Phage Langmuir-Blodgett Films for Biosensing Applications

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***Abstract*—The microstructure and functionality of methicillin-resistant *Staphylococcus aureus* (MRSA) biosensors prepared from novel Langmuir-Blodgett monolayers of lytic bacteriophage were characterized using scanning imaging ellipsometry (SIE) and scanning electron microscopy (SEM). SEM revealed uniform distribution of phage monolayers immobilized to biosensor substrates. SIE indicated monolayers were 49.8 ± 18.3 nm thick on average. SIE charged couple imaging analysis of biosensors yielded an average intensity of 159±7 and 194±13 for 10^8 and 10^9 CFU/ml MRSA concentrations, respectively.**

I. Introduction

The ability to transfer homogeneous, well-controlled nanoscale Langmuir films of affinity bioreceptors from an air-water interface to a wide variety of substrates such as gold, mica and glass makes the Langmuir-Blodgett (LB) technique highly suitable for nanotechnology applications such as biosensors [1,2]. The functionality of bioreceptors as capture agents can be directly related to the experimental aspects of film preparation and transference; subphase pH, bioreceptor molecular orientation and spacing, and substrate properties [3,4] are all critical considerations. Thus, nanoscale characterization is an important consideration to understanding and optimizing self-assembly strategies for LB-based biosensors.

Scanning Imaging Ellipsometry (SIE) and Scanning Electron Microscopy (SEM) are two optical techniques that can help advance the design and fabrication of LB-based biological sensors. These methods afford non-invasive characterization of sufficiency and adequacy of transference of phage monolayers to substrates, including film thickness analysis, 3D mapping and monolayer surface imaging and visual examination, in comparison to other techniques such as atomic force microscopy, scanning tunneling microscopy, scanning force microscopy and x-ray diffraction. High-lateral resolution SIE promotes non-destructive quality control analysis during sensor fabrication and expedient biosensing using charged couple device (CCD) imaging or ellipsometry, where changes in color intensity profile or thin-film thickness profile are proportional to the amount of bound target analyte, respectively.

We prepared and characterized stable, insoluble Langmuir films of methicillin–resistant *Staphylococcus aureus* (MRSA) bacteriophage by surface pressure-area (Π-A) isotherms then immobilized monolayer films by LB technique onto gold plated substrates to prepare MRSA biosensors and analyzed them by SEM and SIE.

II. Experimental

A. Biological and Chemical Components

Bacteriophage possessing lytic activity against type strain *S. aureus* (ATCC 12600) and other *S. aureus* strains was used as a bioreceptor for substrates [5,6]. 1,2-diphytanoyl-sn-glycero-3-phosphocholine ($C_{48}H_{96}NO_8P$) (phospholipid) in chloroform (1 mg/ml) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Stearic acid ($C_{18}H_{36}O_2$) powder (99% purity) was obtained from Alfa Aesar (Ward Hill, MA) and prepared as a solution (1 mg/ml) by dissolving into anhydrous hexane (Sigma Aldrich, St. Louis, MO).

B. Substrates

Silica wafers (WaferNet Inc., San Jose, CA) were immersed in a piranha solution [3:1 NH_4OH:H_2O_2)] for 20 minutes at 55°C then rinsed in deionized water and air-dried [7]. Clean wafers were coated with a ~225 nm thick gold layer over a ~50 nm thick chromium adhesive interlayer using a Denton™ (Moorestown, NJ) high-vacuum RF sputter coater. The gold-plated substrates were cut into 20 mm × 10 mm strips and stored in a desiccator until use then rinsed in hexane prior to Langmuir film deposition.

C. Surface Pressure-Area Isotherms and Langmuir Film Deposition to Substrates

The KSV 2200 LB film balance, experimental setup, and general procedures for preparing monolayers have been thoroughly described [8]. Stearic acid and phospholipid were

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used as standards for isotherm analysis in comparison to phage. Phage monolayers were formed by spreading 200 µl phage (2.94×10^{12} plaque forming units/ml) in suspension onto the subphase via a partially immersed glass rod. Similarly, phospholipid monolayers were prepared with 30 µl of phospholipid suspension and stearic acid monolayers were prepared with 20 µl of stearic acid suspension. The film was equilibrated for 20 minutes then isotherm analysis was conducted by compressing the monolayer over the defined area of the trough at constant rate (30 mm/min) and temperature (30 mm/min, 20 ± 0.1°C) while surface pressure measurements were recorded. Isotherms of each material (phage, phospholipid or stearic acid) provided the optimal pressure in mN/m for deposition to substrates.

