

Cell and Protein Compatibility of Parylene-C Surfaces

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Parylene-C, which is traditionally used to coat implantable devices, has emerged as a promising material to generate miniaturized devices due to its unique mechanical properties and inertness. In this paper we compared the surface properties and cell and protein compatibility of parylene-C relative to other commonly used BioMEMS materials. We evaluated the surface hydrophobicity and roughness of parylene-C and compared these results to those of tissue culture-treated polystyrene, poly(dimethylsiloxane) (PDMS), and glass. We also treated parylene-C and PDMS with air plasma, and coated the surfaces with fibronectin to demonstrate that biochemical treatments modify the surface properties of parylene-C. Although plasma treatment caused both parylene-C and PDMS to become hydrophilic, only parylene-C substrates retained their hydrophilic properties over time. Furthermore, parylene-C substrates display a higher degree of nanoscale surface roughness (> 20 nm) than the other substrates. We also examined the level of BSA and IgG protein adsorption on various surfaces and found that surface plasma treatment decreased the degree of protein adsorption on both PDMS and parylene-C substrates. After testing the degree of cell adhesion and spreading of two mammalian cell types, NIH-3T3 fibroblasts and AML-12 hepatocytes, we found that the adhesion of both cell types to surface-treated parylene-C variants were comparable to standard tissue culture substrates, such as polystyrene. Overall, these results indicate that parylene-C, along with its surface-treated variants, could potentially be a useful material for fabricating cell-based microdevices.

1. Introduction

Polymeric biomaterials are widely used in therapeutics^{1,2} and diagnostics^{3,4} as micro- and nanobiosensors for cell-based assays, drug delivery, and tissue-engineering applications.⁵ Polymeric microdevices are capable for analyzing cells and proteins,^{6–8}

generating tissue-engineering scaffolds,^{9–11} and miniaturizing bioassays for high-throughput experimentation.¹² With the recent emergence of soft lithography, elastomers, such as poly(dimethylsiloxane) (PDMS), have become enabling materials for the widespread fabrication and the use of microfabricated systems. PDMS offers numerous advantages over traditional biomaterials. It is relatively inexpensive, inert, nontoxic, and can be easily molded to form microstructures.¹³ Despite these desirable characteristics, PDMS has a number of shortcomings. For example, although PDMS has been shown to be compatible for short-term culturing of cells,¹⁴ little is known of its long-term stability in tissue-engineering applications and in vivo diagnostics. Therefore, it may be important to explore alternative biomaterials that can be used to fabricate biomedical microdevices. Poly-

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(chloro-*p*-xylylene), also referred to as parylene-C, is one such potential candidate for fabricating biomedical devices.

Parylene-C is a thermoplastic, crystalline, and transparent polymer that is extensively used as a coating for insulating implantable biomedical devices.¹⁵ In addition, parylene-C is chemically inert and nonbiodegradable. Parylene-C is synthesized from a low-molecular weight (MW) dimer, dichloro-di(*p*-xylylene), using a process that involves the decomposition of *p*-xylylene to yield chloro-*p*-xylylene, followed by the polymerization of chloro-*p*-xylylene to parylene-C.¹⁶ Parylene-C can be vapor-deposited onto substrates to generate uniform, pinhole-free membranes that can be subsequently dry-etched using oxygen plasma to yield microscale features and patterns that are ideal for culturing cells.¹⁷ The all-carbon structural backbone, high-MW, and nonpolar entities make parylene-C highly resistant to most chemicals, as well as to fungal and bacterial growth. In addition to having conducive biochemical properties, parylene-C has a Young's modulus of ~ 4 GPa¹⁸ (compared to 0.75 MPa for PDMS¹⁴)—making it mechanically robust and highly suitable for fabricating stable and reusable microfluidic devices or stencils.^{17–22} Recent studies have shown parylene-C to be more hemocompatible and less thrombogenic than silicon.²³ Parylene-C has also demonstrated high stability in vivo for a variety of applications, such as cardiovascular implants.^{24,25} Furthermore, parylene-C is a potentially useful material for in vitro cell culture studies. For example, we have developed the use of parylene-C stencils for patterning cells and proteins and for generation of cocultures with control over the degree of homotypic and heterotypic cell–cell interactions.^{26,27} Another recent study provides the methodology for making nanoscale sculptured thin films (STFs) out of parylene-C.²⁸ Due to the high surface area to volume ratio of the STF, the parylene-C STF supports high level of cell adhesion.²⁸ However, despite the apparent biocompatibility of parylene-C, there has been no direct comparison of parylene-C to PDMS and other materials commonly used in BioMEMS.

