Nanoparticle Arrays in Freely Suspended Vitrified Films

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We introduce a new methodology to vitrify thin, freely suspended liquid films containing a single layer of nanometer sized particles. The procedure relies on the precise optical control of the stepwise film thinning (stratification) process. We illustrate the applicability of the method to three different types of particles. Stratifying films from surfactant solutions were found to contain nonordered layers of micelles. The experiments with larger particles (lipid or protein vesicles and latex spheres) showed that by controlling the liquid film one can produce well ordered particle arrays.

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Cryoelectron microscopy of thin vitrified films of particulate suspensions offers a variety of unique advantages for high resolution structural analysis of samples, which are unstable when dried [1]. Vitrification ensures the excellent structure preservation of delicate hydrated molecules and molecular complexes. In addition, the radiation damage is reduced by working at low temperature [2] while adverse effects on spatial resolution and on molecular conformation are avoided by dispensing with staining and fixation reagents. During the last decade, the cryomicroscopy has yielded valuable structural information for membrane proteins [3,4], viruses [5], protein complexes [6], lipid vesicles [7,8], microemulsions [9], and micelles [10].

Two procedures for the formation of vitrified films are widely used. In the first, a suspension film is spread on a hydrophilic carbon layer deposited on an electron microscope grid [1,3], while in the second "bare grid method" [5], a film is formed without any support. A disadvantage of both techniques is that they employ liquid films of micrometer diameter. This precludes the explicit control and direct optical observation of film formation and thinning.

We present here a novel method for the controlled preparation of freely suspended films of large diameter ($\approx$100 μm) whose thickness is precisely measured by direct video microscopic observation. It allows vitrification at an exactly known moment of the film thinning process, as well as ordered array production from a variety of particles.

Freely suspended liquid films are formed in the central hole of a disk-shaped holder (60 μm thick, 3 mm in diameter)—see Fig. 1. This hole is connected by a narrow channel with the periphery of the holder. During film formation and subsequent thinning, a thin glass capillary is introduced into the outer end of this channel. The capillary is connected to a microsyringe to allow for the precise control of the amount of liquid suspended in the holder, which in turn determines the radius of the film. When observed in reflected monochromatic light (λ = 600 nm) by means of a long-focus magnifying lens attached to a charge coupled device (CCD) video camera, a planar film appears as a circular area whose brightness strongly depends on film thickness (Fig. 2). The following equation is used for accurate calculation of the film thickness from the intensity of the reflected light [11–13]:

$$h = \left(\frac{\lambda}{2\pi n}\right) \arcsin\left\{\frac{\Delta}{1 + [4R/(1 - R)^2](1 - \Delta)}\right\}^{1/2} \quad (1)$$

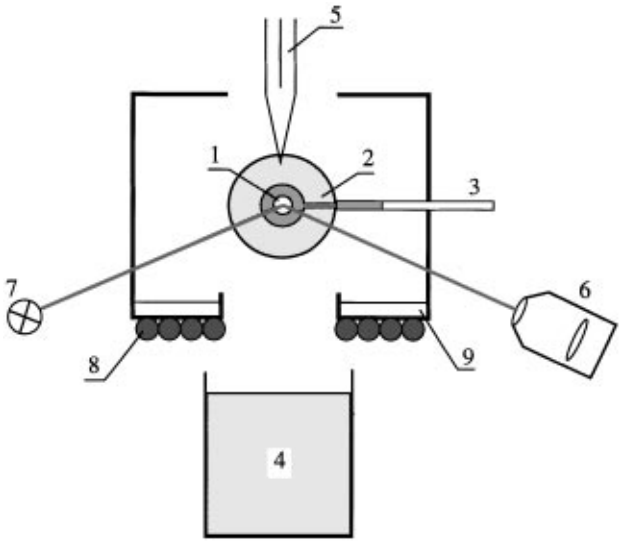

FIG. 1. Scheme of the experimental setup. A liquid film (the thinnest planar portion of the suspension layer) (1) is formed in a disk-shaped film holder (2) by gentle suction of liquid through a glass capillary (3) that is connected to a microsyringe. When a film of required radius and thickness is obtained, it is plunged for vitrification into a cooling liquid (4) by means of a rapidly falling guillotine (5). The liquid film is monitored by a CCD camera (6) in reflected monochromatic light (7). To control the water evaporation from the film we have to adjust the relative humidity of the air inside the experimental cell. For that purpose a heater (8) and a water pool (9) are used.

