Dynamic Light Scattering Measurements for Soft Materials on Solid Substrates: Employing Evanescent-wave Illumination and Dark-field Collection with a High Numerical Aperture Microscope Objective

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We develop an instrument that allows us to measure dynamic light scattering from soft materials on solid substrates by avoiding strong background due to the reflection light from substrates. In the instrument, samples on substrates are illuminated by evanescent-light field and the resultant scattered light from the samples is collected with a dark-field optical configuration by employing a high numerical aperture microscope objective. We apply the instrument to measure the dynamic properties of supported lipid bilayers (SLBs), which have been widely utilized in industries as functional materials such as biosensors. From the time course of the scattered light from the SLBs, the power spectrum with the broad peak ranging from 10 kHz to 20 kHz is observed. The use of the microscope objectives enables us to apply the instrument to future light scattering imaging for dynamic properties of soft materials supported on various substrates by combining with conventional microscope systems.

**Keywords:** dynamic light scattering, evanescent wave, dark-field, microscope objective, soft material
Introduction

Dynamic light scattering (DLS) is a useful method for the studies on the dynamic properties of soft materials, such as polymer gels, liquid crystals, colloids, and lipid assemblies.$^{1-7}$ Since it probes molecular relaxations that occur in the wide time region ranging from $10^{-3}$ s down to $10^{-6}$ s, DLS enables us to extract the information on the multi-scale dynamic properties derived from the fluctuations from collective (e.g., entangled polymer networks) to individual (e.g., single polymer chains) motions.$^{1,3,5-7}$

For the last two decades, soft materials supported on solid substrates, such as polymer brushes and supported lipid membranes (SLBs), are increasingly important in the development of organic transistors, implantable materials such as artificial joints, and sensor devices for the microanalysis of gases, cells, and blood components.$^{8-12}$ The static properties of soft materials supported on solid substrates, such as domain structure, thickness, and molecular conformations, have been widely investigated using atomic force microscope, sum frequency generation spectroscopy, and X-ray or neutron scattering methods.$^{13-15}$ On the other hand, the dynamic properties of soft materials supported on substrates, such as diffusion behavior and collective fluctuation, are also important for the expression of their functions, such as re-construction of
self-assembling structures according to environmental changes, matter transports both in lateral and normal directions, and interactive and repulsive interactions in molecular recognitions. These dynamic properties and resultant functions have been expected to be applied to various devices such as biosensors. Thus, the measurements on the dynamic properties of soft materials supported on solid substrates, especially on various types of substrates, have been required for the evaluation of the functions.

However, few studies on the dynamic properties of soft materials supported on solid substrates with DLS have been reported, although there are many DLS measurements on the dynamic properties of soft materials in bulk, such as hydrogels and free-standing black lipid membranes. In contrast to soft materials in bulk, in the application of DLS to soft materials supported on solid substrates, the intensity of the reflected light from substrates is extremely high, which hinders the detection of the small changes in the intensity of scattered light derived from the fluctuations of the soft materials at the interfaces. In addition, in the supported soft materials, their inhomogeneous domains are expected to be formed on substrates depending on the compositions and concentrations of material components. Thus, measurements on the dynamical properties of the supported soft materials at arbitrary points are
also desired.

Here, we developed an instrument that allowed us to measure DLS from soft materials supported on solid substrates with the avoidance of strong background from the reflection of substrates at arbitrary points. The schematic depiction of the instrument is shown in Figure 1. In the instrument, samples on substrates were illuminated by evanescent-light field and the resultant scattered light was collected with a dark-field optical configuration by employing a high numerical aperture (N.A.) microscope objective. The illumination and the collection with the same microscope objective allowed for the measurements of the supported soft materials at arbitrary points with combining conventional microscope systems. We applied the instrument to the measurements on the dynamic properties of SLBs, which have been widely utilized in industries as functional materials such as sensors, bioinspired interfaces, and organic transistors.\textsuperscript{10,12,20,21} The bilayers composed of cationic lipids were formed on glass substrates with the vesicle fusion method.\textsuperscript{22} The time courses of the signals from the SLBs were successfully measured in spite of the strong hindrance from the reflectance of the substrates. From the power spectra obtained by the Fourier transformation of the time courses, the dynamic properties of the SLBs were investigated.
Experimental

Optical setup of the instrument for the DLS measurements of soft materials supported on solid substrates

The optical setup of the instrument is illustrated in Figure 2(left). The polarization direction of a laser beam (He-Ne laser, 632.8 nm, 35 mW, Melles Griot Ind.) for illumination was set to p-polarization with a gran laser prism. After the beam was reflected with a polarizing beam splitter, it transmitted a quarter-wave plate. The transmitted beam was irradiated onto a SLB with a total reflection configuration with a microscope objective (Olympus, TIRFM, 60×, N. A. = 1.45). The incident angle was calculated to be ca. 72°. The diameter of the incident beam was estimated to be ca. 100 μm at the focal plane from a CCD image acquired with a microscope objective (Olympus, PlanFL, 10×, N. A. = 0.3) (Fig. 2(right)). The backscattered signals from a SLB were collected with the same objective for the illumination. The signals through two apertures (both diameters were the same: 1 mm) apart in tandem configuration were detected with a photomultiplier tube (PMT) (H10722-20, Hamamatsu Photonics Ind.). The time course of the signals was recorded with 1 μs of time resolution by a data logger.
(MR6000, HIOKI). It was Fourier-transformed with the conventional software (Igor Pro 8, WaveMetrics) to obtain the corresponding power spectrum. All DLS measurements were performed at room temperature (25 °C).