Optical biosensors were prepared by compressing phage, phospholipid or stearic acid films to optimally determined surface pressures of 18, 17 and 47 mN/m, respectively, then vertically dipping clean substrates up and down through the film monolayer at a rate of 4.5 mm/min [9].

D. Monolayer and Biosensor Characterization

LB monolayers on substrates were evaluated with a JEOL-7000F SEM (JEOL USA Inc., Peabody, MA). Samples were air-dried at ambient temperature then mounted onto aluminum stubs with copper adhesive tape (Ted Pella Inc., Redding, CA) and sputter-coated. Micrographs were taken using an accelerating voltage of 5 kV and probe current of 54 µA. Images were captured electronically using JEOL-Imaging software. No biological staining agents were utilized.

LB monolayers on substrates were also analyzed using an auto-nulling SIE (EP^3, Nanofilm Technologies, Göttingen, Germany) (Fig. 1a). Thickness measurements were taken in PCSA (Polarizer, Compensator, Sample, Analyzer) configuration: solid state laser source (λ = 532 nm), a xenon arc lamp with 46 interference filters (λ = 300 to 1000 nm), and 10× objective with 2 µm spatial resolution and 60° angle of incidence to the substrate surface's perpendicular axis. All measurements were taken at room temperature (25 ± 2°C) using air as the ambient medium.

Computerized optical modeling software (EP^3 View) allowed analysis of thickness and refractive index data for immobilized monolayers on substrates (Fig. 1b). Estimated values were assigned to unknown parameters (such as layer thickness) for the purpose of preliminary calculations. These free parameters were varied until a minimum mean squared error was achieved, which was used to verify the consistency of measured and simulated data.

An ellipsometric null condition was induced by detecting minimum intensity at the CCD camera while changing P, C and A component settings. Component angles of P, C and A settings at the null condition were determined by the thin film's optical properties. Biosensors were evaluated using the SIE's CCD camera capability.

Quantitative phage deposition studies were verified using a Quartz Crystal Microbalance (QCM) [10].

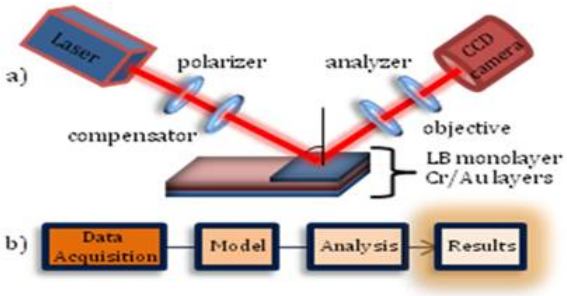

Fig. 1. (a) Schematic of spectroscopic imaging ellipsometer and (b) flow chart for data analysis.

III. Results and Discussion

A. Monolayer Isotherms

Stable Langmuir films of *S. aureus*-specific lytic phage, phospholipid or stearic acid were prepared at an air–water interface. Isotherms were measured by gradually compressing the prepared surface monolayer film at constant rate and temperature into a defined area (LB trough) while monitoring changes in surface pressure. The stearic acid monolayer (Fig. 2a-1) exhibited a moderate gaseous phase of <2 mN/m, requiring only 25% of compression before reaching a liquid-expanded state. The phospholipid monolayer (Fig. 2a-2) also exhibited a very short gaseous phase of <2 mN/m, requiring about 10% compression. These isotherm results principally agree with the literature [11-13] although the stearic acid's monolayer zero pressure (Table 1) was slightly larger than that of published results [14] was and attributed to subphase composition. The phage monolayer (Fig. 2b) was characterized by a long gaseous phase of <10 mN/m, requiring about 60% compression before a liquid-expanded state was reached, and exhibited a shoulder at ~7×10^4 nm^2/phage particles.

