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High Binding Density Coatings for Biomolecules on Plasmonic Gratings and Their Sensing Applications*

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***Abstract*— The COVID-19 pandemic has exposed a critical need for advances in high-throughput and high-resolution epidemiological testing devices with both high clinical sensitivity and specificity. Curbing the rapid spread of emerging diseases requires detection of biomarkers for disease at extremely sparse levels from body fluids. Sample preprocessing prior to detection is often used to increase the amount of biomarker in a patient sample to detectable limits, including polymerase chain reaction (PCR). Alternatively, the sensor might be designed to amplify intrinsic signal, improving the signal generated by sparsely distributed molecules without sample preprocessing. Identifying more stable and highly dense binding surfaces is critical to improving shelf-life, reducing binding protocol complexity, and expanding to a wider range of applications. Polystyrene well plates after exposure to gamma radiation are commonly used for high-density binding substrates. Herein, we present the development of a stable, ultra-thin polystyrene film on plasmonic gratings and demonstrate bioconjugation comparable to commercially available well plates without the need for surface activation (e.g., nuclear irradiation). Testing using Rhodamine Red X-labeled antibody bound to the plasmonic gratings showed a factor of 63.8 times brighter fluorescence signal than commercial Nunc MaxiSorp® plates using the same concentration of biomolecule.**

I. Introduction

Rapid detection of disease biomarkers from noninvasive body fluids (e.g., urine, saliva) is a critical need to advance epidemiological testing and reduce patient suffering worldwide. Biomarkers that might identify specific pathogens often exist in extremely low concentrations in these types of body fluids, the paucity requiring signal amplification prior to measurement. Numerous methods of sample preparation are used to amplify signal by increasing analyte concentration, including sample pre-concentration, polymerase chain reaction (PCR) to amplify pathogen DNA or RNA from a rarified sample, and more. Meanwhile, sensors that provide intrinsic signal amplification improve signal from sparsely distributed molecules, even down to the single molecule level, without the necessity to pre-concentrate or amplify the source sample. One method to enhance optical sensor signals is by utilizing the surface plasmon resonance (SPR) phenomenon between light and a metal-dielectric interface. SPR-based sensors have proven useful for identifying biomolecules at ultra-low levels. Mechanisms include direct measurement of the shift in SPR coupling angle after biomolecular binding as well as SPR-excited fluorescence and Surface Plasmon Coupled Emission (SPCE) of fluorophore-labeled biomolecules. Recently, we have developed a grating-based SPR biosensor capable of detecting single molecules of target bioanalytes of interest using an upright microscope in epifluorescence mode [1]. These have been used to enhance the signals of numerous biological systems, including Holliday Junction DNA hybrids, cancer cell imaging, and detection of tuberculosis disease biomarker lipoarabinomannan at the single molecule level. Surface modification of the alumina capping layer allows for a number of surface binding chemistries to be used, including the well-known carbodiimide crosslinker chemistry between 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) [2]. However, the plasma processing technique required to generate the necessary carbonyl groups for the EDC coupling step results in an unstable, highly reactive surface that degrades rapidly in ambient conditions, making it imperative to perform the binding assay as soon as possible after functionalization. Finding a more stable binding surface with a less complicated binding protocol would benefit this biosensor by increasing its shelf-life beyond the few hours plasma processing provides and expanding its utility to a wider range of users and applications.

