# Visualization of PEO-PBLA-Pyrene Polymeric Micelles by Atomic Force Microscopy

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***Purpose.*** To directly visualize and evaluate the aqueous block copolymeric micelles, poly(ethylene oxide)-poly(β-benzyl L-aspartate) (PEO-PBLA) chemically conjugated with pyrene fluorescence molecule, by nanotechnology of atomic force microscopy (AFM).

***Methods.*** The block copolymers' PEO-PBLA-Pyrene was first synthesized by reacting with pyrene sulfonyl chloride and PEO-PBLA in tetrahydrofuran (THF) solution and were identified by GPC reflect index, UV and fluorescence detectors. The characterization of physical and chemical properties of PEO-PBLA-Pyrene polymeric micellar solution were examined by the dynamic light scattering (DLS) and critical micelles concentrations (CMC). In addition, the nanotechnology of AFM was used to directly visualize the size and shape of nanopolymeric micelles.

***Results.*** The pyrene fluorescence molecule were successfully conjugated at the amino group of the end of PBLA chain by GPC with three different detectors. The size of the aqueous PEO-PBLA-Pyrene polymeric micelles was detected around 57 nm with unimodal distribution by DLS measurement. As a result of this finding, the CMC test was also found out that the fluorescence intensity was increasing around 0.01 ~ 0.05 mg/ml. Using AFM evaluation of polymeric micellar solution, the morphology of aqueous PEO-PBLA-Pyrene polymeric micelles was observed on round shape and with the narrow dispersity of size range 50 ~ 80 nm.

***Conclusions.*** The presence of PEO-PBLA copolymers with pyrene in an aqueous system formed in a spherical and nano range of polymeric micelles.

**KEY WORDS:** polymeric micelles; AFM; DLS; pyrene.

## INTRODUCTION

The development of an effective nano-carrier drug delivery system has become an enormous challenge in many areas of modern science and engineering (1–3). Some of the basic characteristics, as well as functional properties, of nanoparticles stem to a large extent from their submicron size and, consequently, from their large surface to volume ratio. Of particular interest in the application of nanotechnology in the pharmaceutical area are polymeric micelles, having hydrophilic poly(ethylene oxide) (PEO) chains as palisade regions, which have been found to prohibit protein absorption and cellular interaction as well as directly avoiding the liver to other sites (4–5). For example, biodegradable block copolymers produced from PEO and poly(asparate) (pASP) with doxorubicin exhibited good stability after intravenous injection in female ddy mice by Kwon *et al.* (4) and the pharmacokinetic parameters of half life ($t_{1/2}$) were calculated to be around 6 to 7 hours with 24-hour measurement. In addition, the evaluation of solid tumor bearing mice with the same polymeric micelles showed a ten-fold increase in accumulation of polymeric micelles in the tumor side as well as about a 2.9% decrease in the side effect of heart absorption.

At the same time, these types of polymeric micelles display good potential as drug delivery systems, due to the ease of both their preparation and incorporation with drug molecules as well as a potential for high drug loading and possibilities for sustained systemic release. Kwon *et al.* (6) have shown that using polymeric micelles, PEO-PBLA with a 40 nm particle size range by dynamic light scattering measurement, could enhance the low solubility of adriamycin to 10% w/w and prevent high binding of serum albumin. In addition, the physically entrapped adriamycin was gradually released over 100 hours, indicating that the release from polymeric micelles was due to diffusion control and not a result of the dissociation of the micelles. However, the effectiveness and mechanisms with which PEO polymeric micelles are absorbed by the therapeutic target tissue or system presents a major obstacle to the use of such vehicles for site-specific drug delivery. Thus, extensive investigations will be needed to determine if these particles interact with other components of the environment when released in physiological fluids (e.g., blood) or organs (e.g., the intestine or lungs).

It is very difficult to observe these copolymer structures in an aqueous solution as well as see their interaction within a living tissue or membrane. Thus, the application of a fluorescence molecule, pyrene, to PEO-PBLA copolymers (Fig. 1), can improve the observation limit as well as enhance detection using a regular reflect index and UV detectors. Therefore, the purpose of this project was to understand the nature of the association complexes formed by PEO-PBLA-Pyrene copolymers in an aqueous dispersion as well as to observe the structure of polymeric micelles by AFM.

