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Regiospecific Orientation of Single-chain Antibody and Atomic Force Microscope (AFM) Images

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An antibody containing a genetically engineered lipid group at the N-terminus and a hexahistidinyl tag at the C-terminus (Lpp-scFv-His6) was immobilized in an oriented manner on the surface of liposomes. Liposomes, consisting of antibody and phosphatidylcholine, have been prepared and imaged by AFM. For AFM visualization, the resulting liposomes were bound on the surface of mica by two different mechanisms. The histidine tags present in the antibody molecules of the immunoliposome were anchored to the $NiCl_2$ treated mica surface. Alternatively, the immunoliposomes were immunochemically bound on antigen-coated mica surface. Both approaches yielded liposomes which were clearly imaged without damage by AFM in ambient condition.

Key words: lipid tagged single chain antibody(Lpp-scFv), AFM, antigen, antibody

INTRODUCTION

Since its invention by Binnig *et al.* [1], the continuing development of AFM has provided a powerful new method for understanding of biological phenomena by direct observation of biomolecules under nearly physiological conditions [2,3]. The most outstanding advantage of AFM is that the probe is almost in contact with the sample and thus capable of measuring physical properties of the specimens in real time, especially when they are soft and deformable, rather than imaging them from a distance [4,5]. A contact mode AFM can be used to image particles that are attached firmly to the surface through covalent bonds. One limitation of a contact mode AFM is the possibility of damage of the soft sample surface as the probe is scanned [6]. Recently, a new imaging mode called tapping mode has been developed, in which a cantilever is oscillated at very high frequency as it scans the sample [7]. Tapping mode is especially useful for imaging soft samples including biological specimens [8,9]. In this operation, a cantilever is oscillated with a high amplitude (typically 10-20 nm) near its resonance frequency as it is scanned laterally. The amplitude of the oscillation is clipped and reduced by the tip contact with the sample. A feedback loop is used to keep this amplitude reduction constant during scanning and tracking of the topography of the sample.

Liposomes are closed vesicular structures that natural and synthetic lipids form spontaneously when mixed with water. As foreign molecules can be readily incorporated in liposomes during their preparation, liposomes have been studied extensively as vesicles for targeted drug delivery [10,11], and vaccination and as immunoreagents. Liposomes vary in size from 25 nm up to several micrometers in diameter and they can be unilamellar, containing a wall consisting of only a single lipid bilayer, or multilamellar. Scanning electron microscopy and transmission electron microscopy have been used to study the structure and dimensions of liposomes, but the method suffers from such serious problems as the need of freeze fracture and high vacuum treatments.

Recently, AFM becomes available as a tool to study the structure and properties of liposomes. But in study of AFM [12], dipalmitoyl phosphatidyl ethanolamine (DPPE) vesicles were adsorbed to the mica surface and showed that the AFM probe had damaged some of the large vesicles that were formed. When rinsed in distilled water, the vesicles were washed away.

In this study, we have employed tapping-mode AFM to visualize liposomes which were derived by incorporating a genetically engineered single-chain antibody on the liposomal surface [13]. The lipid-tagged antibodies harbored also a hexahistidinyl tag at the C-terminus. Accordingly, we could use the methods to bind the liposomes on the mica surface in a manner, which is expected to limit their lateral mobility and deformability. The histidine tags present in the antibody molecules were used to bind the liposomes to nickel-chloride-treated mica surface. In addition, immunochemical binding based on antibody-antigen interaction was exploited to bind the liposomes to antigen-coated mica surface. In both cases, exceptionally clear images of bound liposomes were obtained under ambient conditions. We conclude that immobilization of the liposomes by specific molecular interactions is crucial for successful imaging and that tapping-mode AFM should be useful for structural analysis of liposomes and of immunoliposome-antigen interactions under mild conditions.

MATERIALS AND METHODS

Purification of Lipid-tagged Antibody and Antigen

Lipid-tagged single-chain antibody against 2-phenyl-

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oxazolone containing a C-terminal hexahistidinyl tail for immobilized metal affinity purification (Ox lpp-scFv-H6, lpp:lipoprotein) was produced in *E. coli* HB 101 in shake flask culture, and purified as described in elsewhere [14], with the exception that Pharmacia HiTrap Chelation Sepharose columns were used instead of batch-wise binding to Ni^{+2}-charged Chelating Sepharose. Protein concentration of soluble antibodies were determined by using the measured absorbance value and the calculated molar extinction coefficient at 280 nm.

