# Bovine serum albumin film as a template for controlled nanopancake and nanobubble formation: In situ atomic force microscopy and nanolithography study

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## ABSTRACT

Air nanobubbles and nanopancakes were investigated in situ by both tapping mode atomic force microscopy (TM AFM) and atomic force nanolithography techniques employing bovine serum albumin (BSA) film supported by highly oriented pyrolytic graphite (HOPG). The BSA denaturation induced by the water-to-ethanol exchange served for conservation of nanobubble and nanopancake sites appearing as imprints in BSA film left by gaseous cavities formerly present on the interface in the aqueous environment. Once the BSA film was gently removed by the nanoshaving technique applied in ethanol, a clean basal plane HOPG area with well-defined dimensions was regenerated. The subsequent reverse ethanol-to-water exchange led to the re-formation of nanopancakes specifically at the nanoshaved area. Our approach paves the way for the study of gaseous nanostructures with defined dimensions, formed at solid–liquid interface under controlled conditions.

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## 1. Introduction

Nanobubbles are sub-microscopic gas cavities usually detected on solid/liquid interfaces [1]. Theoretical calculations [2] predict that nanobubbles should be short-living due to an immense capillary pressure, which forces the trapped gas to dissolve in a surrounding liquid according to Henry's law. Nevertheless, stable nanobubbles were proven to exist at interfaces [3–5]. Employing the surface force apparatus, Parker et al. [6] were the first to report on the existence of sub-microscopic bubbles in 1994. The authors observed steps and discontinuities in the long-range attraction forces measured between two neutral hydrophobic surfaces in water and attributed them to the presence of nanobubbles.

The invention of scanning probe techniques, particularly the tapping-mode atomic force microscopy (TM AFM), allowed the interfacial nanobubbles to be observed directly. In 2000, Lou et al.

[7] and Ishida et al. [8] independently reported on the existence of nanobubbles by the TM AFM technique. Six years later, Ishida and Higashitani [9] showed that the long-range attractive forces are indeed related to the presence of interfacial nanobubbles.

The nanobubbles were detected on hydrophobic surfaces only when a polar liquid phase (pure water or aqueous electrolytes) was saturated by the air [1]. Therefore, the observed nanostructures appear to be cavities filled by ambient gas and solvent vapor respectively, not interfacial impurities. Direct immersion of a substrate into a liquid is the most straightforward way of the nanobubble formation. Lou et al. [10,11] later developed the solvent exchange protocol leading to reproducible appearance of nanobubble-containing interfaces. In it, the surface is first immersed into a liquid that well dissolves the air. Ethanol is routinely employed. Subsequently, ethanol is replaced by miscible liquid exhibiting lower air solubility, such as water. Gradual mixing of ethanol and water causes a part of the air dissolved in ethanol to "precipitate" leading to the formation of nanobubbles that can be trapped and subsequently detected at solid/water interface. When water is replaced back by ethanol, the interfacial nanobubbles dissolve and disappear.

Zhang et al. [12,13] investigated the liquid degassing and temperature effects on the nanobubble occurrence, employing mica

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and highly oriented pyrolytic graphite (HOPG) as the solid surfaces. When air-saturated solvents (ethanol or methanol and water) were used, the nanobubbles were observed with the surface concentration depending on the temperature. No nanobubbles were reported to appear with the solvents degassed. In a related study [14], the TM AFM technique was employed to determine the contact angle of nanobubbles on HOPG and silicon wafers in water. The obtained values, measured through the gaseous phase, were considerably smaller than those found for macroscopic bubbles at the same interfaces. This suggests that the real nanobubble radius is much larger than expected. According to Young–Laplace equation, an increase in the nanobubble radius reduces the capillary pressure and contributes to its interfacial stability. By the aid of infrared spectroscopy, Zhang et al. [15] provided an elegant proof that the inner space of nanobubbles is composed of molecules in the gaseous state rather than of dissolved ones ($CO_2$ gas was chosen due to its IR activity).

Recently, the flat gaseous structures called nanopancakes were observed and described on HOPG [16–18]. Besides nanobubbles and nanopancakes, the solvent exchange technique was applied for the preparation of nanostructures composed of an immiscible liquid such as oil. These structures were called nanodroplets [19,20].

Detail studies focusing on the interaction of nanobubbles with solid/liquid interfaces and nanostructures present on them are very rare. Janda et al. [21] employed in situ TM AFM, transmission electron microscopy as well as Raman spectroscopy to demonstrate that the air nanobubbles induce the exfoliation of the top graphene sheets from the HOPG surface. Wu et al. investigated how the air nanobubbles influence the bovine serum albumin (BSA) adsorption on mica [22] and HOPG [23]. The interfaces with embedded nanobubbles formed by the solvent exchange were exposed to protein solutions. When aqueous environment was replaced by ethanol, the nanobubbles vanished. In a related study [24], the electrochemically generated hydrogen nanobubbles were employed to clean the HOPG surface covered by the BSA film.

