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Co-immobilization of Biomolecules on Ultrathin Reactive Chemical Vapor Deposition Coatings Using Multiple Click Chemistry Strategies

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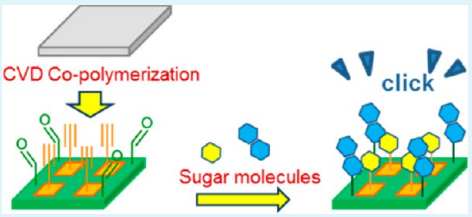
ABSTRACT: Immobilization of biomolecules, such as proteins or sugars, is a key issue in biotechnology because it enables the understanding of cellular behavior in more biological relevant environment. Here, poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) coatings have been fabricated by chemical vapor deposition (CVD) polymerization in order to bind bioactive molecules onto the surface of the material. The control of the thickness of the CVD films has been achieved by tuning the amount of precursor used for deposition. Copper-catalyzed Huisgen cycloaddition has then been performed via microcontact printing to immobilize various biomolecules on the reactive coatings. The selectivity of this click chemistry reaction has been confirmed by spatially controlled conjugation of fluorescent sugar recognizing molecules (lectins) as well as cell adhesion onto the peptide pattern. In addition, a microstructured coating that may undergo multiple click chemistry reactions has been developed by two sequential CVD steps. Poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) and poly(4-formyl-*p*-xylylene-*co*-*p*-xylylene) have been patterned via vapor-assisted micropatterning in replica structures (VAMPIR). A combination of Huisgen cycloaddition and carbonyl-hydrazide coupling was used to spatially direct the immobilization of sugars on a patterned substrate. This work opens new perspectives in tailoring microstructured, multireactive interfaces that can be decorated via bio-orthogonal chemistry for use as mimicking the biological environment of cells.



KEYWORDS: *biointerfaces, chemical vapor deposition polymerization, click chemistry, microstructuring, polymer, surface modification*

INTRODUCTION

Over the last decades, advanced biomaterials have been widely developed for medical applications.[1] The surface functionalities of the materials determine interactions between an abiotic material and a biological organism. Ubiquitous in nature, peptides, sugars, and other recognition ligands play a significant role in numerous biological events. For example, they may trigger some signaling cascades within the cell and modulate cell receptor activities that influence cell adhesion or proliferation.[2] As a result, mimicking this rich biomolecular environment is needed for improved understanding of cellular behavior and mechanisms. The spatially controlled distribution of biomolecules and the development of cell culture substrates require stable, yet designable surfaces.[3] Versatile surface engineering techniques have thus become a major focus of material scientists. Tailoring surface properties, while preserving the inherent physical and mechanical properties of a bulk material, represents an elegant approach for the development of biomaterials. In this context, chemical vapor deposition (CVD) polymerization is a robust solvent-free process that enables surface modification.[4,5] Stable, homogeneous coatings can be produced on a multitude of substrates including three-dimensional scaffolds and thermosensitive materials. Several adaptations of the CVD polymerization process have been reported in the literature,[6,7] which contributes to the versatility of the technique. For instance, Gleason and co-workers have suggested plasma polymerization[8] or initiated chemical vapor deposition.[9] CVD polymerization of substituted [2.2]-paracyclophanes has also been widely studied for the design of biointerfaces.[10] Originally developed by Gorham[11] and commercialized under the parylene brand, coatings made by vapor-based polymerization of [2.2]paracyclophanes are approved by the FDA for biomedical use.[12] However, because of the lack of functional groups in these original parylene coatings, direct immobilization of biomolecules is not possible without subsequent surface modification. In contrast, our group has focused on CVD polymerization of substituted [2.2]-paracyclophanes using a custom-designed CVD system in order to synthesize chemically active surfaces for controlled immobilization of biomolecules.[13] The synthesis of a wide range of [2.2]paracyclophanes with appropriate functional groups is now well-established[14] and substituted poly(*p*-xylylenes) were prepared that contained amines,[15] esters,[16,17] ketones with fluorinated groups,[18] or aldehydes.[19] In addition, CVD polymerization has also been extended to the production

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of multifunctional coatings,[20] including those displaying functional film gradients.[21,22]