In this study, we compared the biocompatibility of parylene-C membranes with PDMS, glass, and optically clear virgin polystyrene by analyzing protein adsorption, cell adhesion, and cell morphology characteristics on each of these surfaces. In addition, we treated parylene-C and PDMS with air plasma and coated the surfaces of these substrates with fibronectin to study the effects of surface treatments on protein adsorption, cell

adhesion, and spreading. Protein adsorption was studied using bovine serum albumin (BSA) and immunoglobulin G (IgG), and cell adhesion and spreading were studied using NIH-3T3 fibroblast and AML-12 hepatocyte cell lines.

2. Methods and Materials

2.1. Fabrication of Parylene-C and PDMS. Three inch silicon wafers were first cleaned for ~ 10 min using a 1:1 piranha solution (equal volume mixture of H₂SO₄ and H₂O₂), sufficiently rinsed with deionized water, nitrogen-dried, and then coated with hexamethyldisilazane (HMDS). Following pretreatment, the silicon wafers were deposited with dichloro-di(*p*-xylylene) by utilizing a Labcoater 2 PDS 2010 chemical deposition system (Specialty Coating Systems, Indianapolis). Inside the deposition system, dichloro-di(*p*-xylylene) is first vaporized at 150 °C and 1 torr and then pyrolyzed at 690 °C and 0.5 torr to form chloro-*p*-xylylene—the monomer of parylene-C. A reduction in the chamber temperature causes chloro-*p*-xylylene to condense onto the wafer surfaces to form parylene-C membranes. Initial loading of dichloro-di(*p*-xylylene) onto the silicon wafers determines the thickness of the parylene-C membrane at a rate of 0.5 $\mu\text{m/g}$. With the use of the aforementioned protocol, 10 μm thick parylene-C membranes were fabricated on silicon substrates.

The PDMS substrates were fabricated by directly curing a Sylgard 184 (Essex Chemical) elastomer in the wells of a Costar 24-well TC-treated cell culture microplate for nearly 2 h at 70 °C, using a 10:1 weight ratio of elastomer to curing agent.

2.2. Preparation of Surfaces. A total of eight types of surfaces were used in this study. Costar 24-well TC-treated cell culture microplates were utilized as optically clear virgin polystyrene substrates. Parylene-C experimental samples were prepared by carefully cutting the 10 μm thick parylene-C membranes (section 2.1) to form square-shaped pieces of ~ 5 mm \times 5 mm. Each cut-out piece of parylene-C was placed and sealed reversibly onto a PDMS-coated well in the microplate. Platinum glass coverslips, 18 mm \times 18 mm in size, were used as the glass samples. Plasma-treated PDMS and parylene-C were obtained by treating the two polymers with air plasma in a Harrick PDC-001 plasma treatment chamber for 2 min. Protein coating to parylene-C and PDMS surfaces was performed by simply incubating a 5 $\mu\text{g/mL}$ fibronectin solution on the surfaces for 1 h.

Each substrate was sterilized prior to the experiments. The sterilization of plain and plasma-treated surfaces consisted of UV irradiation for 30 s, followed by successive washes with 70% ethanol and sterile PBS, respectively. The sterilization of fibronectin-coated surfaces consisted of UV irradiation for 30 s followed by a 1 h incubation of a sterile solution of fibronectin (5 $\mu\text{g/mL}$) on sterilized samples of plain PDMS and parylene-C.