## MATERIALS AND METHODS

### Materials

PEO was purchased from Nippon Oil and Fat (NOF) Co., (Kawasaki, Japan). Spectra/Pro dialysis membrane (Cellulose, molecular weight cutoff 3500) were obtained from Spectrum (Houston, Texas). Disposable ultrafiltration units were obtained from Millipore (Bedford, MA). Pyrene sulfonyl chloride used was from Molecular Probe Inc. (Eugene, Oregon). All other chemicals were reagent or analytical grade and were used as received. Mica were purchased from Comar Instruments (Cambridge, UK).

### Synthesis of Micelle-Forming PEO-PBLA with Pyrene Block Copolymer

The synthesis of PEO-PBLA block copolymers based on PEO and PBLA has been described in detail elsewhere (6).

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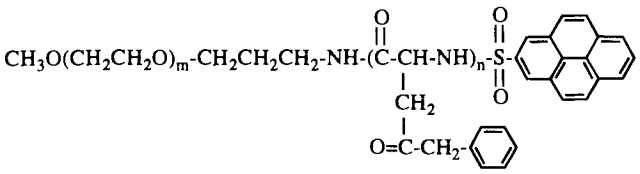

**Fig. 1.** The chemical structure of PEO-PBLA-Pyrene. PEO = 12,000 g/mol, n = 15 units.

Briefly, PEO-PBLA block polymers were obtained by the ring-opening polymerization of β-benzyl L-aspartate N-carboxyl anhydride using α-methoxy-*w*-amino-poly (ethylene oxide) (a gift from NOF Co., Japan) as an initiator. The PEO-PBLA block polymer was precipitated in diethyl ether, collected and dried under vacuum. $^1$H NMR spectra of PEO-PBLA were obtained in 1% dimethylsulfoxide (DMSO)—d6 using an NMR instrument (JEOL EX400, Japan) at 400 NHz. For this study, the block polymer used PEO and PBLA number average molecular weights of 12,000 g/mol and 15 unit of PBLA, respectively. The diameter of micelle-forming PEO-PBLA was measured to be about 50 nm by dynamic light scattering (6).

Chemical conjugation of PEO-PBLA with pyrene moiety at chain end was obtained by reaction with PEO-PBLA and pyrene sulfonyl chloride in distilled THF in the presence of triethylamine for 3 days. The purification of PEO-PBLA-Pyrene block copolymers was accomplished using a membrane dialysis (molecular weight cut of 3,500) method in methanol solvent for 3 days, and subsequent dialysis with DMSO with 10 mM/L of LiBr solution for three days, while constantly stirring at room temperature.

### Preparation of Aqueous Dispersions

Aqueous dispersions of the PEO-PBLA-Pyrene copolymeric micelles were prepared by dissolving the copolymer in pure methanol solution then adding double distilled water for a 1:1 ratio. The copolymer/methanol/water system was generally heated at 70°C for several hours until the required volume was obtained and Hank's buffer solution was used to give a final concentration in the range of 0.01 to 10 mg/ml.

### Gel Permeation Chromatography (GPC)

Molecular weights of the copolymers and their polydispersity were determined by gel exclusion chromatography on two TSK gel G3000H and G4000H columns (30 cm × 7.8 mm), using dimethylformamide (DMF) for the mobile phase at a flow rate of 1 ml/min, with the columns thermostated at 40°C. The peaks were detected by both refractive index (RI-8022, Tosoh, Japan) and UV (UV8020, Tosoh, Japan) with a wave length of 280 nm and analyzed using Tosoh GPCSEC software.

Data for aqueous gel permeation chromatography were derived from preparative runs on a column of TSK gel G3000sw (30 cm × 7.8 mm). Samples (100 μl) were loaded at concentrations ranging from 0.01 to 10 mg/ml and eluted at 1 ml/min with double distilled water. Peaks were detected using a refractive index (830 RI, Jasco, Japan), UV (970UV, Jasco, Japan) and fluorescence detectors (FS8020, Tosoh, Japan).