II. Materials And Methods

A. Plasmonic Grating

Silver plasmonic gratings were prepared by soft lithography process similar to our previous work [1, 3]. Briefly, a silicone stamp was prepared by curing 5:1 ratio Sylgard® 184 polydimethylsiloxane (PDMS, Fisher Scientific) over a halved, cleaned HD-DVD for 24 hours at 50 °C and 55% relative humidity. Meanwhile, plain glass microscope slides (Fisherbrand, Fisher Scientific) were cleaned by a dilute soap solution and deionized water (18.2 MΩ-cm) followed by drying under flowing nitrogen. Cleaned slides were then soaked for 15 minutes in 3:1 H_2SO_4:H_2O_2 (Piranha solution), dip-washed twice in fresh deionized water, rinsed under copious flowing deionized water, and dried under flowing nitrogen. Cured PDMS was cut into 1"×1" square slabs, the grating side was spin-coated with 3% w/w GR650F polymethylsilsequioxane (PMSSQ, Techneglas Inc.) in ethanol, and the PMSSQ ink pressed onto the cleaned glass

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slide. After removing the stamp, the nascent gratings were then vapor-treated with 1:1 3-aminopropyltriethoxysilane (APTES) in ethanol, pre-baked at 60 °C for 3 hours, and baked at 400 °C for 1 hour. An AJA RF Magnetron sputter system was used to deposit 100 nm of silver on the gratings. Finally, a 10 nm alumina layer was deposited by low-temperature (65 °C) atomic layer deposition (ALD) using trimethylaluminum (TMA) and water.

B. Fabrication of Thin Polystyrene Coating

Several silanes were purchased from Gelest and used without further modification: Trichloromethylsilane (TMCS), Phenyltriethoxysilane (P silane), or N-Phenylaminopropyltrimethoxysilane (N silane). As-prepared gratings were exposed to 7 W CO_2 plasma for 30 s to activate the surface, then dip-coated immediately in a 0.1%, 1%, 2% or 5% v/v silane solution in toluene for 5 minutes. Silanized gratings were washed in successive baths of fresh 1) toluene, 2) ethanol, and 3) ethanol for one minute apiece. The gratings were then rinsed in copious 2-propanol, dried under flowing nitrogen, and heated at 120 °C for 10 minutes. Chevron MC3700 polystyrene pellets (Seaview Plastic Recycling, Bridgeport, CT) were dispersed in toluene to 10 mg/mL and sonicated to generate a stock solution. This was further diluted to 1 mg/mL and spin-coated onto the gratings at 3000 rpm for 30 s and vacuum annealed at room temperature for 1 hour [4].

Polystyrene films were characterized by a battery of thin film techniques. Thickness and optical constants were measured by variable angle spectroscopic ellipsometry (VASE, J. A. Woollam) and topography and thickness confirmed where possible by atomic force microscopy (AFM) and optical profilometry. Chemical composition of as-prepared polystyrene layer was compared with commercial plates by FTIR. A tape test for adhesion was performed on a set of 50 nm polystyrene films cast onto either silanized or unsilanized ALD alumina on glass by firmly pressing Scotch® tape on the film and rapidly removing the tape in one even, swift stroke. Failure was indicated using optical profilometry to show the step created by removal of the polystyrene film.

C. Comparative Bioassay

Bioconjugation using polystyrene-capped gratings was compared with as-prepared alumina-coated structure and with two commercially available 96-well polystyrene microplates: Nunc Maxisorp® and Dynex. Immediately prior to use, the as-prepared alumina-coated gratings were exposed to 7 W CO_2 plasma for 30 s to activate the surface [1]. Then, a 24-well slide adapter from Grace BioLab was attached to each of the grating slides with plasma-treated as-prepared grating or with polystyrene-capped multi-layer grating with P or N silane and polystyrene thin film. For the as-prepared grating, a solution of 4 mg/mL EDC and 11 mg/mL Sulfo-NHS was made in 2-(N-morpholino)ethanesulfonic acid (MES, pH 6) buffer and 100 µL of this solution was aliquoted to each well and incubated at room temperature for 15 minutes. Then, antibody labeled with Rhodamine Red X (RRX) fluorescent dye (Ab-RRX, Jackson ImmunoResearch) was diluted to 2 µg/mL in MES buffer pH 8 and 100 µL added to the wells (final concentration and volume were 1 µg/mL and 200 µl, respectively) [1]. For polystyrene surfaced substrates, Ab-RRX was diluted to 1 µg/mL in carbonate buffer pH 8 and 200 µL added to each well so that the final volume and Ab-RRX concentration was aliquoted to each substrate. All samples were stored at 4 °C overnight, brought to room temperature for 30 minutes, and washed in triplicate with 1 M phosphate buffered saline with 0.1% Tween-20 (PBST) and gentle agitation on an orbital shaker. The final wash buffer was replaced with 200 µL 1 M phosphate buffered saline and the samples were measured. All samples were measured using a BioTek H4 Synergy microplate reader in fluorescence mode using 555 nm (± 10 nm) and 600 nm (± 10 nm) monochromator bands for excitation and emission, respectively. Height above the well and gain were optimized to enable measurements at the same gain level across all samples without saturating the detector or achieving zero signal. The final adjusted detector height above the well was 6 mm for the Dynex and Nunc plates and 1 mm for the well-adapted gratings. The gain was held at 135 for all samples.