2.3. Surface Property Characterization. **2.3.1. Contact Angle Measurements.** Contact angles were measured on static drops of water on different substrates by using a contact angle measurement system (Phoenix 300 plus, SEO) to provide information about hydrophobicity of the surfaces (See Table 1). The substrates were measured as-received or as-deposited (plain), and additional measurements were performed with a subset of these substrates (PDMS and parylene-C) that were treated with oxygen plasma and were coated with fibronectin. The contact angle measurements were

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Table 1. Contact Angle Measurements

substrates	contact angle (deg)		
	untreated	plasma-treated	fibronectin-coated
glass	36.3 ± 2.6		
polystyrene	79.1 ± 5.9		
PDMS	105.9 ± 4.5	9.9 ± 1.1 ^a 73.7 ± 3.0 ^b	99.0 ± 6.7
parlyene-C	97.2 ± 4.2	4.4 ± 2.4 ^c	105.0 ± 10.4

^a Measurements made immediately after PDMS was treated in oxygen plasma. ^b Measurements made after 40 min following treatment in oxygen plasma. ^c No significant change observed in measurements made immediately after and following 40 min after plasma treatment.

Table 2. Surface Roughness Measurements

substrate	roughness (nm)
glass	1.6 ± 0.6
polystyrene	1.2 ± 0.2
parlyene-C	19.3 ± 6.3
plasma-treated parlyene-C	19.3 ± 5.4
fibronectin-coated parlyene-C	29.0 ± 11.5
PDMS	2.2 ± 0.6
plasma-treated PDMS	0.4 ± 0.1
fibronectin-coated PDMS	3.2 ± 0.6

performed by dispensing deionized water drops (5–10 μ L) on each substrate with a micropipette (Ted Pella Inc.). Each data point represents an average of >10 independent measurements.

2.3.2. Surface Roughness Measurements. Surface roughness values of four different substrates (glass, polystyrene, PDMS, and parlyene-C) as received were measured with atomic force microscopy (AFM) (Q-Scope 250, Quesant Instrument Corporation) using noncontact mode with a cantilever tip (NCS 16, Quesant). Scan areas of 50 μ m \times 50 μ m were randomly selected on the substrates. To obtain the surface roughness values from an as-deposited thin (10 μ m) parlyene membrane, we first peeled the parlyene off the silicon wafer and then placed it on top of a robust substrate (1 mm thick PDMS slab). Afterward, we performed the AFM measurements. To obtain the surface roughness of the surface-treated parlyene-C, we applied surface treatments (O₂ plasma treatment or fibronectin coating) on the parlyene surface mounted on a PDMS slab and performed AFM surface roughness measurements. Three independent measurements from 5 μ m \times 5 μ m squares of each surface were performed and averaged. Roughness values (mean) acquired from various samples corresponding to the variations in surface heights are summarized in Table 2.

2.4. Protein Adsorption Measurements. Protein adsorption was characterized by incubating 50 μ g/mL of fluorescein isothiocyanate (FITC)-conjugated BSA (Sigma-Aldrich) and 100 μ g/mL of FITC-conjugated IgG (Sigma-Aldrich) on each substrate for 1 h. The substrates were encased in aluminum foil to prevent photodegradation of the FITC. Following incubation, the substrates were rinsed with deionized water and imaged using a fluorescent microscope (Nikon TE 2000) with a constant exposure time of 500 ms. Emitted fluorescence was then measured using ImageJ pixel brightness analysis tool (National Institutes of Health, U.S.A.). The average pixel brightness of each image is an indirect measurement of the protein adsorption onto the substrates. Control substrates were also used to eliminate the effect of autofluorescence from the substrates.

2.5. Cell Culture. NIH-3T3 fibroblasts were cultured in Dulbecco's modification of Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin–streptomycin (MediaTech). AML-12 hepatocytes were preserved in 44.5% DMEM and 44.5% Ham's F12 media (Invitrogen) supplemented with 10% FBS and 1% penicillin–streptomycin (Sigma-Aldrich). The cells were maintained under humid conditions, at 37 $^{\circ}$ C, and in a 5% CO₂ atmosphere.

2.6. Preparation of Samples for Cell Adhesion. NIH-3T3 fibroblasts and AML-12 hepatocytes were trypsinized and resus-

incubated on each of the substrates for 6 h. Each well of the Costar 24-well microplate has a base area of 283.5 mm². This corresponds to a loading density of \sim 353 cells/mm². Next, the substrates were rinsed twice with 1 \times phosphate-buffered saline (Invitrogen), and cells adhering to the substrates were then fixed using 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.2% Triton X-100 (Sigma) for 10 and 5 min, respectively.