### Estimation of the Effective Hydrodynamic Diameter and Polydispersity of PEO-PBLA-Pyrene Dispersions by Dynamic Light Scattering

Dispersions of PEO-PBLA-Pyrene copolymeric micelles (12,000 g of PEO and 15 unit of PBLA) at a concentration of 1 mg/ml were produced as described. A comparison was made with a 1 mg/ml aqueous dispersion of PEO-PBLA as a part of preparation of the aqueous dispersion. Samples were analyzed by dynamic light scattering, using a Photal DLS-7000 light scattering photometer (Otsuka electronics, Japan), with a helium/neon laser light source operating at a wavelength of 632 nm, with an assumed refractive index ratio of 1.33 and viscosity of 0.88. The sample cell was cleaned before each measurement by flushing with double distilled water and drying up with air. The sample time for PEO-PBLA-Pyrene dispersion was 10 μs and the experimental duration 100 s. All measurements were performed at 25°C at a measurement angle of 90°

### Determination of the Critical Micelle Concentration (CMC) of PEO-PBLA-Pyrene Micelles

The fluorescence emission spectra of pyrene were obtained using a fluorometer (FP-770, Jasco, Japan). Experiments with aqueous PEO-PBLA-Pyrene micelles were done with excitation and emission wavelengths of 352 and 382 nm, respectively. The quantity of fluorescence emission of pyrene was examined in PEO-PBLA-Pyrene micellar solution with a concentration range from 1 mg/L to 30 mg/ml. The excitation band width was set up in 5 nm and the emission band width 3 nm. All fluorescence experiments were carried out at 25°C.

### Atomic Force Microscopy Measurement

The micelle-forming PEO-PBLA and PEO-PBLA-Pyrene were put in amounts of 1 to 2 μl on the mica surface without any treatment. The AFM used in this study was manufactured by Nanoscope III (Digital Instruments, USA) and was operated in the constant tapping mode. The cantilevers used were standard Nanoprobe silicon single crystal levers (125 μm) and the constant force mode was used with scan frequencies typically at 5 Hz. A scanner of 1-μm scanning range was used and all images were collected in a 2.5 × 2.5 μm area. All images shown are the raw experimental data subjected only to the normal image processing of leveling, unless otherwise specified.

## RESULTS

The conjugation and purification results of PEO-PBLA-Pyrene are indicated in Fig. 2. The first peak represents the copolymers by increasing intensity following the methanol and DMSO two dialyzing procedures. The second peak in the UV chart is considered to correspond to the pyrene compound isolated from the micelles. Micelle formations of PEO-PBLA-Pyrene in Hank's buffer solution are shown in Fig. 3 using three detectors. The molecular weight (first peak with retention time around 6.5 minutes) corresponding to the gel-exclusion volume is much higher than the molecular weight of this conjugate (ca. 15,000), indicating micelle formation of the conjugate. In addition, the intensity of pyrene fluorescence compound was higher than UV and RI could detect. The second peak, with