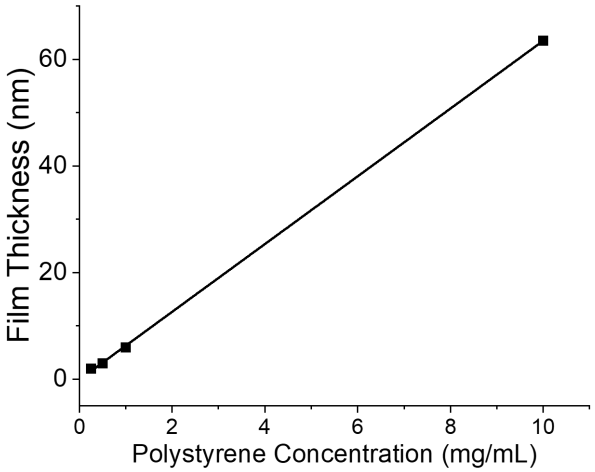
III. Result And Discussion

A. Characterization of Polystyrene Coating

Adhesion between the ultra-thin polystyrene layer and the as-prepared grating is a critical aspect of the current work. The adhesion layer is necessary due to the multiple physical factors at work at the polystyrene interface to create a high-density binding, high-enhancement substrate. First, the total dielectric stack above the silver layer must be as thin as possible since plasmonic enhancement decays off exponentially with increasing distance from the silver-dielectric interface [5]. The thickness of the ALD alumina layer is set by results from prior work to inhibit damage to the sensitive silver layer by ions in the aqueous buffers used for biological research. Therefore, the polystyrene layer should be ultra-thin (< 10 nm) to minimize further decay of plasmonic enhancement. Meanwhile, the polystyrene layer should be of sufficient thickness to be continuous in order to provide adequate protein binding sites for proper function as an ultrasensitive biosensor. Moreover, the surface must be resistant in order to survive without delaminating from the underlying substrate in unmodified complex patient body fluid samples and aqueous washing buffers containing surfactant to remove nonspecific binding.

The thickness of polystyrene films was characterized by spin-coating various concentrations on alumina coated glass slides. The concentration and the thickness showed a linear relationship (Fig. 1). The study of adhesion was performed on a surrogate substrate of 10 nm ALD alumina deposited on flat glass using the same deposition parameters as were used for the silver gratings described above. Polystyrene films spun directly on alumina (Fig. 2a) and onto TMCS-treated alumina (Fig. 2b) were easily removed by tape test, as indicated by the regions labeled (2) in Fig. 2a and 2b.