Soluble hapten (6-aminocaproic acid derivative of 2-phenyloxazolone, Ox-CA) and hapten-BSA conjugate (2-phenyloxazolone-BSA, Ox-BSA) were used as a competing ligand. Phenyl-4-ethoxymethylene-5-oxazolone (ph-Ox) is a reactive compound which can be coupled covalently to proteins with concomitant loss of ethoxymethylene group.

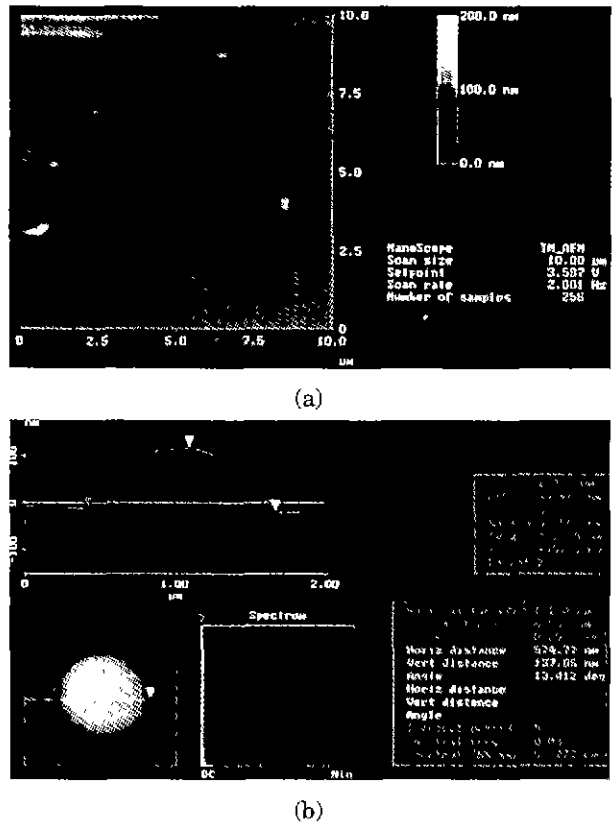
Preparation of Liposome

Liposomes were prepared by a detergent-dialysis method using a cellulose dialysis membrane (cutoff: 12 ~14 kDa) essentially as described previously [14]. Pure egg yolk phospholipid (10 mg) was dissolved in 2 mL of 20 mM HEPES buffer (pH 7.4) containing 1% (w/v) OG, with 20 mM of Lpp-scFv-H6 antibodies in HEPES buffer. The detergent was then removed by dialyzing against 20 mM HEPES buffer overnight in cellulose dialysis bags with two buffer changes at 4℃. After detergent removal, the liposomes were collected by ultracentrifugation (150,000 g, 2 hr, 4℃) and suspended in 1 mL of 20 mM HEPES buffer.

AFM Images

All AFM images were obtained by a tapping mode of method using a commercial instrument (Nanoscope IIIa; Digital Instruments, Santa Barbara, CA) with a D type scanner. An etched silicon cantilever with 10 nm of radius of curvature was employed. The cantilevers have high resonant frequencies (50-500 KHz) and high spring constants. In this experiment, all the images were recorded under ambient conditions. The fresh mica surface was cleaved by pressing some adhesive tape against the mica surface, and peeling off the tape. Mica surface was treated with 10 mL of 1 mM nickel chloride dissolved in Milli Q water for 5 min, rinsed three times with Milli Q water, and allowed to air dry. A solution containing the immunoliposomes was dropped on the nickel treated mica surface and allowed to interact for 5 min at room temperature. Unbound immunoliposomes were removed by rinsing with Milli Q water and the mica surface was exposed to air.

The immunoliposomes were also adsorbed on the mica surface immunochemically as described in the following. Antigen (10 μg/mL of Ox-BSA) was adsorbed on a fresh mica surface by incubation overnight at room temperature. The immunoliposomes were incubated for 30 min in the presence or absence of various concentrations of Ox-CA as a soluble competing hapten. Thereafter, the mixture was dropped on the antigen coated mica surface. After 10 min, the unbound immunoliposomes were removed by rinsing with HEPES buffer and the mica was kept in air condition. To test the reusability of the immunoliposome-treated mica surfaces, these were treated with a solution containing Triton X-100 (to dissolve the liposomes) and glycine buffer, pH 2.5 (to disrupt the antigen-antibody interaction) (TX/Gly; 1% Triton X-100 and 0.05 M glycine, 0.15 M NaCl, pH 2.5).



