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ABSTRACT

In this paper, we present a universal microfluidic liquid chamber device platform for atomic force microscopy (AFM), which enables to fabricate the uniform lipid bilayer on the hydrophilic surface using the solvent-assisted lipid bilayer formation method. Using this device enables us to acquire the various properties of delicate soft matter, including morphological data, and mechanical property measurements, using high-resolution AFM systems. The proposed technology is expected to provide an understanding of complicated biological materials.

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Supported lipid bilayer (SLB) has been used as a simplified mimic model of an artificial membrane platform since it comprises two-lipid leaflet structure same as the biological membrane.^{1–3} Although the atomic force microscopy (AFM) has shown great promise in the successful characterization of high-resolution imaging capabilities with biological samples in liquid condition, performing the measurement of SLB was a challenge due to the lack of the appropriate liquid chamber system.^{4–7} Conventional AFM measurement chambers do not facilitate solution-exchange within the flow cell, thereby limiting utility for biological measurements.

The solvent-assisted lipid bilayer (SALB) formation method^{8–12} is not available in the conventional AFM chamber because there is no proper chamber system to support the solvent exchange process. In addition, the conventional chamber does not allow *in situ* multitask operations: flowing the solution with a constant flow rate in microliter level, exchanging the solvent using the peristaltic pump with tubes, and availabilities of opening and closing of the top cover multiple times for the AFM measurement in different experimental conditions. In other words, the ordinary chamber cannot apply the precision flow control by a pump with tubes, enabling the measurement of the dynamic processes of functional biological molecules.

Especially, controlling the flow speed takes a significant role in the formation of the uniform SLBs.^{2,12–14} The vesicle fusion, which is the conventional method for lipid bilayer formation, has shown the shortcomings using the open-ended petri dish or commercial liquid cell chamber with a large volume. Unfortunately, due to the lack of robust fluidic chamber, the results without controlling the flow rate have often created defects in SLBs that cause the complexity of the data analysis because of its signal instability, making it difficult to extract useful information of planar bilayer.¹⁵

Furthermore, conventional liquid cells are typically one time usable for filling the channel area with the solvent hence the solvent-exchange step cannot be performed. Moreover, there is often a limitation for designing a microfluidic chamber system with the conventional AFM because the reducing volume of the device hinders the approach of the AFM tip to the substrate for imaging [Figs. 1(a) and 1(b)]. For example, the channel dimension of the conventional microfluidic chamber for fluorescence microscope is 6 mm × 45 mm with 0.1 mm height [Fig. 1(c)]. However, it cannot be opened after assembling the coverslip to the chamber; as a result, the AFM tip cannot approach the surface from the top. In addition, expanding the volume of the channel area of the chamber causes generation of an air bubble that interferes with the high-resolution image

FIG. 1. (a) Schematic of the bilayer structure comprising two lipid leaflets with the polar head groups facing outward and the hydrocarbon chains facing inward; the AFM tip is able to approach from the top to take measurements of the bilayer. (b) The conventional method for AFM using coverslip inside of the petri dish. (c) Disposable micro-fluidic chamber for a fluorescence microscope. (d) Three-dimensional drawing of each part of the newly designed fluidic chamber for AFM. (e) Top and (f) side views of the assembled chamber. (g) Final product of the microfluidic chamber for AFM.

quality because it could be an obstacle between AFM tip and soft samples.

In order to overcome these disadvantages of the conventional liquid chamber for the AFM system, we developed the microfluidic-based liquid cell chamber that enables the constant flow in the reservoir area without the interference of AFM measurements. The designed chamber facilitates to investigate the layer of molecules because of its perfectly sealing structure and reusability for multiple solvent exchange processes without leakage problem. We also investigated the AFM imaging of the SLBs on the bare glass substrate which contains different concentrations of cholesterol (20% and 40%). In addition, to compare the mechanical properties in each condition, we demonstrated the force-distance (f-d) curves with the proposed fluidic chamber for AFM.

The advantages of building a microfluidic chamber are that it can be modular, uses less solvent volume, forms a uniform bilayer repeatedly, and is able to be used in combination with the AFM system. For the preliminary experiments, the microfluidic chamber device for AFM can satisfy the perfect sealing condition, and it was manufactured as shown in Figs. 1(d)–1(g). The top-cover of the chamber is tightly bound to the chamber part with bolts to prevent the solvent leakage. Two O-rings made of rubber with different sizes (20 mm and 25 mm) absorb the pressure to the coverslip from the bottom cover, and they act as a sealant for the channel area where the actual solvent is placed. The outer dimension of the chamber part is 35 mm of diameter and 6 mm of the thickness, which can be mounted on the XY stage of the AFM system. The design can be easily tweaked to fit the sample stage with different dimensions for other AFM models. The entire chamber consists of six parts [Fig. 1(d)] and made from stainless except the top-cover. The top-cover is made from transparent acrylic material for observing

the solvent flow during the SALB process. The inner dimensions of the flow chamber are $9 \times 9 \times 0.5 \text{ mm}^3$, and the SALB flow rate is $0.83 \text{ mm}^3/\text{s}$. For these parameters, there is full SALB formation, which is supported by a theoretical analysis, presented in the [supplementary material](#).