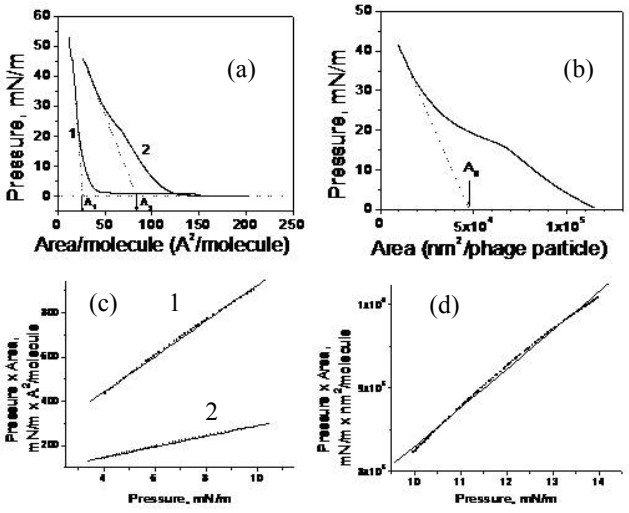

Fig 2. Π-A isotherms: (a) stearic acid (1) and phospholipid (2). The estimated zero-pressure areas for stearic acid and phospholipid (A1and A2) were equal to 23 ± 1.0 and 81 ± 2 A2/molecule, respectively. (b) Phage monolayer. The estimated zero-pressure area for phage (A0) was 47 ± 2×103 nm2/phage particle. Volmer presentation of isotherms: (c) stearic acid (1) and phospholipid (2). Points are experimental data, lines are linear

regressions (1: R +0.999, p<0.0001, slope = 24.1 ± 0.2 A^2/molecule; 2: R +0.999, p<0.0001, slope = 80.6 ± 0.4 A^2/molecule). (d) phage monolayer. Points are experimental data, line is a linear regression (1: R +0.998, p<0.0001, slope = 46.4 ± 0.26 ×10^3 nm^2/molecule).

The specific areas were estimated by extrapolating "zero-pressure" areas [14] and a two-dimensional modification of the van der Waals equation of state [15-17]:

$$(\Pi + a/A^2)(A - A_e) = kT \quad (1)$$

where A represents a specific molecular area, a is intermolecular interaction term, A_e is an excluded specific area, k is the Boltzmann constant, Π is the surface pressure, and T is the absolute temperature. At small interactions and large specific areas, (1) can be reformed as a Volmer-type equation [18-20]:

$$\Pi A = kT + A_e\Pi \quad (2)$$

Equation (2) can be represented as a linear plot of the left-side expression versus the surface pressure and yields an estimate of A_e. The excluded area is derived from the slope of the line and represents the area around a single phage inaccessible to other phage due to structural hindrances.

Calculated excluded molecular areas for stearic acid (24.1 ± 0.2 A^2/molecule), phospholipid (80.6 ± 0.4 A^2/molecule) (Fig. 2c) and phage monolayers 46.4 ± 0.26 × 10^3, nm^2/molecule) (Fig. 2d) concur well with observed zero pressure data from isotherms and literature (Table I), indicating that when lytic phage was deposited to the water-air interface, a stable monolayer film was formed. A single phage capsid volume is ~6000 times larger than that of a phospholipid molecule yet the phage monolayer produced a stable, reproducible isotherm that withstood surface pressures up to ~40 mN/m. This is both novel and unexpected.

Π-A isotherm data was further analyzed to determine the compressibility modulus (elasticity) of films, which describes compactness and film stability (Fig. 3). Taken together, isotherm and elasticity analysis indicated that constant surface pressures of 18, 17, and 47 mN/m were optimal for transference of phage, phospholipid and stearic acid, respectively, to substrates.