(a)

(b)

Fig. 1. AFM images of the immunoliposomes adsorbed on the mica surface for 5 min reaction; (a) AFM image of the immunoliposome distribution, (b) AFM image of single immunoliposome. The scan speed was proportional to the scan size, and the corresponding line frequency was 2.0 Hz. The size of liposomes is ranged from 0.5 to 1.2 μm in diameter.

RESULTS

AFM Imaging for Immunoliposomes on the Fresh Mica Surface

Fig. 1 (a) (b) show the AFM images of the [Ox lpp-scFv-H6]-bearing immunoliposomes that were incubated on untreated, fresh mica surface for 5 min. Fig. 1 (a) shows structures that with size 0.5 to 1.2 μm diameter are seen on mica surface, likely representing the liposomes. Fig. 2 (b) illustrates a hemispheric shape of a liposome adhered on the mica surface. Section analysis shows that this hemisphere structure has a diameter of 1,200 nm and a height of 137 nm. The hemispheres collapsed during the drying stage of sample preparation [12]. When the mica surface was rinsed in HEPES buffer, the liposomes were washed away, indicating the weak nature of the interaction between the mica surface and the liposomes.

AFM Imaging for Immunoliposomes on the Ni-treated Mica Surface

Next, we examined by AFM liposome samples which were bound to treated mica surface. The mica surface is negatively charged and is expected to bind Ni^{+2} ions

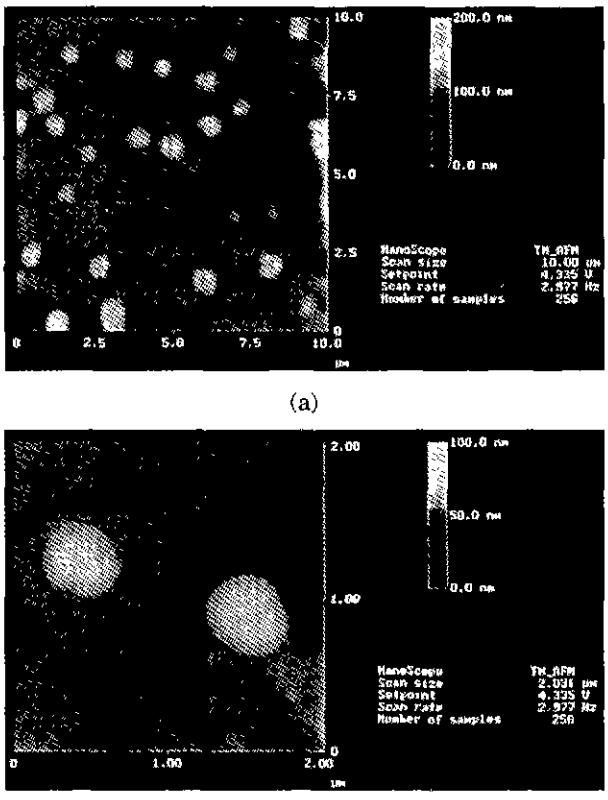


Fig. 2. The immunoliposomes were adsorbed to Ni^{+2}- treated mica surface and imaged by AFM at room temperature and in air condition. (a) image is 10 μm by 10 μm areas in air condition. The scan speed was 2.98 Hz. It shows the globular structures with size from 300 to 1,000 nm diameter. (b) shows an zoom image of the middle part in (a).

through electrostatic interactions. The bound nickel may form a chelate with the hexahistidine tag of the lipid-tagged single chain antibody present on the surface of the immunoliposomes (Fig. 2 (a), (b)). Fig. 2 (a) shows a clear image of immunoliposomes that were bound to the nickel mica surface. The diameters of the globular structures seen in the image range from 300 to 1,000 nm. The central part in Fig. 2 (a) is enlarged in Fig. 2 (b), allowing visualization of two types of structures on mica surface: globular and flat. Section analysis indicates that the globular structure has a diameter of 642 nm and height of 30.1 nm. The average thickness of flat layers is 11 nm, consistent with the thickness of a layer between the mica surface and the flat.