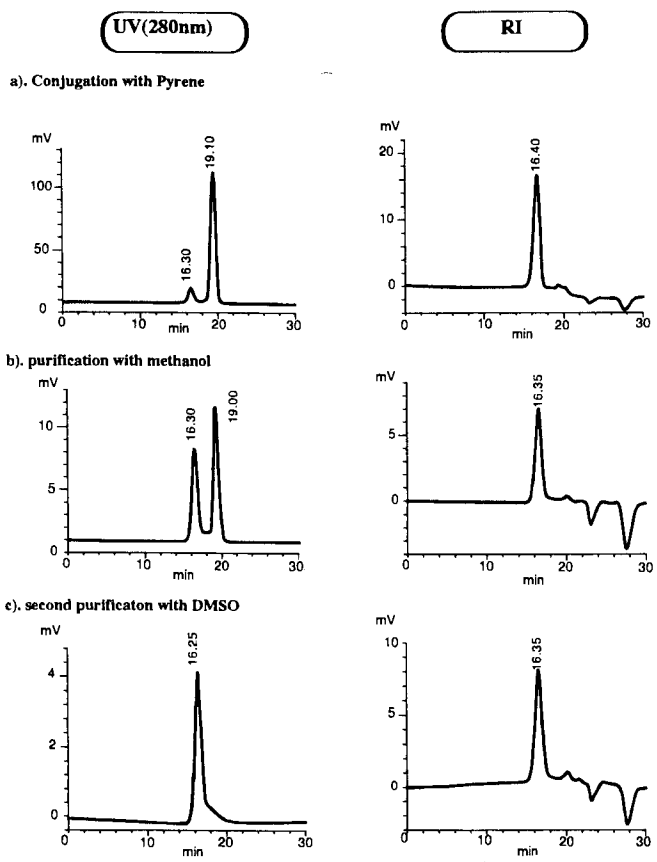


**Fig. 2.** The purification chromatograms of gel permeation chromatography of the synthesis of PEO-PBLA with pyrene under two UV (280 nm wavelength) and reflect index detectors. a) The first chart of conjugation of PEO-PBLA with pyrene compound. b) The purification of PEO-PBLA-Pyrene using methanol solvent in a membrane dialyzer (MW cutoff: 3,500). c) The second purification of PEO-PBLA-Pyrene using DMSO solvent in a membrane dialyzer (MW cutoff: 3,500). The GPC's were performed using 50 μl injections of 2 mg/ml of polymeric with a DMF for the mobile phase at a flow rate of 1 ml/min. (Column; TSK gel G4000$H_{HR}$, G3000$H_{HR}$, mobile phase; DMF with 10 mM LiBr; flow rate: 1 ml/min in 40°C).

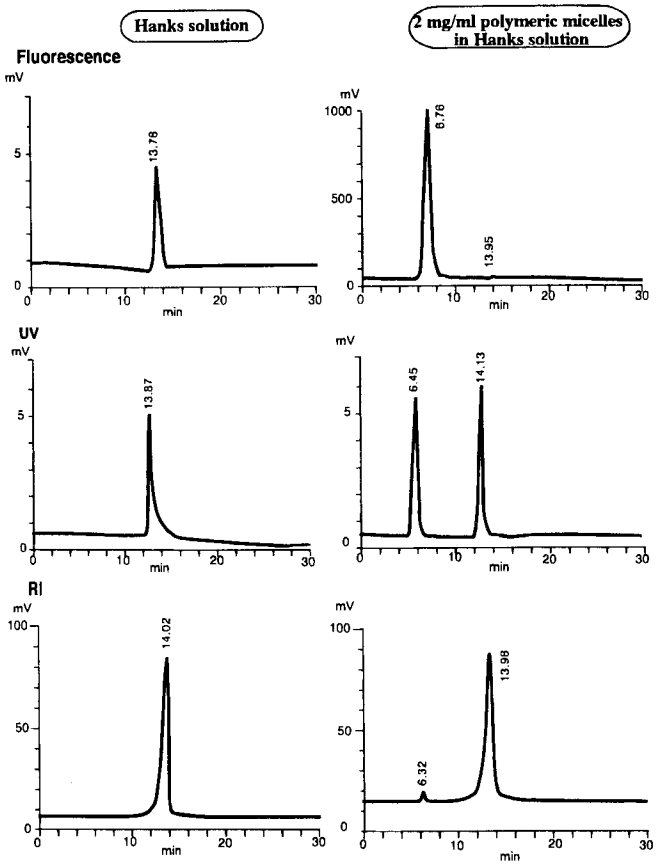


**Fig. 3.** The chromatograms of gel permeation chromatography of PEO-PBLA-Pyrene polymeric micelles in Hank's buffer solution. Peaks were, detected by a fluorescence, UV and RI, using 50 μl injections of 2 mg/ml of polymeric micelles solutions with a double distilled water for the mobile phase at a flow rate of 1 ml/min.

retention time around 14 minutes, was influenced by the composition of Hank's buffer solution. In the meantime, the micelle-forming of PEO-PBLA-Pyrene solutions were testing under on RP-18 Lichrosphere column (5 μm with endcapped 250-4 mm, E Merck Co., Darmstadt, Germany) using double distilled water for the mobile phase at a flow rate of 1 ml/min. The peaks were detected by both refractive index (ERC-7515A, ERC Inc., Japan) and UV (Spectra100, Sepctra-physics, Japan) with a wave length of 280 nm. The purification results of PEO-PBLA-Pyrene micellar solution only observe one peak with retention time around 11 minutes on the charts (data did not show).