The role of the CVD coatings is the presentation of chemically addressable functional groups for targeted biomolecular immobilization. To obtain a stable interface, covalent bonding of biomolecules with reactive groups present at the surface is the preferred strategy. The functional groups, natively or synthetically present on the biomolecules, may be exploited as reactive partners for immobilized ligands. Such biomolecular immobilization benefits from chemical reactions that are quantitative, highly specific, and tolerant to many functional groups, such as click reactions.[23] Click chemistry was introduced by Sharpless and co-workers in 2001[24] and has since been widely used by material scientists for surface modification.[25−27] There are numerous click reactions including, but not limited to, Huisgen 1,3-dipolar cycloaddition, hydrazone, oxime, Diels−Alder, Staudinger, thiol−ene, and Aza-Wittig reactions.[28] However, the most commonly used click chemistry reaction is the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between azido- and alkyne-terminated compounds. As a result of this coupling reaction, triazoles are formed, which are stable linkers under physiological conditions.[29] For instance, immobilization of azido-terminated sugars has been successfully achieved onto alkyne-functionalized self-assembled monlayers (SAMs) via Huisgen cycloaddition.[30−34] Other recent studies reported spatially directed immobilization by Huisgen cycloaddition of biotin,[23,35] carbohydrate,[36] and peptide[37] derivatives. To consider surface functionalization by click chemistry on a broader range of substrates, CVD polymerization of alkyne-substituted [2.2]-paracyclophanes has been studied.[23] Azido-functionalized biotin was successfully immobilized on poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) and the spatially controlled copper-catalyzed Huisgen cycloaddition has also been enabled by dip-pen nanolithography (DPN).[38] This process led to nanostructuring of a broad range of substrates, including soft polymers. Copper-free click chemistry has also been investigated on CVD coatings for bio-orthogonal "double-click" reactions;[35] thereby combining copper-catalyzed and copper-free click chemistry for the immobilization of biotin and Oregon 488 on a single multifunctional surface. Very recently, a copolymer coating containing alkyne and aldehyde groups for the coimmobilization of two different biomolecules has been synthesized by CVD polymerization.[39] However, to date no study has combined spatio-selective microstructuring with multiple, distinct click chemistry reactions on a single CVD coating.

Herein, we first prepared ultrathin poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylelene) films by CVD polymerization as a proof of principle of using homogeneous coatings to explore the effect of single biomolecules on a specific biological event. For this purpose, we tuned the thickness of the polymer coatings in a controlled fashion and established the dependence of the film thickness on the monomer feed amount. In order to show the functionality of such thin films, several biomolecules were immobilized on CVD coatings including azido-terminated saccharides and peptides. Their influence on lectins conjugation and cell adhesion respectively has been investigated. Then, we produced microstructured coatings combining two types of functional parylenes on the same substrate that may undergo subsequent, orthogonal click reactions. The micropatterning of the CVD coatings has been performed by sequential deposition of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylelene) and poly(4-formyl-*p*-xylylene-*co*-*p*-xylylene) *via* vapor-assisted micropatterning in replica structures (VAMPIR).[40] Finally, the orthogonal reactivity of the CVD coating was confirmed by the selective bonding of saccharides through Huisgen cycloaddition and aldehyde-hydrazide coupling. This work is an important demonstration of the immobilization of various biomolecules on a microstructured CVD coating via multiple orthogonal click chemistry reactions. The selective immobilization of different sugars may be a promising means of assessing ligands/cell receptor interactions and synergic effects of a cascade of reactions on biological events.

EXPERIMENTAL SECTION

CVD Polymerization. 4-Ethynyl[2.2]paracyclophane[41] and 4-formyl[2,2]paracyclophane[19] precursors were synthesized as described elsewhere. Both precursors were sublimated at 90−110 °C under low pressure (<0.07 Torr) before entering the pyrolysis furnace, maintained at 660 °C, at an argon flow rate of 20 sccm. Polymerization of the precursors occurred by vapor-deposition on the substrate, kept at 15 °C, in the deposition chamber. Micropatterns containing two different CVD coatings were prepared via the previously developed VAMPIR technique.[40] Briefly, a layer of poly(4-formyl-*p*-xylylene-*co*-*p*-xylylene) was first homogeneously coated on the substrate. Then a patterned PDMS microstencil (squares of 200 μm × 200 μm), fabricated as previously described[40] was placed on the coated substrate, and a second layer of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) was subsequently deposited onto the surface not covered by the microstencil to generate the micropatterns.

Characterization of CVD Films. Fourier transform infrared spectroscopy (FTIR) was performed on a Nicolet 6700 spectrometer at a grazing angle of 85°. The thicknesses of the polymer layer were measured using an EP^3−SW imaging ellipsometer (Nanofilm AG, Germany) at a wavelength of 532 nm and at an angle of incidence of 65°. An anisotropic Cauchy parametrization model was used for curve fitting. The error bars on the thickness of the polymer films were calculated from three different CVD experiments, where each experiment was based on three parallel samples. For the mapping mode, data was recorded by an imaging scanner with a lateral resolution of 1 μm with a field of view of about 100 μm × 500 μm.

Poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene):[23] FTIR (cm^{-1}) = 833, 894, 1158, 1251, 1411, 1454, 1493, 1513, 1605, 1699, 1900, 2102, 2859, 2926, 3015, 3286.

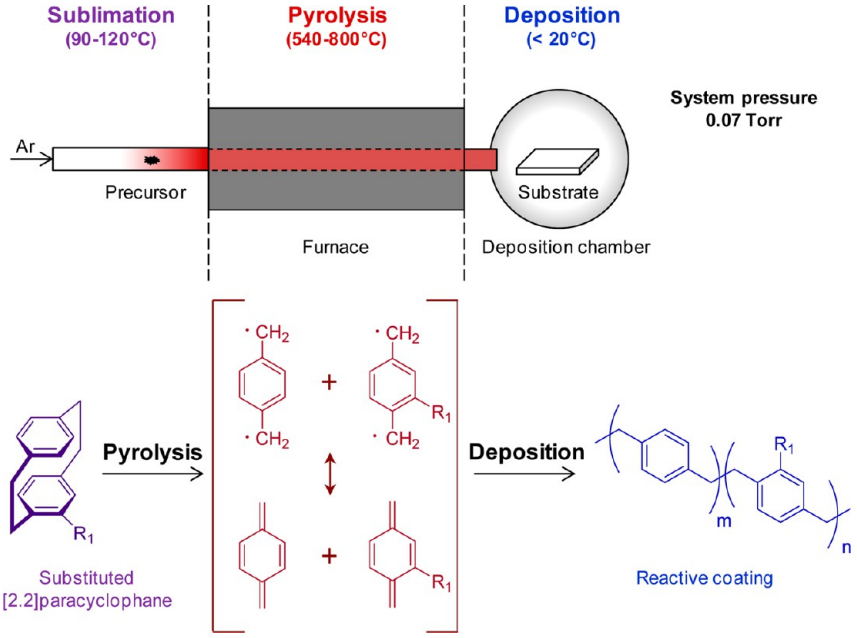
Poly(4-formyl-*p*-xylylene-*co*-*p*-xylylene):[19] FTIR (cm^{-1}) = 839, 905, 1158, 1235, 1453, 1500, 1567, 1608, 1688, 2732, 2922.

Immobilization of Azido-Functionalized Biomolecules by Microcontact Printing. A solution containing azido-saccharides (1-azido-1-deoxy-β-D-glucopyranoside or 1-azido-1-deoxy-β-D-galactopyranoside, 10 μg/mL) or azido-peptides (YIGSR-N_3, 20 μg/mL) and sodium ascorbate (1 mM) in a 2:1 mixture of water:tert-butyl alcohol was prepared. These azide solutions were spread on poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) coatings and the substrates were dried using N_2. Patterned PDMS stamps (squares of 50 μm x 50 or 250 μm × 250 μm) were created as previously described.[17] Stamps were oxidized for 20 min using a UV-ozone cleaner (Jelight Co. Inc., Irvine, CA) before use and inked with $CuSO_4$ solution (1 mM in methanol). The stamps were then kept in contact with the CVD coating for 12−18 h for the saccharides and for 3 h for the peptide.

For the peptide-patterned substrate, a similar microcontact printing procedure was repeated (after peptide microcontact printing) with an unpatterned flat PDMS stamp to immobilize azido-functionalized poly(ethylene glycol) (PEG-N_3, M_W = 5000 g/mol, 50 μg/mL) on the remaining areas. Substrates were then washed with aqueous phosphate buffer (PBS) containing 0.02% (v/v) Tween 20, PBS and distilled water.

Immobilization of Mannobiose and Azido-Saccharide on Patterned CVD Coatings (obtained via VAMPIR). The patterned substrate was first incubated in an aqueous solution containing adipic acid dihydrazide (100 mM, pH 4−5) overnight. After washing, the

Scheme 1. CVD Polymerization Process of Functional Poly(*p*-xylylene) Polymers



surface was incubated in an aqueous solution (pH 4−5) of mannobiose (10 mM) and sodium periodate (40 mM) for 5 h, followed by repeated washing with distilled water. Subsequently, the substrate was incubated in an aqueous solution containing 1-azido-1-deoxy-β-D-galactopyranoside (1 mg/mL), $CuSO_4$ (1 mM), and sodium ascorbate (3 mM) overnight, followed by repeated washing with distilled water.