AFM Images of the Immunoliposomes Immunochemically Adsorbed on Mica Surface

Finally, we studied immunoliposomes that were adsorbed on mica surface by using antigen-antibody interaction. For this purpose, freshly cleaved mica was preincubated with Ox-BSA (10 μg/mL in PBS) overnight, followed by brief treatment in 1% BSA to prevent any nonspecific binding of immunoliposomes on mica surface. Fig. 3 (a)-(c) show that use of this immunochemical procedure, resulted in stable adherence and orientation of the immunoliposomes. Fig. 3 (a) shows the mica surface after coating with Ox-BSA. An even surface is seen with occasional particles of 70-150 nm in diameter. Fig. 3 (b) shows a sharp AFM image of immunoliposomes adsorbed on the Ox-BSA coated mica surface. The diameter of the globular structures varies from 200 to 500 nm, in agreement with the expected size of liposomes.

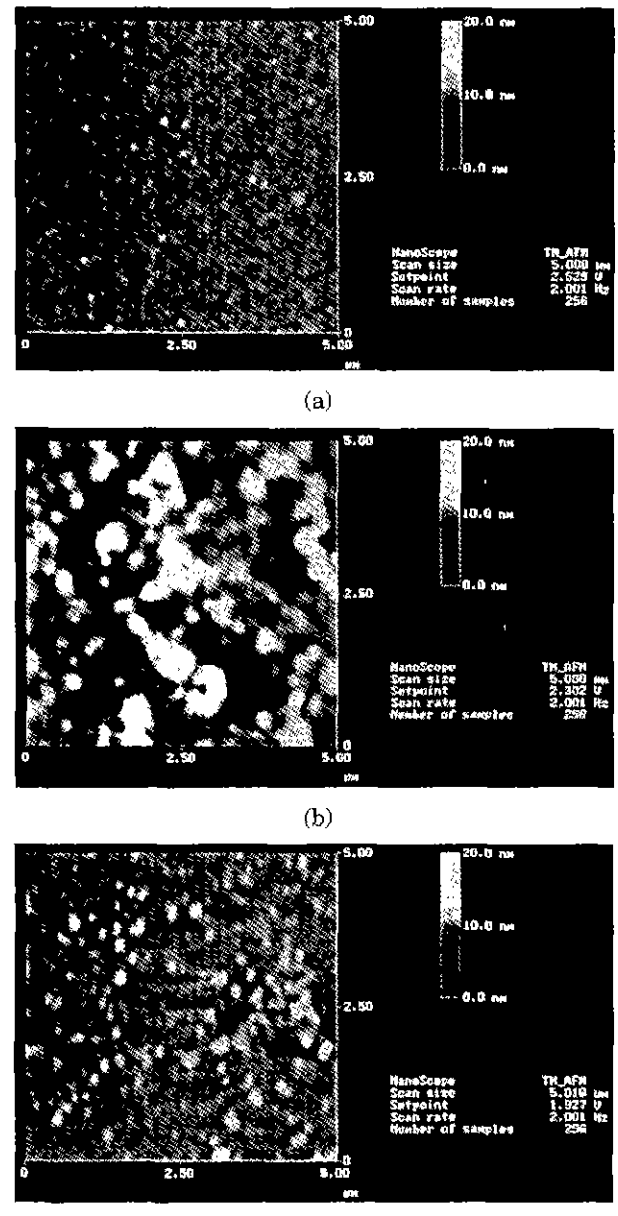
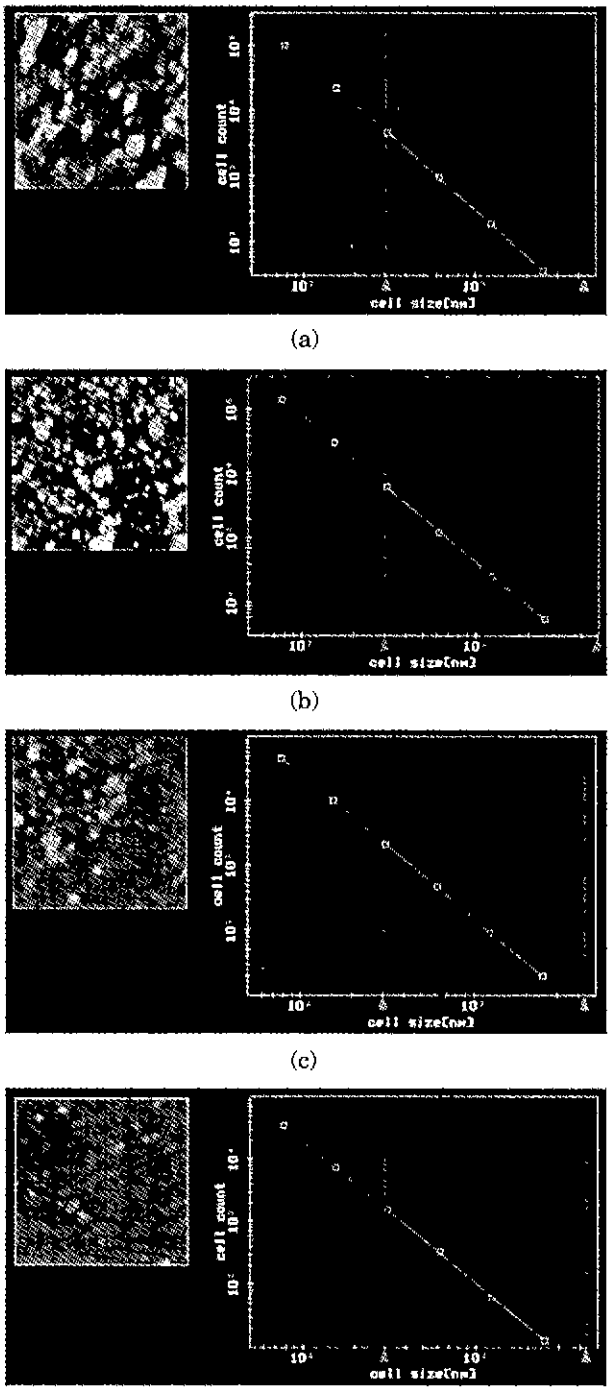