The total fluorescence intensity increases of a fluorescent probe upon micellization have been utilized to determine CMC for a host of surfactants and for PEO-PS, PEO-PBLA. Fig. 4 illustrates the plotting of total fluorescence intensity as a function of the logarithm PEO-PBLA-Pyrene concentration. At low concentrations of PEO-PBLA-Pyrene (below 0.01 mg/ml), negligible changes in the total fluorescence intensity were observed. All the particle sizes and shapes measurements of polymeric micelles solution were carried out to at least 1 order of magnitude above the CMC of the polymers.

The PEO-PBLA-Pyrene polymeric micelles solutions (1 mg/ml) exhibited one single modular population of particle distribution by dynamic light scattering (Fig. 5). The particle

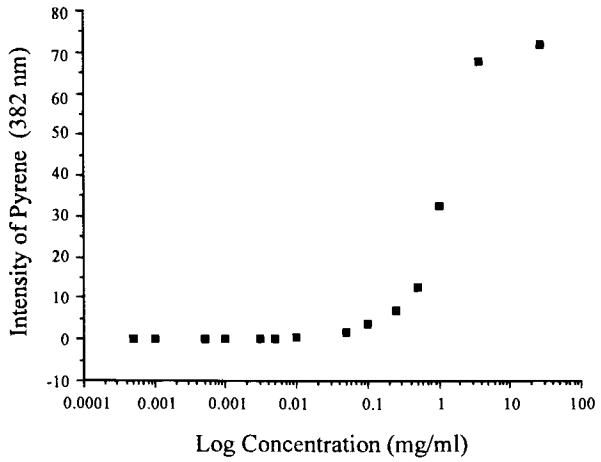


**Fig. 4.** Total fluorescence intensity of pyrene emission versus the logarithm of PEO-PBLA-Pyrene concentration.

weight average size (Gwt) indicated was 48 nm and the number average size (Gno) shown was 41 nm. The polydispersity (Gwt/Gno) value of the micelles was calculated to be around 1.17, which indicated that PEO-PBLA-Pyrene polymeric micelles have a narrow size distribution.

In order to visualize the morphology of polymeric micelles, atomic force microscopy was used. As a control, the mica images contained without polymeric micelles, in the 5.5 × 5.5 μm range, obtained commonly showed surfaces with smooth or flat topography (Fig. 6a). The polymeric micelles readily attached to the surface of mica and remained sufficiently tightly bound as imaged with an AFM tip (Fig. 6b). The shape of polymeric micelles was typically observed as single, smooth and round. The average diameter of polymeric micelles measured from one edge across the center to the other edge was found to be 56.4 (±10.3) nm. In the preparations with a high concentration of the polymeric micelles (5 mg/ml), there appeared to be aggregated formation on the mica surface (Fig. 6c). These morphology were still observed as individual and round shapes, except they were compactly arranged on the mica surface. Also, the average size of polymeric micelles did not change significantly and no fusing polymeric micelles were found on AFM observation.

## DISCUSSION

As the concentration of PEO-PBLA-Pyrene was increased, at a certain polymer concentration (i.e., CMC), the total fluorescence intensity in the emission spectrum (382 nm) increased dramatically in a sigmoidal manner. The PEO-PBLA-Pyrene block copolymer with a 12,000 PEO and 15 unit PBLA had a low CMC value (ca. 5 mg/L). The low CMC values of PEO-PBLA-Pyrene block copolymers are consistent with known low CMC values for AB block copolymers, i.e., PEO-PBLA, which range from 0.0035% to 0.1% (w/v). Furthermore, Kwon *et al.* (7) using a partition pyrene method in PEO-PBLA polymeric micelles, demonstrated the CMC values had a similar range (5 to 10 mg/L) compared with our data. As was noted during fluorescence measurement, there was a change in the emission of pyrene upon micellization of PEO-PBLA. The vibrational structure of pyrene monomer emission is known to be dependent on local polarity (i.e., the Ham effect) and the ratio of the (0,0) band, I, to the intensity of the (0, 2) band, III, exhibits sensitivity toward local polarity (7). In the PEO-PBLA-Pyrene polymeric micellar experiment, two of five bands disappeared, leaving three bands. This may be due to the conjugation of pyrene through a electron accepted sulfonyl group.