The detailed experiment methods are described in the [supplementary material](#). The morphological and mechanical properties of the bilayer were recorded using the newly designed microfluidic chamber for AFM. The AFM images and force-distance (f-d) curves in the bare glass substrate and the bilayers using SALB technique are shown in Fig. 2. The bilayer formation was also confirmed by measuring the thickness of the square area after scratching (Fig. S2). Additionally, we investigated the bilayer properties of mixed lipid systems. To differentiate and test the bilayer-forming capability of the microfluidic chamber for AFM, DOPC-cholesterol

FIG. 2. Comparison of AFM images of the bare glass substrate in liquid condition (a) and the bilayer with different concentrations of cholesterol [(b) pure DOPC lipid bilayer, (c) 20% of cholesterol, and (d) 40% of cholesterol]. (e) f-d curve on the bare glass substrate, (f) on the pure DOPC bilayer, (g) with 20% of cholesterol, and (h) with 40% of cholesterol. The bilayer formation was performed via SALB method. From the images, the domain area has increased as the cholesterol concentration is higher (0%, 20%, and 40%).

systems were tested. Cholesterol is known to alter the membrane organization in many lipid systems,^{8,16} at high cholesterol concentrations, the lipid bilayer attains the in-homogenous condition and then it forms micrometer-sized domains. The study of cholesterol-induced domains has been of great interest because of their roles in biological processes,^{8,10,17} particularly in re-organizing membranes and regulating membrane trafficking.^{17,18} Apart from the biological implications, we believe that cholesterol-lipid systems are ideal for AFM studies as the size and width of the domains are significantly different from those in the fluid phase lipid bilayer. In other words, cholesterol-lipid systems can serve as an ideal sample for the morphologic studies that are required to test the proposed microfluidic chamber for AFM. Two compositional studies were performed on bilayers with varying cholesterol concentrations. To test the performance of the AFM microfluidic chamber, the properties of the bilayers formed by SALB technique with 0%, 20%, and 40% of the cholesterol in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were compared. At this concentration, domains (rafts and detergent-resistant membranes) were clearly visible [Figs. 2(c) and 2(d)]. Indeed, domains are typically formed in the presence of sphingomyelin and cholesterol.^{3,19} From the results of the f-d curves, the different peak values in each case with 0%, 20%, and 40% of cholesterol are also shown in Figs. 2(f)–2(h). In the case of the bare glass substrate, the f-d curve does not show the peak on it [Fig. 2(e)] which means that it does not have a specific interaction between the tip and surface which is different from the case of bilayers.²⁰ In general, bilayer formation is not reliable or reproducible under high cholesterol concentrations using a vesicle fusion method because the small unilamellar vesicles (SUVs) often do not rupture on the substrate.²¹ By contrast, surprisingly, the SALB method resulted in the formation of a uniform bilayer with domains under this high cholesterol concentration [Fig. 2(d)]. The roughness values of the bilayers formed with different concentrations of cholesterol by SALB complement each other (Fig. S3); however, the higher cholesterol concentrations led to higher roughness due to the formation of domains. These results clearly indicate that the AFM equipped with the proposed microfluidic chamber designed based on the SALB process can provide a facile tool to form uniform bilayers on substrates even in the presence of high concentrations of cholesterol.

In this study, the optimized microfluidic liquid cell chamber was designed based on the solvent-assisted lipid bilayer (SALB) process for AFM imaging. The bilayer AFM images were taken after formation by the SALB method at the different concentrations of cholesterol. Bilayers could be formed successfully in both concentrations with 20% and 40% cholesterol. Additionally, the mechanical properties of the cholesterol-enriched bilayer have been investigated using the force-distance curves. We believe that the proposed AFM-compatible microfluidic device can be used to conduct detailed morphological and mechanical studies and extract

other parameters, such as diffusivity and viscosity of soft biological systems. This system can provide a complete platform to extract many useful parameters for handling fragile soft matter and understanding complicated biological membrane systems.

See [supplementary material](#) for the experimental, bilayer depth measured by the square test and histograms of the roughness values measured in the bilayer.

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