Fig. 3. The AFM images (256 × 256 pixels) were collected in air with the tapping mode using a commercial silicon tip. (a) is a image of Ox-BSA preincubated with the freshly cleaved mica at a concentration of 10 μg/mL in PBS during overnight. (b) shows a image that by using immunoliposome combined with Ox-BSA immobilized mica surface. (c) shows a image that (b) was treated by TX/Gly solution. The scan rate was about two lines per second. Low pass filtering was applied to reduce high frequency noise. All images are 5 μm by 5 μm areas.

Fig. 3 (c) shows an image of the mica surface after treatment with low-pH detergent solution (TX/Gly) to remove the immunoliposomes. Almost a total disappearance of immunoliposomes is obvious, and only some particles-like those present already in the antigen-treated surface (Fig. 3 (a)) with a diameter between 70 and 150 nm can be seen on the surface.



(a) (b) (c) (d)

Fig. 4. Images of fractal analysis on competitive bioaffinity sensing for Ox-CA. The fractal dimension is defined as the slope of the line obtained by plotting the log of the cell size versus the log of the cell count. Cell Z max-min is 30 nm and scan size is 5 μm by 5 μm areas. (a) 120 μm Ox-CA and immunoliposome solution, (b) 15 μm Ox-CA, (c) 3 μm Ox-CA and (d) 0.5 μm Ox-CA.

AFM Imaging of Immunoliposomes and Free Hapten Interaction on the Antigen-immobilized Solid Surface

To prove that the firm binding of immunoliposomes to the antigen-coated mica surface is due to specific antibody-antigen interaction, we incubated the immunoliposome-reacted antigen-coated mica surface with free hapten, Ox-CA, followed by a rinse with HEPES buffer (pH 7.4) and drying of the sample. This treatment resulted in the removal of immunoliposomes in a concentration-dependent manner, as indicated by fractal and grain anaylsis. The fractal command of AFM manual evaluates a surface geometric complexity. The fractal dimension is defined as the slope of the line obtained by plotting the log of the cell size versus the log of the cell count. For three-dimensional surfaces, the fractal value varies from a minimum of 2.00 for a flat surface to a maximum of 3.00 for an extremely rough surface. It matters not what Z height is used in scaling cell dimensions, as long as images being compared use the same Z height. Similarly, varying the scan size will give different fractal values; therefore, it is crucial that parameters used in computing fractals (cell Z max-min and scan size) be held constant whenever comparing two or more samples. In this study, cell Z max-min is 5, 30, 200 nm and scans size is 5 μm by 5 μm areas. The Gain Size analysis defined grain boundaries based on the height and slope of the data. Grains are defined in terms of conjoined scan lines having defined min. and max. grain size above a certain threshold height. It shows that binding of immunoliposomes to the hapten-coated mica surface was inhibited specifically by the pre-incubation with Ox-CA in a concentration-dependent manner. The concentration range from 10^{-4} to 10^{-7} M of Ox-CA, the competitive adsorption of immunoliposomes on the Ox-BSA immobilized mica surface was imaged by AFM as shown in Fig. 4 (a)-(d) and Table 1.