2.7. Visualization and Imaging of Adhered Cells. To count the number of adhered cells on each surface, images of fluorescently labeled nuclei were collected using a fluorescent microscope (Nikon Eclipse TE 2000). Three pictures per well and three wells per substrate were analyzed and counted using ImageJ software.

2.8. Visualization and Imaging of Cells for Estimating Shape Factors. To analyze cell spreading on various surfaces, data was collected from at least 70 adhered cells per sample. To effectively analyze cell shape, the dimensionless shape factor, S , was used to compare the spreading of cells. It is computed as

$$S = 4\pi A/P^2$$

where A is the area occupied by the cell and P is the perimeter of the cell. A shape factor of 1 corresponds to a perfect circle, whereas a shape factor of 0 represents a line. Cell shape factors were computed utilizing the calibration and measurement features of the SPOT Imaging Software.

3. Results and Discussion

We evaluated the surface properties of parlyene-C stencils in comparison with other commonly used biomedical materials, such as PDMS, glass, and polystyrene. In addition, we analyzed the effect of two common surface treatments, oxygen plasma and protein coating on these substrates. The surfaces were characterized for their hydrophobicity and roughness as well as for protein adsorption, cell adhesion, and cell morphology. Particular attention was paid to the differences between parlyene-C and PDMS, due to their emerging applications in biomedical microfabrication.

3.1. Surface Analysis. Surface hydrophobicity and surface roughness are important factors in cell adhesion and the resulting cellular morphology.^{14,29–31} In addition, hydrophobicity has also been shown to affect protein adsorption.^{32–35} Therefore, it is important to evaluate these properties in parlyene-C membranes to understand the interaction of mammalian cells with these substrates. To assess the hydrophobicity of the surfaces, we measured contact angles of as-deposited and treated parlyene-C surfaces and compared the values to control surfaces (Table 1). The substrates varied greatly in their water contact angles, from \sim 36 $^{\circ}$ for glass to \sim 111 $^{\circ}$ for PDMS. As-deposited parlyene-C and plain PDMS were both hydrophobic as they exhibited contact angles of \sim 100 $^{\circ}$, which is consistent with our previous study.³⁶

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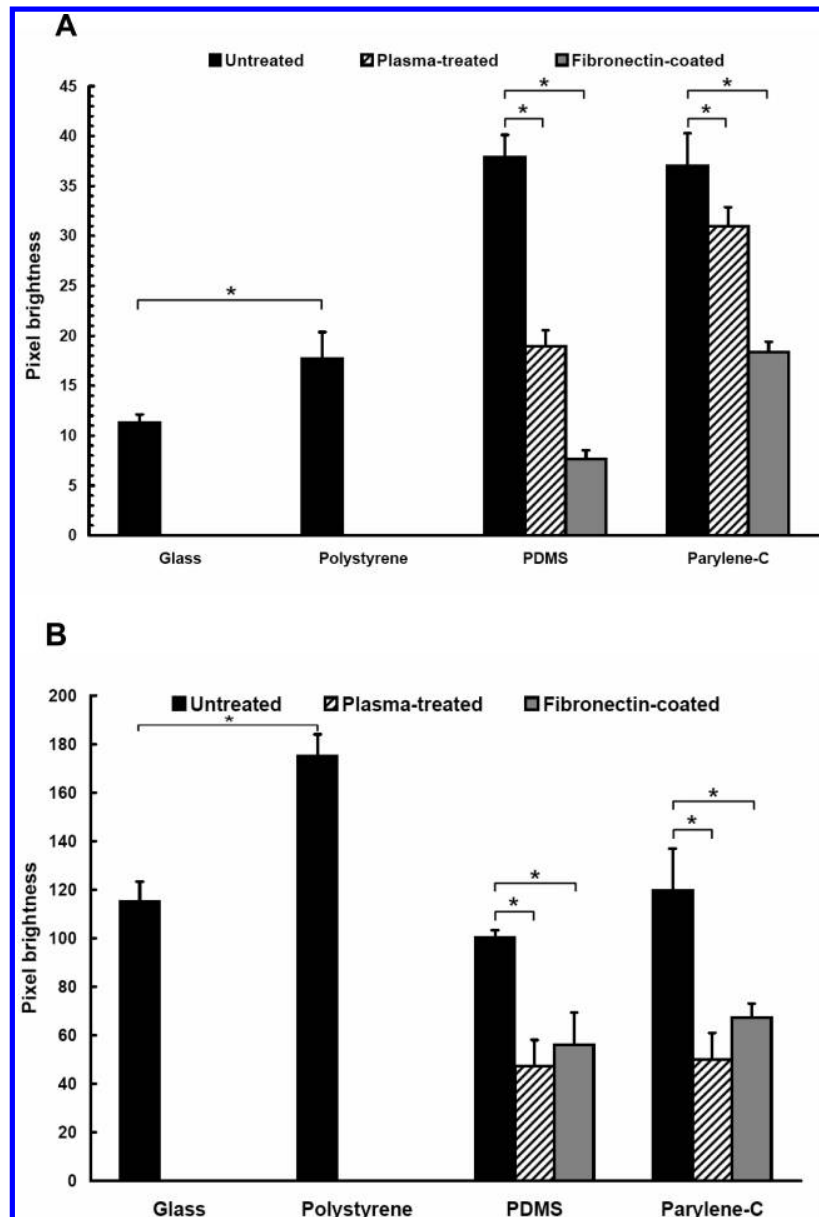