Materials

Dimethyldioctylammonium bromide (DOAB) (> 97.0%) (Fig. 3(a)), dimethylditetradecylammonium bromide (DTDAB) (> 97.0%) (Fig. 3(b)), and dimethyldioctadecylammonium bromide (DODAB) (> 98.0%) (Fig. 3(c)) were purchased from Tokyo Chemical Industry Co., Ltd and were used without further purification. H$_2$SO$_4$ (95%, Wako Chemicals) and H$_2$O$_2$ (30%, Wako Chemicals) were also used without further purification.

Ultrapure water, produced from the Millipore ELIX system, was used for all sample preparation.

Preparation of a SLB on a glass substrate with the vesicle fusion method

For the formation of vesicles, 1 mM of lipid aqueous solution was prepared. The solution was stirred for 1 hours under the temperature above the gel-to-liquid crystalline phase transition temperature of each lipid (DOAB: < 10 °C, DTDAB: 25–27 °C, DODAB: 42–45 °C). After its dissolution, the solution was slowly cooled until room temperature
(25 °C). For the removal of organic substances on a glass substrate, a piranha solution (3 :1 = 98 % H$_2$SO$_4$ : 30 % H$_2$O$_2$ (v/v)) was deposited onto a cover glass (Matsunami Glass Ind.) and the glass left to stand for 30 minutes (Fig. 4(a)). The 100 µL of vesicle dispersion solution was deposited onto the glass (Fig. 4(b)). After still standing for 30 minutes for the formation of a SLB, the glass was washed three times with 100 µL of water for the removal of remaining vesicles in the solution (Fig. 4(c)). The vesicle solution-deposited glass was installed on a glass bottom dish (Matsunami Glass Ind.) (Fig. 4(d)).

Results and Discussion

We investigated the dependence of the DLS from a SLB on the length of alkyl chains of lipids by using the developed instrument. In the principle of DLS, light is scattered by the inhomogeneity of refractive index in bodies and the temporal changes in the inhomogeneity. In our experimental conditions, the density fluctuation of both the lipid bilayer and water (solvent) on a SLB should give DLS. To reduce the contribution from the density fluctuation of the water on the SLB to DLS, we adopted the total reflection illumination configuration. For the estimation of the contribution of the water to DLS, we measured the time course of the
DLS derived only from the water at the same conditions as a reference (Fig. 5(a)). Owing to the total reflection illumination configuration, the contribution from the water was remarkably reduced.

Figures 5(b)–(d) show the time courses of DLS derived from each SLB and the corresponding power spectrum, respectively. For DOAB, the signal amplitude in the time course and the spectrum were similar to those for only the water (Fig. 5(b)). For DTDAB, the two patterns of time courses were observed for the different SLBs (Fig. 5(c, left)). One is that the signal amplitude was slightly larger than that for only the water (Fig. 5(c, left, black line)). The other is that the amplitude was enough larger compared with that for only the water (Fig. 5(c, left, gray line)). In the latter case, however, the peak position was not clearly discriminated in the corresponding spectrum (Fig. 5(c, right, gray line)). To the contrast, for DODAB, the signal amplitude was large as with that for DTDAB (Fig. 5(d, left)) and the corresponding spectrum clearly showed the broad peak ranging from \( \text{ca.} 10 \text{ kHz to } 20 \text{ kHz} \) (Fig. 5(d, right)).

As the increase in the length of alkyl chains, the peak for the power spectrum just started to be observed for DTDAB (Fig. 5(c, right, gray line)), then the peak became clearer for
DODAB (Fig. 5(d, right)). In addition, the frequency region (ca. 10 kHz to 20 kHz) of the observed peak for DODAB was similar to that for a free standing black membrane of phospholipids doped with DODAB observed by DLS.\textsuperscript{17} Although the kind of lipids was different, the DLS for several types of free standing black lipid membranes was characterized by the frequency ranging from several kHz to several 10 kHz.\textsuperscript{1,24} It has been reported that those frequencies were derived from the collective motion of the black membranes.\textsuperscript{1,17,24} We assumed that the observed peak originated from the collective motion of DODAB bilayer on the glass substrate. It was expected that the longer alkyl chains of SLBs were able to maintain their bilayer structure through stronger hydrophobic interaction between lateral packing of the alkyl chains and to surpass the interaction between their head groups and the surface of substrates. To the contrast, from the small amplitude in the time course for DOAB, it was expected that DOABs did not form bilayer structure or the bilayer did not fluctuate due to the strong electrostatic interaction between its head group and the surface of the substrate. For DTDAB, the observation of the two signal patterns might be due to the formation of different phase states in the bilayer. The gel-to-liquid crystalline phase transition temperature for DTDAB molecule is 25–27 °C.\textsuperscript{23} Because the temperature for the DLS measurements (25 °C)
was close to the phase transition temperature, we expected that the phase states of the DTDAB bilayer might be different due to slight difference in surrounding temperature. Based on these results, we suggested that the maintenance of lipid bilayer structure for DODAB allowed its collective fluctuation due to strong lateral packing of alkyl chains even on the solid substrate, leading to the appearance of the characteristic peak to the collective fluctuation (Fig. 5(d)).