TABLE I. MOLECULAR AREA OF MONOLAYERS

Monolayer	Zero pressure area	Excluded area	Literature data
Stearic acid	23±1.0 A^2/molecule	24.1±0.2 A^2/molecule	21 A^2/molecule [10,11]
Phospholipid	81±2 A^2/molecule	80.6±0.4 A^2/molecule	81 A^2/molecule [12]
Phage	4.7±0.2×10^4 nm^2/phage particle	4.6±0.26×10^4 nm^2/phage particle	Not available

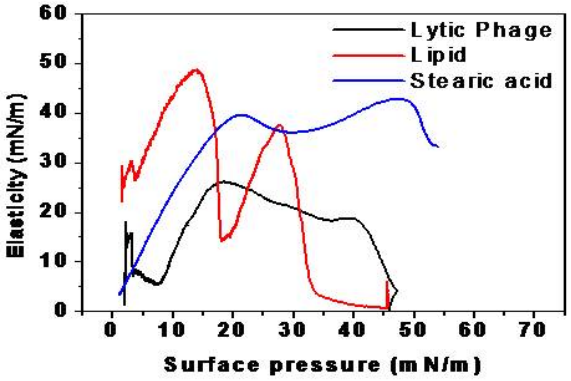

Fig. 3. Elasticity of lytic phage, lipid and stearic acid monolayers as a function of surface pressure at 20 ± 0.1°C.

B. Modeling of Phage at the Air-Water Interface

Isotherm analysis yields insights to phage positioning at the air-water interface under differing conditions of pressure (Fig. 4). At low concentrations, phage are widely-spaced and behave as a gaseous phase and most likely assume a horizontal orientation similar to that of tobacco mosaic virus particles [21,22]. However, as the film is compressed, phage become densely packed and space is reduced at the plane of the air-water interface, resulting in many reorienting to an inclined or strictly vertical position, capsid-down and tail-up. Phage tail orientation in the film is critical since it is responsible for capturing the target (MRSA) in the virus's natural infective process.

At the liquid expanded phase, the monolayer consists of a mixture of horizontal and inclined phages, which coincides with the shoulder observed in the Π-A isotherm (Fig 2b). Similar shoulders have been observed in other monolayers near the liquid-expanded/liquid-condensed phase transition at the air-water interface. It has been shown that this transition is accompanied by a reorientation of the molecules and that the different phases are separated by an inhomogeneous coexistence region on the isotherms [23,24].

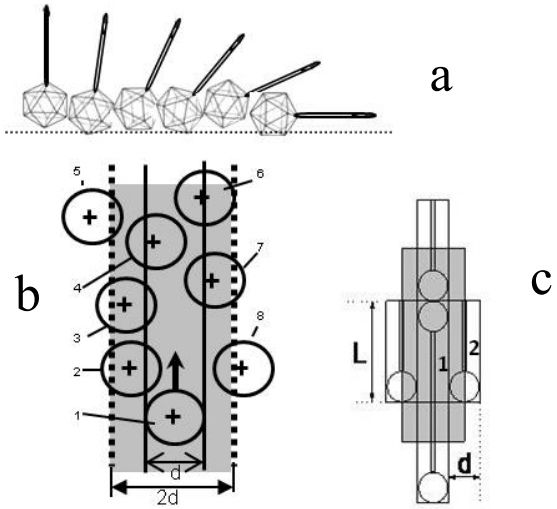

Fig. 4. Packing models of phage on the water/air interface. **(a)** One dimensional model of phage assuming that phages can change orientation from horizontal to oblique, and to vertical as surface pressure change from small to high (from right to left). **(b)** The sketch for excluded area of phage in vertical orientation. The phage heads are approximated by discs with diameter, *d*. The phage, 1, occupies an area $A=\pi d^2/4$. The excluded area is estimated by an area of interacted phage. When phage 1 is moving in direction indicated by an arrow, it will collide with phage (2, 3, 4, 6, and 7) whose centers (+) are inside the *2d* diameter circle. The excluded area (shaded region in Figs. 4b and 4c) equals πd^2. Two phage in close proximity can share the excluded area; therefore, the excluded area per phage equals half of the inaccessible area ($\pi d^2/2$). **(c)** The sketch for excluded area of phage in horizontal orientation. The central phage, 1, occupies area A=Ld, where L is a length of a whole phage.