Figure 1. Adsorption of FITC-BSA (A) and FITC-IgG (B) onto each of the substrates. (A) Adsorption of FITC-BSA onto parylene-C and PDMS exceeds that on glass and polystyrene. Surface modifications of parylene-C and PDMS show a negative effect on FITC-BSA adsorption. (B) FITC-IgG adsorbs onto glass, parylene-C, and PDMS in a comparable manner. Adsorption onto polystyrene is the highest. Surface modification of parylene-C and PDMS reduces their affinity for FITC-IgG. In general, plasma treatment and fibronectin coating the two polymers reduces their ability to adsorb proteins. The * indicates $p < 0.05$.

Furthermore, fibronectin-coated parylene-C and PDMS were also hydrophobic (contact angles of $\sim 100^\circ$). This hydrophobic property of fibronectin-coated PDMS is confirmed by results obtained by other groups.³² Even though there has not been investigation in the past on fibronectin-coated parylene-C, it is logical to expect it to be hydrophobic. Because fibronectin coatings have no electron donor components and have low surface energy,³³ materials coated with fibronectin would not form hydrogen bonds with water molecules, so they would become hydrophobic. Furthermore, air plasma treatment reduced the contact angle of both parylene-C and PDMS substrates to less

than 10° . This finding agrees well with the previous findings that the formation of hydroxyl groups from the O_2 plasma treatment process significantly increases the hydrophilicity of surfaces.⁸

One of the main drawbacks of using PDMS for fluidic devices is that the plasma-induced hydrophilicity of the PDMS surfaces is short term.⁷ In many applications involving fluidics and cells, the ability to generate substrates that remain hydrophilic may be beneficial. To compare the stability of plasma-treated surfaces, we measured the contact angles of plasma-treated parylene-C and PDMS surfaces immediately and 40 min after plasma treatment. It was observed that although the plasma treatment initially decreased the contact angle values, the hydrophilicity of a PDMS substrate deteriorated rapidly (Table 1). This is due to the viscoelastic properties of PDMS, in which the surface