The power spectrum for DODAB also contained the fluctuation modes over higher frequency regions (Fig. 5(d, right)). In the application of DLS techniques to the fluctuation measurements of black lipid membranes, the fluctuation modes have been analyzed from correlation functions of signals.\textsuperscript{1,17,24} However, the analysis is based on hydrodynamic theory and is applicable to systems such as black lipid membranes, where surface tension and bending elasticity act as restoring forces. On the other hand, in SLBs on solid substrates, not only these forces but also the complicated interactions between the membranes and the adjacent substrates should be taken in account for additional restoring forces and also for frictional ones. To our knowledge, the relational theoretical treatment including these interactions to the frequencies of the collective fluctuations in SLBs has not been reported yet in spite of its relevance. Further development of analytical methods for the fluctuation modes on SLBs is now under
The obtained results suggested that SLBs with the strong hydrophobic interaction between long alkyl chains collectively fluctuated in the similar order in frequency with those of free standing black lipid membranes. This suggestion might be in contrast to a general assumption that SLBs do not fluctuate due to their strong interaction to the substrates. Our measurements on DOAB, DTDAB, and DODAB systems might cast a doubt on whether such assumption always holds true or not. X-ray or neutron scattering methods have been also applied to measure dynamic properties of soft materials supported on solid substrates and they yield fruitful information. These methods have advantage especially for probing local structural fluctuation derived from the segmental motions of polymer chains or lipids. To the contrast, DLS has advantage for the measurements on the dynamic properties in wider temporal range including collective motion of soft materials. This nature will largely contribute to the study on the dynamic properties of the soft materials interacting with their substrates and to the development of functional materials and devices such biosensors with SLBs. In a living system, the dynamic properties of biological membranes supported by three-dimensional protein networks play a crucial role for the functional expression, such as membrane fusion and
molecular recognition.\textsuperscript{28,29} The present results also suggested the applicability of the developed instrument to the studies of model lipid bilayers for the examination of both spatially and temporally complex of cell systems, where the composition of biological membranes is dynamically changed and the resultant changes in their dynamic properties should take an important role for emerging various cell activities.

Summary

A DLS instrument for measuring weak signals from soft materials supported on solid substrates at arbitrary points was developed. To reduce strong background, a high N.A. microscope objective was applied to achieve evanescent-field illumination and dark-field signal collection at the same time. The instrument was applied to measure the DLS signals from SLBs of DOAB, DTDAB and DODAB. The characteristic broad peak ranging from \textit{ca.} 10 kHz to 20 kHz was observed for DODAB system and its assignment was suggested to the collective fluctuation of the membrane. Combination of the analytical method to conventional microscope systems will allow us to measure DLS of soft materials on solid substrates and will provide a useful tool for studying biological systems and developing various industrial materials and devices.
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Figure captions

Fig. 1  Schematic depiction for the evanescent-wave illumination of a SLB and the dark-field collection of resultant DLS with a high N.A. objective (not to scale).

Fig. 2  (Left) Optical setup of the developed instrument for the DLS measurements of soft materials supported on solid substrates. (Right) CCD image of the incident beam at the focal plane.

Fig. 3  Chemical structures of (a) DOAB, (b) DTDAB, and (c) DODAB.

Fig. 4  Schematic depiction for the preparation of a SLB on a glass substrate.  (a) Cleaning of a cover glass with a piranha solution (3 : 1 = 98 % H₂SO₄ : 30 % H₂O₂ (v/v)) for 30 minutes.  (b) Deposition of the 100 µL vesicle dispersion solution onto the glass.  (c) Still standing for 30 minutes for the formation of a SLB, followed by the wash with 100 µL water three times for the removal of remaining vesicles.  (d) Installation of the SLB-formed glass on a glass bottom dish.

Fig. 5  (Left) Time courses of the scattered light generated from (a) the water and from each sample prepared from (b) DOAB, (c) DTDAB, and (d) DODAB.  The time courses were ones after treated with a high pass filter with 500 Hz of cut-off frequency installed into IGOR Pro 8.  (Right) Corresponding power spectra for the time courses.
Fig. 1
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