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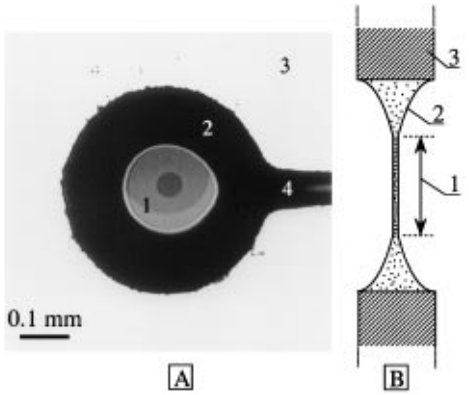

FIG. 2. (a) Photograph of a film that is undergoing stratification (stepwise thinning). The film was made from a micellar solution of SDS ($0.1M$). In reflected light the film (1) is seen as a bright circular area (in the center) surrounded by a very dark meniscus region (2). The central part of the film holder (3) and a portion of the channel (4), connecting the meniscus with the glass capillary, are also seen. Three regions of different thickness are discernible in the film: the slightly darker circular spot in the film center shows a thickness $h \approx 27$ nm, the region around this spot shows $h \approx 36$ nm, and the brighter band at the bottom of the film shows $h \approx 46$ nm. (b) Schematic cross section of the central part of the film holder (3) with the meniscus (2) and film (1) regions.

for $h \leq \lambda/4n$, where h is the film thickness, λ is the wavelength of light, n is the refractive index of the film substance, $R = (n - 1)^2/(n + 1)^2$, and $\Delta = (I - I_{\min})/(I_{\max} - I_{\min})$. Here I is the instantaneous value of the light intensity, reflected by the film in the process of thinning, while $I_{\max}$ corresponds to the last interference maximum and $I_{\min}$ is the background intensity. A major advantage of this method is that it requires only relative values of the light intensity. These are readily extracted by image analysis of the video record of the film thinning process by using an image statistics computer program. The accuracy of the film thickness measurement is around ± 1 nm in the range between 10 and 95 nm, and is thus superior to thickness determination of the frozen film in the electron microscope [14].

When the liquid film attains its desired radius and thickness, the capillary is detached from the film holder and the film is plunged into a cooling liquid (1:1 mixture of ethane and propane, $-190\,^{\circ}$C) for vitrification. The frozen film is transferred into the electron microscope for observation at low temperature by means of CT3500 cryotransfer stage (Oxford, UK). The micrographs were taken using a JEM-1200 EX JEOL transmission electron microscope (120 kV) at $-190\,^{\circ}$C and total electron dose less than $200e/\text{nm}^2$.

We have applied our method of vitrification to a variety of samples including viruses, large protein molecules, lipid vesicles, and micelles, some of which have been previously examined with the bare grid method. In addition to the precise film thickness monitoring, the method has several new features which we now describe in three specific illustrative applications.

An important feature of our film preparation procedure is the ability to produce well ordered arrays of particles within vitrified films without recourse to specific binding agents and supporting substrates. We have recently demonstrated [15] that thin liquid films on solid substrates may serve as a two-dimensional (2D) matrix for the formation of large ordered arrays of polystyrene latex spheres under the action of nonspecific interactions of long range. This procedure requires a fine control of the film formation process and this idea was utilized here to generate vitrified films containing ordered arrays of nanometer sized latex particles and of vesicles made of bacteriorhodopsin.

Figure 3 displays an electron micrograph of a vitrified film containing a well ordered monolayer of latex spheres (144 nm in diameter). At the periphery of the monolayer region ordered bilayers and triple layers are observed (not shown in Fig. 3). In fact, a lower rate of film expansion generally favors the formation of bilayer and triple-layer regions at the expense of the monolayer. Similar results were obtained with smaller latex spheres (66 nm).

Remarkably, our method is readily applied to form ordered arrays of "soft" particles. The micrograph in Fig. 4 shows a well ordered vitrified monolayer of vesicles formed from the membrane protein bacteriorhodopsin and lipid [16,17]. Such ordered arrays are produced only at an appropriate film thickness (slightly larger than the vesicle diameter) and precise control of the film expansion process. The mean diameter measured from digitized images is 38.8 nm with small polydispersity (standard deviation ± 2.2 nm). The mean center-to-center distance is 44.7 nm. While some features in the vesicle structure are discernible in the micrographs, we initiated more systematic structural analysis. As far as we know, this is the first observation of ordered array of vesicular type particles in vitrified films.

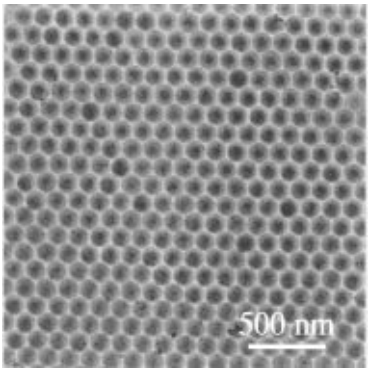

FIG. 3. Ordered monolayer of polystyrene latex spheres (144 nm in diameter) in a vitrified film of thickness close to the particle diameter. In this case the film thickness cannot be precisely measured, because it is too large and Eq. (1) is not applicable.