Though the studies dealing with the interactions between the nanobubbles and proteins exist [22,23], there is, to the best of our knowledge, no work focusing on the interactions between nanopancakes and proteins. Here, we show that interfacial nanopancakes suppress the protein adsorption and cause the presence of vacancies in the protein films. Therefore, they considerably decrease the protein surface coverage. We suggest that the nanopancakes should be (together with the nanobubbles) added to the list of factors that significantly affect the nanomorphology of interfacial protein layers. When present in large quantities, the nanopancakes may severely reduce the amount of adsorbed proteins, which may cause errors in the surface-based biochemistry assays. As a model protein, BSA was employed due to its ability to adsorb on various surfaces and interfaces, including HOPG [25–33].

As mentioned above, the AFM imaging was already employed to investigate the structure of interfacial nanobubbles and nanopancakes [7–18], Besides the imaging, we further employ the techniques of atomic force nanolithography [34–37], namely the nanoshaving and nanografting. Nanoshaving is a technique, in which an interfacial film is intentionally removed ("nanoshaved") by an AFM probe. Nanografting resembles the nanoshaving, but takes place in a solution of adsorbable species different from that already adsorbed at the interface. The newly exposed substrate is refilled by the species from the solution bulk, forming thus a two-component film with morphology dictated by the shape of the nanoshaved area. Though both nanoshaving and nanografting techniques have already been applied to investigate and pattern the structure of self-assembled monolayers [38–49], there is no work using these advanced and smart techniques in the research of gaseous nanocavities.

The scientists reporting on the existence of nanopancakes so far described them as spontaneously formed nanostructures [16–18]. On the contrary, our approach allows their intentional formation. We are the first to show that nanoshaving and nanografting techniques combined with the solvent exchange are able to form gaseous nanopancakes at defined surface locations and with prescribed shapes and dimensions.

In future, this technique might be employed in the fabrication of well-defined gaseous arrays. We believe that the ability of controlling the gas nanocavity dimensions will help to gain a deeper knowledge about their formation, stability and properties, which may have serious implications in heterogeneous catalysis at solid/liquid interfaces as well as in the studies of transmembrane gas exchange processes in general.

## 2. Materials and methods

Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (fraction V, >96%). Absolute ethanol (99.8%) was obtained from AppliChem, Darmstadt, Germany. Deionized water with a minimum resistivity of 18 M$\Omega$ cm was obtained by means of a Milli-Q RG purification system (Millipore Co., USA). The water solution of BSA (40 ppm) was prepared by dissolving solid BSA in deionized air-saturated water. Highly oriented pyrolytic graphite (HOPG, Structure Probe Inc., USA) was cleaned by ethanol, dried and cleaved by the scotch tape immediately prior to use.

The gaseous nanostructures formed on the HOPG/water interface were inspected by the in situ tapping mode AFM (TM AFM) technique. The interface was then exposed to 40 ppm water solution of BSA for 30 min [23]. The BSA solution was subsequently removed by extensive rinsing with pure water and the formed interfacial structures were re-inspected by in situ TM AFM. Afterwards, the aqueous environment was replaced by ethanol and the interface was investigated by TM AFM imaging and was further modified by atomic force nanolithography techniques (nanoshaving and nanografting).

All AFM measurements were carried out in situ employing Agilent 5500 SPM (Agilent Technologies) equipped by a flow liquid cell (Agilent Technologies). The probes "Type II MAClevers" (Agilent Technologies) were employed, with the nominal resonant frequency $f_N$ = 75 kHz (in the air, reference range 45–115 kHz) and nominal force constant $k_N$ = 2.8 N/m (reference range 0.5–9.5 N/m). The actual force constant $k$ was determined by measuring the resonant frequency $f$ and employing the cubic interpolation in the $k$ vs. $f$ dependence.

All AFM images were obtained by the TM AFM technique. The probes were oscillated close to the resonant frequency by an external oscillating magnetic field.

The relative probe amplitude $A_r$ (the ratio of the set-point amplitude $A$ maintained when imaging and the amplitude of the freely vibrating probe retracted from the interface $A_0$) is related to the pressure that is exerted by the probe onto the sample. For the relative amplitude, we employed the value 95% throughout the measurements. Besides the topography image, the phase shift between the driving magnetic field and the resulting cantilever oscillation was recorded. The negative changes in the phase shift value are related to the retardation of the probe, which is caused by pronounced interactions between the probe and interfacial nanostructures.

The AFM nanoshaving was performed in a constant repulsive force contact mode AFM (CM AFM). Prior to each experiment, the force spectroscopy measurement was performed in situ to determine the exact loading force applied. The sensitivity $S$ of a given laser/cantilever configuration, defined as the ratio of the laser beam deflection $D$ (in volts) and the cantilever vertical position $z$ (in meters), was determined as a slope in the repulsive part of