Figure 1. A linear relationship between the thickness and concentration of polystyrene

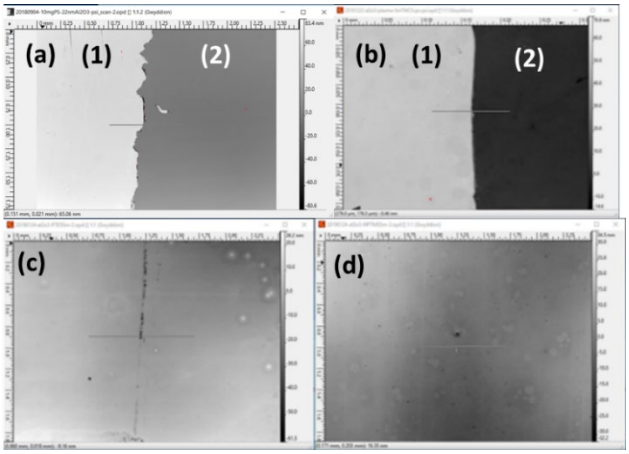


This suggests that hydrophobic-hydrophobic interaction was not a strong contributor to adhesion. On the other hand, survival of the polystyrene films over the phenyl-terminated P and N silanes (Fig. 2c and 2d) suggests π-π stacking as the mechanism of adhesion, whereby phenyl-terminated silanes interact with the phenyl groups of polystyrene to form numerous quadrupole interactions across the length of the polymer. The degree of interaction in this arrangement is determined by the silane packing density over the alumina surface and the orientation of the silane active group with respect to the normal from the plane of interaction and reactive surface groups.

B. Comparative Bioassay

After undertaking a thorough characterization of the system to ensure that the polystyrene thin film was adhered to the surface with appropriate thickness, morphology, and chemical composition, the binding of protein was compared between several available substrates in a fluorescence microplate reader.

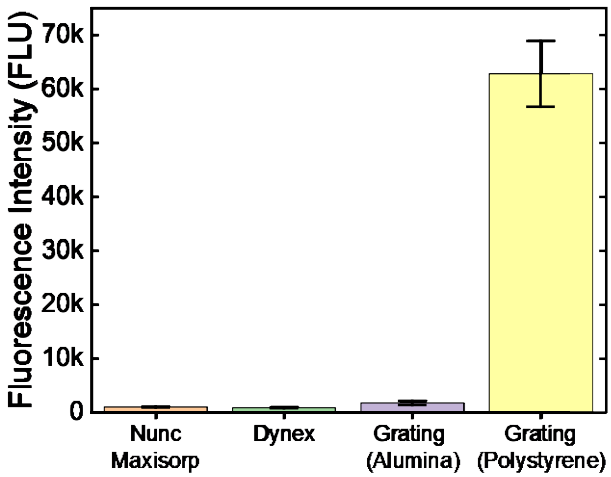
Figure 2. Optical profilometer images after tape test: 50 nm polystyrene films on (a) unsilanized alumina, (b) TMCS-treated alumina, (c) P-silane-treated alumina, and (d) N-silane-treated alumina. In (a) and (b), the areas labeled (1) were not exposed to tape while the areas (2) were exposed to tape, resulting in complete removal of the polystyrene films. The vertical line in (c) shows residue from the tape edge with no change in height on the tape-exposed righthand side.



Ab-RRX of the same final concentration and volume per well was incubated and bound to each of four substrates: Nunc Maxisorp® γ-irradiated polystyrene microwell plate, Dynex unmodified polystyrene microwell plate, our in-house generated silver plasmonic grating with alumina top surface, and silver plasmonic grating with polystyrene-coated alumina top surface. Microplate reader experimental conditions were tuned such that meaningful fluorescence values could be extracted across all four substrates under the same set of conditions without under- or over-saturating the detector, including the light source intensity, excitation and emission windows, and instrument gain. Meanwhile the height above the substrate was tuned on a plate-by-plate basis since the 24-well adapter by Grace Bio-Lab has a different well height and geometry than the commercial microwells. As can be seen in Figure 3, RRX-labeled protein on the Nunc Maxisorp® plate generated an average 984 FLU and Dynex generated 855 FLU after background subtraction. Normalizing to the Nunc Maxisorp® plate, the Nunc Maxisorp® plate resulted in 15% higher fluorescence than the Dynex plate.

