger, brighter positive phase shift to a stiffer surface, having a higher elastic modulus (Magonov et al., 1997; Leclère et al., 1996). Some materials, especially polymers and lipids, have been found to show viscoelastic behavior; consequently, these substances tend to display both solid- and liquid-like characteristics when subjected to the tip approach (Nguyen et al., 2001; Dubourg and Aimè, 2000; Wang et al., 2003; Scott and Bhushan, 2003). Particularly, the phase shift of soft liquid-like samples was affected primarily by the wetting behavior of the samples (Tamayo and Garcia, 1996; Luna et al., 1998). Concerning this, Dong and Yu (2003) analyzed the effect of condensed water and organic coating on the nanoparticle surface and clearly correlated the superficial properties with the phase imaging obtained by using the tapping mode AFM. In the same way, the surface properties of our liposomes can be considered different from those of stiff and soft materials, as liposomes are nanovesicles formed by the phospholipid layer enclosing an aqueous core. The phase shift could be affected primarily by the wetting and the bilayer hydration. Therefore, the AFM informations of liposomes could be related to the surface viscoelastic properties, rather than the hardness of the material. As liposomes are soft and the water is not only present into the core, but also hydrates the bilayer and condenses on the surface, the dark contrast in the phase image (negative phase shift) of sample 1a well describes the higher viscous/attractive forces between tip and sample. Practically, the AFM tip worked in the attractive regime with a dark phase contrast when it approached very viscous samples. Similarly, Haugstad and coworkers (Haugstad and Jones, 1999; Haugstad et al., 2000) and Dong and Yu (2003) described the correlation between bright and dark contrast in the phase image and the humidity in the environment affecting the amount of condensed water on the sample surface.

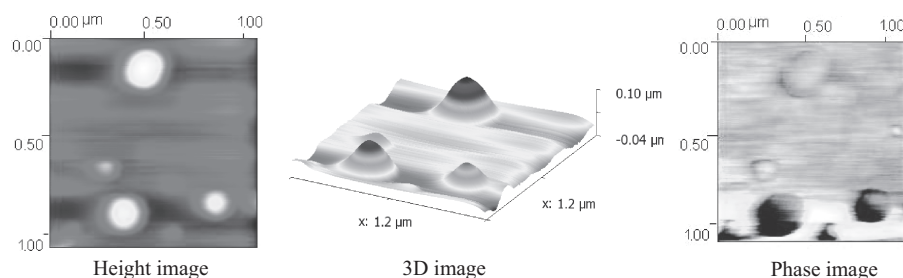


Figure 5. Tapping mode height image, 3D reconstruction, and phase image for mixed match of liposomes (sample 1a/sample 2). Area of scansion, $1.2 \times 1.2 \mu\text{m}$.

The presence of PEG in liposome formulation leads the conversion from dark to bright frame in phase images (Figure 4, panel B). The bright phase contrast on the particle surface could be explained considering the presence of PEG chains on the liposome surface, along with the very high capacity of these hydrophilic molecules to bind water (Tirosch et al., 1997). The amount of condensed water can modify the properties of the sample surface. In our opinion, the massive water condensation at the surface of PEG-grafted liposomes resulted in a relative low viscosity environment. The bright contrast due to a larger amount of condensed water can be noted as one of the characteristics of the samples containing a very liquid-like surface with low viscosity. In fact, the high level of hydration of PEG-grafted liposomes leads to the reduction of the attractive forces between the tip and the samples, while repulsive ones are increased. The low viscosity of the surface produced by the PEG hydration induces the tip to work in a repulsive regime. Besides, considering that the interaction between the tip and the surface could be due also to a different contact angle, the higher amount of condensed water on the liposome surface could increase the contact area. As described by Magonov et al. (1997), considering the polyethylene surface, both the large contact area and soft surface resulted in the bright phase contrast. Therefore, it is possible to conclude that the large contact area between the tip and our liquid-like sample could favor the phase conversion from negative (dark contrast/soft sample) to positive contrast (bright phase/well-hydrated soft samples with a low viscosity). This result gives evidence that the amount of water condensed on a hydrated lipidic surface significantly contributes to the phase shift.

To confirm the ability of AFM phase image to describe the surface modification of liposomes, we observed the mixed match of our preparation (containing both PEG-coated and conventional liposome); the phase image reported in Figure 5 describes two different populations of liposomes, while the sample containing a very liquid-like surface with low viscosity (PEG liposomes) is easily recognizable by bright contrast, in comparison with the very viscous conventional liposomes characterized by dark contrast.

Conclusions

The present study reveals that the local information about the surface of liposomes can be evaluated by using tapping mode AFM. Particularly, the phase contrast on the detected AFM phase image can be useful to successfully describe the surface modification introduced in order to obtain pegylated liposomes. This approach completes the morphological, dimensional, and surface investigations of samples applied in the pharmaceutical and medical field, strengthening the versatility of AFM.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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