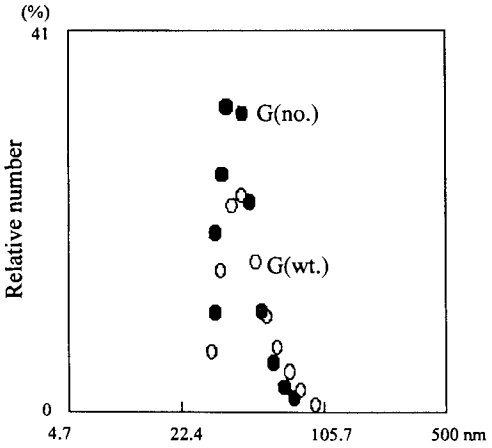


**Fig. 5.** Particle size distribution of PEO-PBLA-Pyrene polymeric micelles using dynamic light scattering. The solid circle represents distribution of particle number average size (Gno) and the open circle represents distribution of particle weight average size (Gwt).

When compared to the polydispersity and size distribution before the conjugation of pyrene with PEO-PBLA polymeric micelles, there is not much different dispersity of polymeric micelles (Gwt/Gno = 48 nm/42 nm = 1.14). These results also were consistent with previous fluorescence measurement of the formation of polymeric micelles. The micellar molecular weight was estimated to be much higher (Fig. 3) as compared to the free polymer chain (ca. 15,000 g/mol). These are agreed with the closed association of single polymer chains into micelles (7).

In the observation of round, single polymeric micelles morphology by AFM, such edge-to-edge data, it was similar to polymeric micelle size obtained by DLS examination. The circumference of the polymeric micelles formed on mica were also consistent with the observation of Hagan's *et al.* (8) by the transmission electron microscopy (TEM). Although the structure of polymeric micelles was similar, the average diameter of polymeric micelles was reported to be around 17 nm by the TEM method. This discrepancy could be due to the preparation of polymeric micelles under the TEM procedure using an air-dried approach or due to the different composition of each polymeric micelle.

These polymeric micelle shapes were stable with continuous scanning under experimental conditions. However, the height measurements obtained by AFM, which were examined at around 6 nm, are not seen with a similar latitude. This may be due to the polymeric micelles shrinking in thickness during the examination or there could be compression by the cantilevers. The other possibility is that the surface of mica has a hydrophilic property similar to the outer hydrophilic surface property of polymeric micelles. However, it is undergoing to investigate interaction between surface of mica and different outside regions properties of polymeric micelles as well as inside interaction of polymeric micelles. Furthermore, after repeated scanning of a limited region on the surface at different times (1 hr), the shape of the polymeric micelles still could be seen as clearly (data not shown) as before, indicating that molecules did not desorb or were not swept away due to interactions with the tip. Quist *et al.* (9) have reported that some protein could desorb and adsorb on the tip and subsequently change the field of view of the microscope over time. However, this was not the case in our study. In Fig. 6c, these polymeric micelles (5 mg/ml) morphology were still observed as individual and round shapes and this could be due to decrease surface area of boundary of two segments and increase steric repulsion of between each polymeric micelle. On the other hand, it may be due to the concentration of polymeric micelles not being high enough to form a different shape or fuse to each other, or the kinetic equilibrium time was not long enough. The thickness of polymeric micelles appeared around 5 nm and this could be also relative to the physical properties of individual structures (e.g., compressibility).