Table 1. AFM analysis data of grain size and fractal dimension

(1) Grain size analysis

OX-CA concentration (M)	5×10^{-7}	3×10^{-6}	1.5×10^{-5}	1.2×10^{-4}
Threshold Height (nm)	8.116	8.043	7.826	6.957

(2) Fractal dimension

Cell Z max-min (nm)		OX-CA concentration (M)			
		5×10^{-7}	3×10^{-6}	1.5×10^{-5}	1.2×10^{-4}
Fractal dimension	5	2.359	2.281	2.302	2.300
	30	2.334	2.267	2.259	2.306
	200	2.273	2.223	2.411	2.330
Cell count at 300 nm cell size	5	4×10^{4}	3×10^{4}	1×10^{4}	8×10^{3}
	30	5×10^{3}	6×10^{3}	2×10^{3}	1×10^{3}
	200	1×10^{3}	1×10^{3}	5×10^{2}	14×10^{2}

DISCUSSION

Several factors in the sample preparation influence the quality of AFM images: the affinity of the imaged molecules for the support, the concentration and amount of the deposited solution, and the length of incubation time allowed before the surface is rinsed to get rid of unbound molecules [15]. Freshly cleaved mica produces a surface well-suited for high resolution

AFM. It is known, however, that proteins do not adhere well to mica and that the anchoring of proteins to withstand the force of the AFM is a major hurdle for high resolution imaging [16]. Fig. 2 shows that by using the hexahistidine tag of the immunoliposome combined with nickel treatment of the mica surface stable adherence and orientation of the liposome was possible.

The AFM generates an image by providing the surface of the sample with a sharp tip attached to the end of a flexible cantilever. Small forces of interaction between the sample and the tip produce a deflection of the cantilever that is detected and converted into height information [17]. By plotting the heights for each position of the tip over the sample, the topography of the sample can be reconstructed. For future application on the AFM as an imaging tool, higher structural resolution will probably result from instrumental improvements such as operation at much lower forces combined with sharper tips, and from further improvements in sample preparation techniques. The resolution capability of the AFM is directly related to the tip geometry, since surface profiling is always with a stylus of finite sharpness. The first tips were fabricated in a batch process using Si micromachining techniques, based on Si_3N_4 with a pyramidal shape with an effective tip diameter of approx. 50 nm at the apex [18]. Using electron beam induced deposition (EBD) of vapor contaminants in a vacuum chamber of a conventional scanning electron microscope, tips became available with an apical diameter 10 nm [19]. A single carbon nanotube attached to the pyramidal tip of a silicon cantilever is able to resolve atomic scale resolution of AFM [20]. The tip can be also used as a force-sensing and manipulation tool, provided that the tip-sample interaction can be controlled appropriately.

CONCLUSION

The purpose of the present study was to improve the current methods for the attachment of liposomes to a solid surface in order to enhance the quality of AFM images of liposomal and other soft samples. Specifically, liposomes were adsorbed on mica surface by using two different specific molecular interactions. Immunoliposomes consisting of lipid-tagged single-chain antibody have been prepared and successfully imaged by AFM. Images of these immunoliposomes on a nickel treated mica substrate and antigen immobilized mica surface were obtained. In AFM images, immunoliposomes bound specifically to immobilized antigen on a mica surface, and this binding was inhibited by soluble hapten in a concentration-dependent manner. By anchoring the immunoliposomes under non-denaturing conditions it should retain its biological activity. Anchored, immobilized, active immunoliposomes should provide a system well suited for the examination of real time antigen-antibody interactions by direct visualization by AFM.

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