The ~15% increase in fluorescence intensity of Nunc Maxisorp® over Dynex plates was consistent across multiple plates and experimental conditions performed with freshly prepared aliquots over several days while tuning the conditions prior to the current results. Meanwhile, the alumina-topped silver grating provided 1748 FLU signal, an enhancement of 1.78× with respect to the Nunc Maxisorp® plate. Finally, the polystyrene-coated alumina-topped silver grating provided a signal of 62839 FLU after background-subtraction, resulting in an enhancement factor of 63.8× with respect to the Nunc Maxisorp® plate. This extraordinary increase in fluorescence is attributed to a combination of the plasmonic enhancement of fluorescence signal and the high binding density of the polystyrene surface. Both Nunc Maxisorp® and Dynex plates have relatively flat, black microwells that suppress reflection and scattering of excitation light and provide a dark background on which to visualize the isotropic fluorescence. Meanwhile, the plasmonic grating is highly reflective, which increases the background signal by allowing for scattering of excitation light. Moreover, fluorescence is not isotropic due to the physical principles guiding light coupling to the grating structure. Both excitation and emission light are captured/coupled to the grating in a directional way. RRX was chosen as the reporter dye due to the spectral overlap between the excitation wavelengths of RRX and the near-normal incidence coupling angles between light in aqueous buffer and the polystyrene-coated alumina-topped silver grating. This selection allowed experiments to be performed using the relatively tall microwell adapter as opposed to a microscope cover slip thickness of less than 1 mm. Similarly, light that would be emitted from reporters very close to the surface is coupled back to the grating and reemitted directionally at the appropriate 'coupling' angle by surface-plasmon-coupled emission (SPCE). Again, for RRX, this wavelength is near zero degrees from normal and may be captured by the fluorescence reader.

Figure 3. The comparison of fluorescence enhancement for the different substrates.



IV. Conclusion

Herein, we have demonstrated the integration of polystyrene thin film coatings to plasmonic silver gratings to generate a biosensor surface with enhancement of biomolecule binding compared to the as-prepared alumina-topped plasmonic silver gratings. The uniqueness of this design is the integration of all these various parts in a mutually beneficial way while leaving out gamma irradiation or other modification after polystyrene deposition to hypothetically improve biomolecule immobilization. After coating with polystyrene thin film, the substrate is ready for packaging and use. Aside from just enhancing binding density or improving fluorescence signal enhancement over a functionalized flat surface, improving molecular immobilization density improves the efficiency of any downstream capture, enzymatic, or other interactive activity with analytes of interest. In that case, the higher binding density means a higher probability of visualizing the analytes or interactions of interest from any given sample, regardless of concentration, and will notably extend the dynamic range and improve the lower limits of detection and mean accurate maximum concentration. Another issue with polystyrene thin films is the long-term stability of the film on a surface of non-ideal surface energy, functionality. Direct-deposited polystyrene may be modified by gamma, beta, or even alpha radiation. This may have longer shelf life due to the thicker polystyrene layer and the surface modification due to irradiation will be permanent. Nanoscale films have a natural tendency to restructure in order to minimize internal stresses and optimize surface energy with the constantly changing local environment. This restructure and reflow leads to film discontinuity through dewetting into spheroid islands to minimize surface tension with the underlying surface, environment, or both. An adhesion layer promotes adhesion to the base layer and introduces a stable surface functional group that attracts and reacts with the thin film through chemical bonding and attractive noncovalent interactions like pi stacking. The primary goals for the future are the automation of fabricating individual grating substrates and the application of the plasmonic substrates to sensitive biomarker detections including COVID-19 and tuberculosis, with tuberculosis detection having been performed previously on alumina-topped gratings [1]. Additional improvement is planned by using machine learning algorithms to identify optimized conditions of polystyrene film fabrication.

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