In conclusion, the presence of PEO-PBLA-Pyrene copolymers in the aqueous system did form a spherical and nano-range polymeric micelles. The fluorescence copolymers obtained are of interest as an instrument for directly locating polymeric micelles in physiological fluids or organs and for future studying

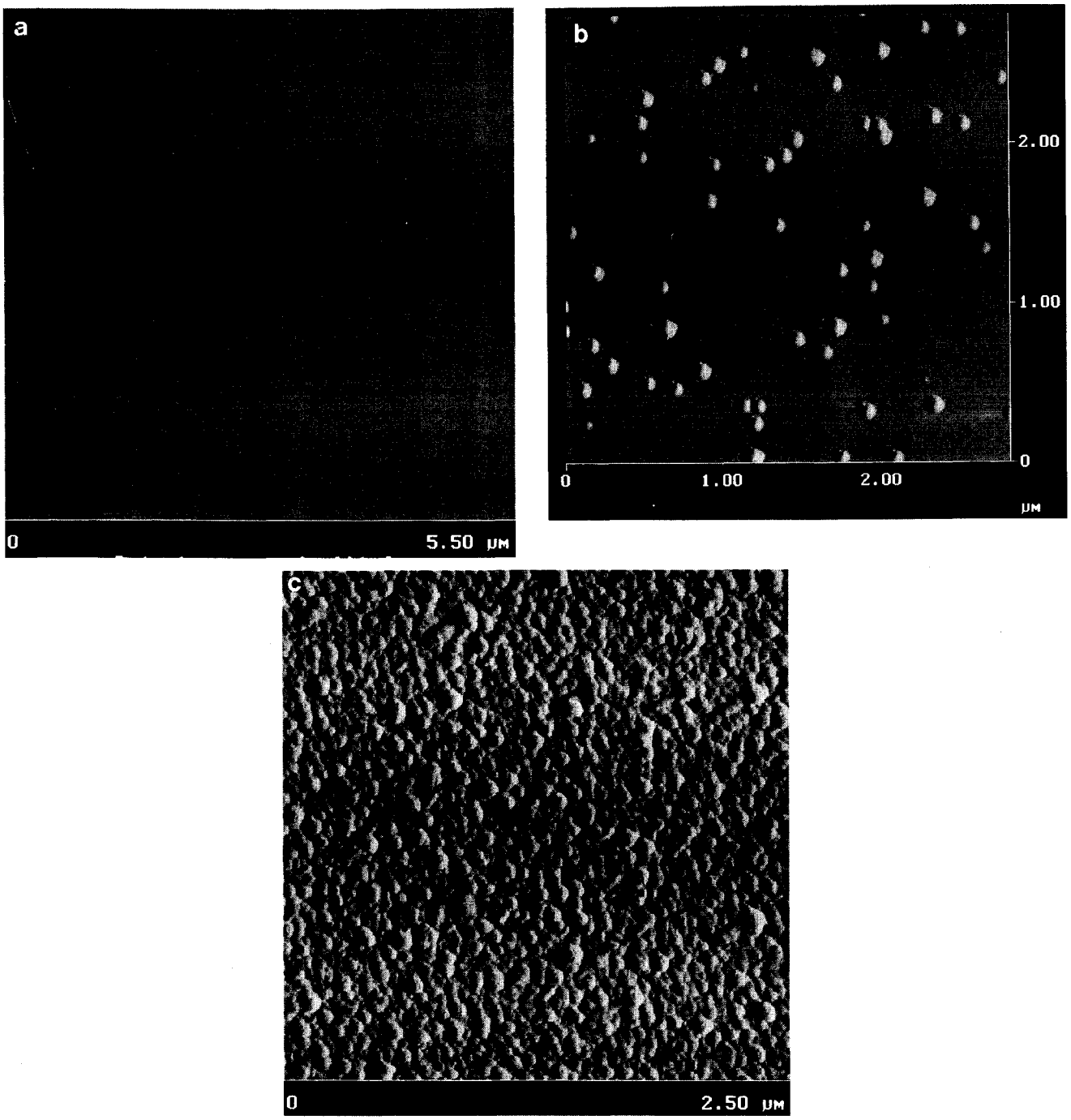


**Fig. 6.** AFM images of PEO-PBLA-Pyrene polymeric micelles on mica surfaces. a) without polymeric micelles; b) with one drop of PEO-PBLA-Pyrene polymeric micelles solution (0.1 mg/ml); c) with one drop of PEO-PBLA- Pyrene polymeric micelles solution (5 mg/ml) showing the micelles' compact arrangement with each other on the surface.

the mechanism of interaction of polymeric micelles with tissues or membranes.

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