C. SEM

SEM revealed nearly homogenous phospholipid (Fig. 5a) and stearic acid (Fig. 5b-1) monolayers on substrates with no visible discontinuities. In comparison, phage (Fig. 5c) had an estimated average (n = 10) adsorption of 8.84 ± 0.6 phage per square micron on substrates. This correlated well with quantitative QCM studies (Fig. 6) that showed an adsorption of 9.6 phage per square micron on gold plated resonators. Monolayers appeared as patches covering ~10% of substrates on average. The LB method increased substrate surface coverage 100% in comparison to phage immobilization through biotinylation [25]. The LB method is much more effective and efficient than simple physical adsorption (Fig. 5d) for immobilizing phage in the correct orientation to substrates [10].

SEM analysis indicated that phage possessed slightly oval heads measuring 90 ± 6 nm × 108 ± 7 (SE) nm and tails measuring 196 ± 8 (SE) nm in length and 21 ± 1 (SE) nm in diameter. The cross-sectional area of a phage through the middle of head and tail was equal to ~1.2 $\times 10^4$ nm^2. These dimensions agree well with those found for staphylococcal phage [26-28]. The average thickness of phage monolayer estimated from micrographs was equal to 39.0 ± 8.0 nm.

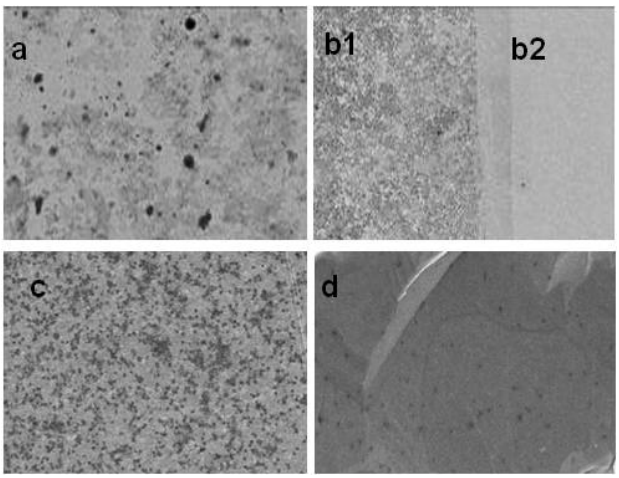

Fig. 5: SEM micrographs of a) phospolipid, b1) stearic acid and c) phage deposted to substrates by LB method in comparison to b2) clean substrate devoid of monolayer and (d) phage adsorbed to substrate by physical adsorption.

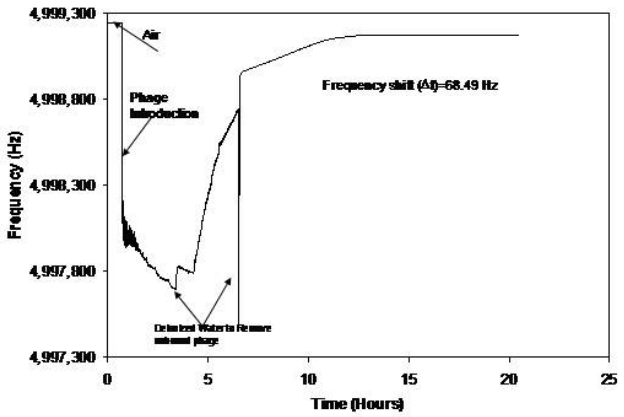

Fig. 6. Representative QCM graph depicting frequency change as a function of phage adsorption to gold-plated resonator over time. Application of phage in suspension to a clean, dry resonator at steady state: 4,999,242 ± 0.5 Hz. Unbound phage were removed by gently washing with deionized water followed by air-drying of resonator. Dried resonator with adsorbed phage at steady state: 4,999,174 ± 0.5 Hz. Frequency shift caused by phage (Δf) = −68 ± 0.5 Hz, which equates to frequency shift caused by $1.21*10^9$ bound phage particles or 9.6 phage per square micron.