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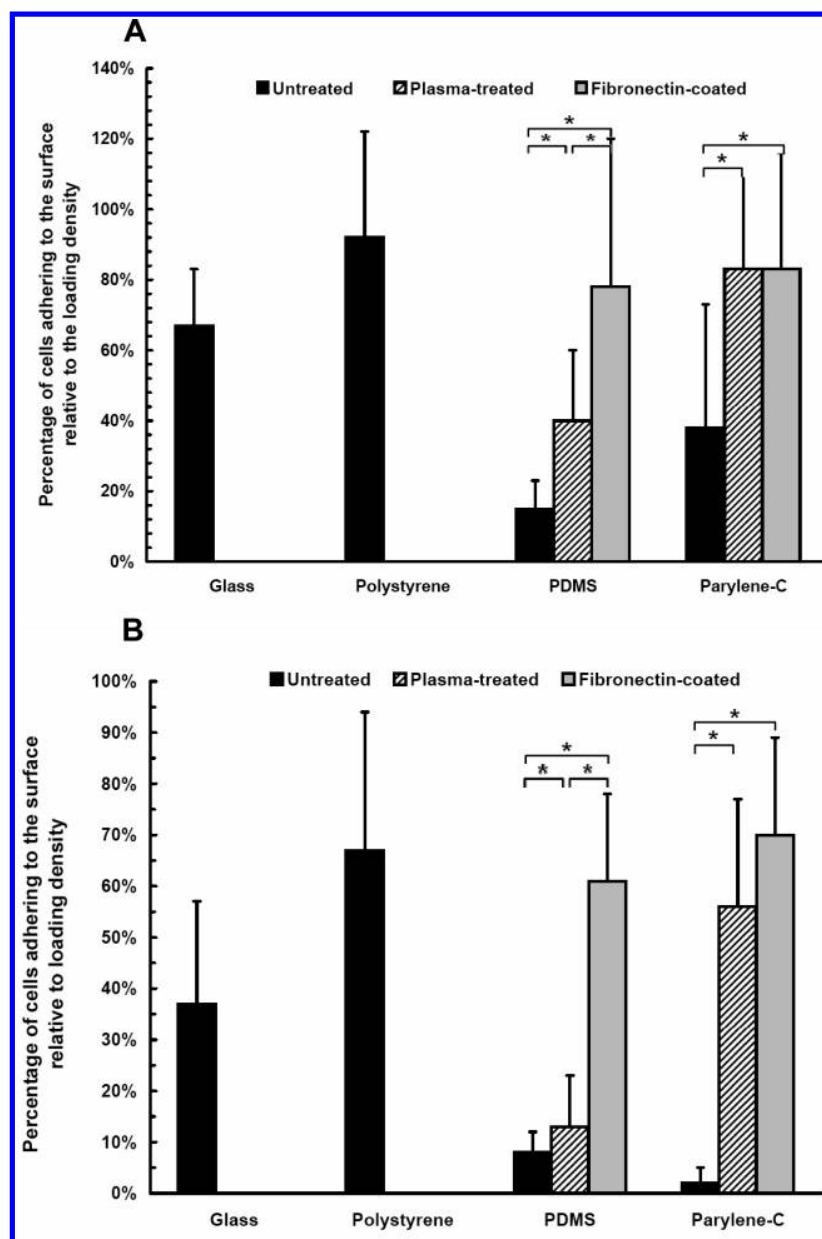


Figure 2. Adhesion of (A) NIH-3T3 fibroblasts and (B) AML-12 hepatocytes on the various substrates. (A) The cells do not adhere to as-deposited parylene-C and plain PDMS. Furthermore, plasma treatment and fibronectin coating of the two polymers increase their adhesiveness to NIH-3T3 cells. (B) Similar trends are exhibited by AML-12 adhesion to the various substrates. The * indicates $p < 0.05$.

contact angle for plasma-treated parylene-C did not change significantly after 40 min. The fact that the plasma-treated parylene surface stays hydrophilic for longer periods could be advantageous for various biological applications. We next examined the surface roughness of the parylene-C and PDMS substrates by using AFM. As shown in Table 2, as-deposited and treated parylene-C substrates were significantly rougher compared to other substrates, including glass, PDMS, and polystyrene. Fibronectin-treated parylene-C surfaces had the highest roughness values of ~ 30 nm. The higher surface roughness values of parylene-C may be due to the irregularities in the deposition process, which were further increased with fibronectin molecules adsorbed onto the surface. On the other hand, PDMS, glass, and polystyrene were much smoother with surface roughness values of < 3 nm. An increase in surface roughness enhances the protein adsorption

more integrin receptors on the cells will bind to the proteins and, therefore, mediate the attachment of cells.²⁹

3.2. Protein Adsorption. To generate substrates that are favorable for cell adhesion, a routine procedure is to coat a layer of adhesive proteins on the substrates. To measure protein adsorption properties of parylene-C relative to other substrates, we incubated each sample with fluorescently labeled BSA and IgG. First observation we made was that significantly more BSA and IgG adsorbed to polystyrene relative to glass. This finding is consistent with other studies that BSA and IgG adsorb more onto highly hydrophobic surfaces like polystyrene, compared to relatively hydrophilic surfaces like glass.^{14,29,31,33} Similarly, BSA adsorption level on plain PDMS and as-deposited parylene-C were 3 times higher relative to glass (Figure 1A). On the other

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