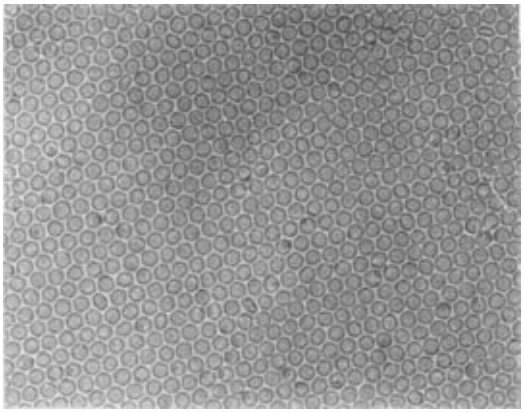

FIG. 4. Ordered monolayer of bacteriorhodopsin vesicles in a vitrified film. The liquid film thickness was 59 nm just before vitrification.

The third system we studied was the thinning film of surfactant micellar solutions. It has been known for many years [18] that in a certain range of concentrations liquid films prepared from micellar solutions thin in a stepwise manner—see Fig. 2. This "stratification" phenomenon has no explanation in the framework of the classical Derjaguin-Landau-Verwey-Overbeek theory [19]. Several alternative explanations have been proposed which differ in the assumed surfactant arrangement within the film. Some of them presume that the film contains more or less ordered layers of spherical micelles [12,20,21], while others accept that continuous surfactant bilayers are formed in the neighborhood of the film surfaces, along with the two adsorbed monolayers [13,22]—see Fig. 5. The stepwise film thinning is then attributed to the consecutive decrease in the number of these (micellar or continuous) layers in the film interior.

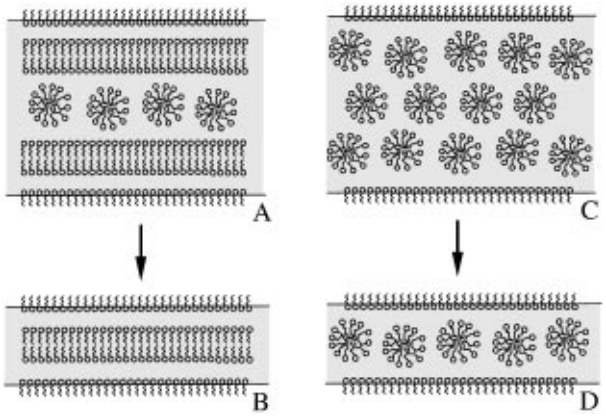

FIG. 5. Two alternative hypotheses for the stratification of films made from micellar solutions are discussed in the literature. In the first explanation [13,22], the film contains at least one continuous surfactant bilayer (along with the adsorbed monolayer) close to each of the film surfaces (a), and the last step in the process corresponds to one continuous bilayer (b). According to the second hypothesis [12,20,21], the film contains more or less ordered layers of spherical micelles (c), and the last step corresponds to one layer of micelles (d).

Cryoelectron microscopy permits the direct visualization of micelles and surfactant bilayers and it is particularly suitable to address this issue. Our method of film preparation offers one important advantage compared to previous cryomethods: We directly observe the stratification and are able to determine exactly at what "step" of the film evolution vitrification occurs, which is important because the surfactant structure inside the film could depend on the film thickness (Fig. 5).

To study the film stratification phenomenon, we performed experiments with solutions of the surfactant sodium dodecyl sulfate (SDS) at concentrations $0.03M$ and $0.1M$. In this concentration range SDS forms spherical micelles of diameter 4.8 nm in bulk solution, each containing about 67 surfactant molecules [23]. We took electron micrographs of vitrified films of thicknesses 36 nm ($0.03M$ SDS) and 27 nm ($0.1M$ SDS). These thicknesses correspond to the last step of thinning [12,13] when presumably either one layer of micelles, or one continuous bilayer, is present inside the film—see Figs. 5(b) and 5(d). The micrographs revealed small particles, around 5 nm in diameter, which apparently are SDS micelles confined within the film. In some parts of the film poorly ordered "chains" and clusters of micelles can be discerned, but there is no long-range ordering. The average distance between the micelles in the film determined from micrographs (more precisely, this is the projection of the intermicellar distance on the film plane) appears slightly smaller than the average intermicellar distance in the bulk, which is calculated from the surfactant concentration and aggregation number. The fact that the thinnest films contain micelles (similar in size to those in the bulk solution) shows that no bilayers are present in the neighborhood of the film surfaces in this concentration range. These observations support previous models [20,21] of stratifying films, suggesting that their stability originates in the oscillatory structural forces created by the micelles. Obviously, for other surfactants and conditions the stratification could have a different origin and our method presents an appropriate tool for studying such systems.

In conclusion, the method for preparation of vitrified films we have developed offers advantages which make it promising for application in several areas. In colloid science the method becomes fascinating with the investigation of the mutual relationship between film stratification, thickness, and structure for systems composed of colloid particles (micelles, microemulsions, vesicles, protein molecules, etc.) or of liquid-crystal phases. The large size and precisely known thickness of the films make them suitable for the investigation of the physical-chemical properties of vitrified water (and aqueous solutions), such as its electron scattering cross section, surface tension, and viscosity. Finally, our method provides the scientist with

the advantage to select the most appropriate film thickness for vitrification (best contrast and absence of "flattening" effect) and to produce ordered crystalline or paracrystalline arrays of soft particles (viruses, vesicles, etc.) which could be important for better structural analysis.

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