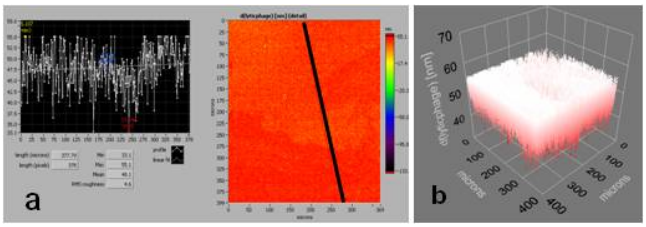

Fig. 7. (a) and (b) are an ellipsometric thickness profile and 3D thickness map of a lytic phage, respectively (effective refractive index = 1.05). Thickness profiles show mean, RMS roughness, minimum, and maximum thickness of the monolayer. The line across the thickness map was drawn to generate the thickness profile

D. SIE

3-D thickness profiles from delta mapped SIE images revealed average monolayer thicknesses of 49.8 ± 18.3 nm, 21.4 ± 2.1 A° and 18.5 ± 1.6 A° for phage (Fig. 7), phospholipid and stearic acid, respectively. Surface substrate profiles showed phospholipid and stearic acid monolayers oriented at 38.9 ± 2.1° and 40.3 ± 4.1° angles, respectively.

E. SIE-based biosensor for MRSA

Formation of a thin film due to bioreceptor (phage)-target (MRSA) interaction at the sensor surface altered the interference pattern of light and was analyzed by SIE either through the charge-coupled device (CCD) imaging (measuring color intensity differences) or ellipsometry (measuring thin film thickness). The changes in intensity profile or thickness profile was proportional to the amount of bound target analyte. Preliminary SIE CCD imaging analysis of post-assayed MRSA-phage substrate yielded an average relative intensity (arbitrary units) of 159 ± 7 (Fig. 8a) and 194 ± 13 (Fig. 8b) for MRSA concentrations of 10^8 and 10^9 CFU/ml, respectively. Visible differences in the bacterial coverage can also be seen in Figs. (8a and 8b-left) due to cell distribution as well as the

intensity profile with varying concentrations of MRSA. After optimizing each step the total assay time could be reduced to less than 10 minutes.

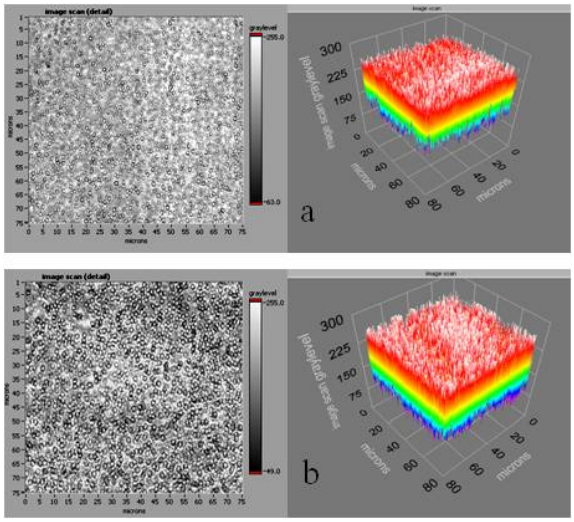

Fig. 8: Representative CCD camera image and 3D intensity profile of MRSA at concentrations of (a) 10^8 CFU/ml, and (b) 10^9 CFU/ml attached to phage bioreceptors immobilized on glass substrates.

IV. CONCLUSION

We demonstrated that stable, insoluble Langmuir monolayers could be formed at an air-water interface using lytic bacteriophage. The phage gradually orient themselves from a horizontal position parallel to the air-water interface to a vertical position with phage capsids immersed into the interface as surface pressure increases. Surface pressure-area dependence was found to be described well by the Volmer equation of state for horizontal orientation to the air-water interface at low surface pressures. Phage specific adsorption in Langmuir monolayers transferred to substrates and measured by SEM is consistent with that calculated independently from Π-A isotherms at the transfer surface pressure of 18 mN/m. Lytic phage, phospholipid and stearic acid monolayers were transferred onto solid gold coated substrates using the LB technique. These monolayers were characterized for quality and thickness with SEM and SIE. SEM showed phage monolayers occupying approximately 10% of substrate, where as stearic acid and phospholipid monolayers showed nearly 100% of area coverage of substrates. Prepared sensors were capable of binding MRSA at 10^8 -10^9 CFU.ml.

We conclude that SIE measurement and SEM micrographs have potential use in quality control measures when LB films are used in the preparation of nanotech devices such as biosensors for the detection of pathogenic bacteria.

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