Lectin Conjugation. For lectin conjugation of β-glucose- or β-galactose-patterned substrates obtained by microcontact printing, samples were incubated for 1 h in buffer solution (PBS with 0.02% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin) containing respectively fluorescein-conjugated concanavalin A (FITC-Con A, 50 μg/mL) and rhodamine-labeled peanut agglutinin (TRITC-PNA, 50 μg/mL). For lectin conjugation of mannobiose- and β-galactose-patterned substrate obtained via VAMPIR technique, the sample was incubated in a buffer solution containing either rhodamine-labeled concanavalin A (TRITC-Con A) or fluorescein-conjugated peanut agglutinin (FITC-PNA) for 3 h. The buffer solution consisted of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (10 mM) with MnCl (1 mM), $CaCl_2$ (1 mM), and NaCl (0.15 M). Substrates were then washed with the incubating buffer, PBS and distilled water. Fluorescence micrographs were captured using a Nikon TE200 fluorescence microscope.

Human Endothelial Cell Culture. Cryopreserved human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD) and cultured in complete Endothelial Growth Medium (EGM) containing 2% fetal bovine serum. Cells were cultured in 75 cm^2 tissue culture-treated polystyrene flasks, maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide and cell culture medium was replaced every other day until 80% confluence was attained. Cells were harvested from the flasks using 0.25% trypsin/ethylene-diaminetetraacetic acid solution and seeded on the substrates as needed.

For the cell patterning studies, peptide-patterned surfaces were rinsed with Dulbelcco's PBS (D-PBS) and placed in a multiwell plate. Cells were harvested from the flask and resuspended at a density of 1 × 10^4 cells/mL in endothelial basal medium (EBM) without serum and 1 mL was added to each well. They were allowed to adhere for 1 h without serum at 37 °C. Subsequently, this media was replaced with medium containing serum (EGM) and cells were maintained for 10 h. Substrates were washed with D-PBS, fixed with formaldehyde (4% v/v in D-PBS), permeabilized with TritonX-100 (0.1% v/v in D-PBS) and stained with rhodamine-conjugated phalloidin. Samples were mounted with Prolong Gold containing DAPI. Substrates were imaged using an Olympus BX-51 fluorescence microscope (Microscopy and Image Analysis Laboratory, University of Michigan, Ann Arbor).

RESULTS AND DISCUSSION

The polymerization of 4-ethynyl[2.2]paracyclophane was performed by the Gorham method in a custom-designed CVD system. As described in Scheme 1, the precursor was sublimated before being pyrolyzed under vacuum in order to form quinodimethane intermediates. Poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) was then deposited by spontaneous polymerization of these intermediates in the deposition chamber at 15 °C. As confirmed by a scotch tape test[19] and by solubility tests in various media,[23] the polymer films coated on silicon and gold wafers adhered to the substrates. FTIR analyses confirmed the presence of alkyne groups on the CVD surface, as indicated by strong stretching modes of the C−H bonds at 3286 cm^{-1} and the carbon/carbon triple bonds at 2102 cm^{-1}.

Depending on the amount of precursor (3−80 mg) used for CVD polymerization, poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) films (Figure 1a) with various thicknesses were obtained. The experimentally observed thicknesses ranged from 2.7 to 90 nm, as shown in Figure 1b. The coating thickness was determined by ellipsometry and the reproducibility of the process resulted in remarkably small error bars. The thickness of the polymer film increased linearly with the amount of precursor used for CVD polymerization. This observation allows for facile fine-tuning of the coating thickness for the requirements of a given application. Furthermore, ultrathin reactive films of few nanometers can be produced, which may further increase the potential applications of this CVD technique, for instance for coating of biosensors. Even below 20 nm, the thickness of the coating can be adjusted by the precursor amount.

The reactivity of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) was confirmed by spatially directed biomolecular immobilization as described in Scheme 2.

The alkyne functionality was tested against an azido-terminated biomolecule, in the presence of copper(II)sulfate and sodium ascorbate at room temperature. Under these

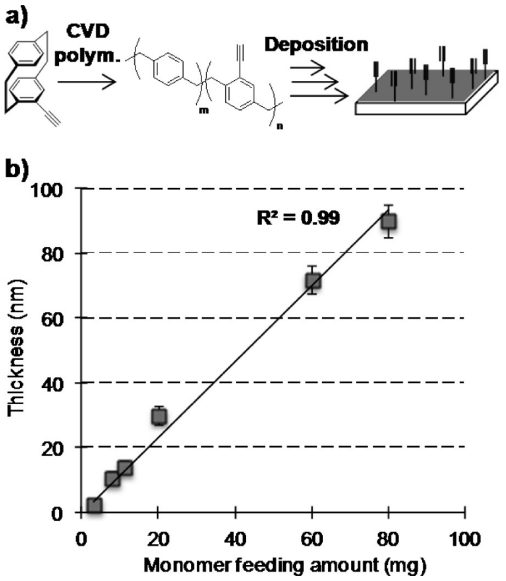
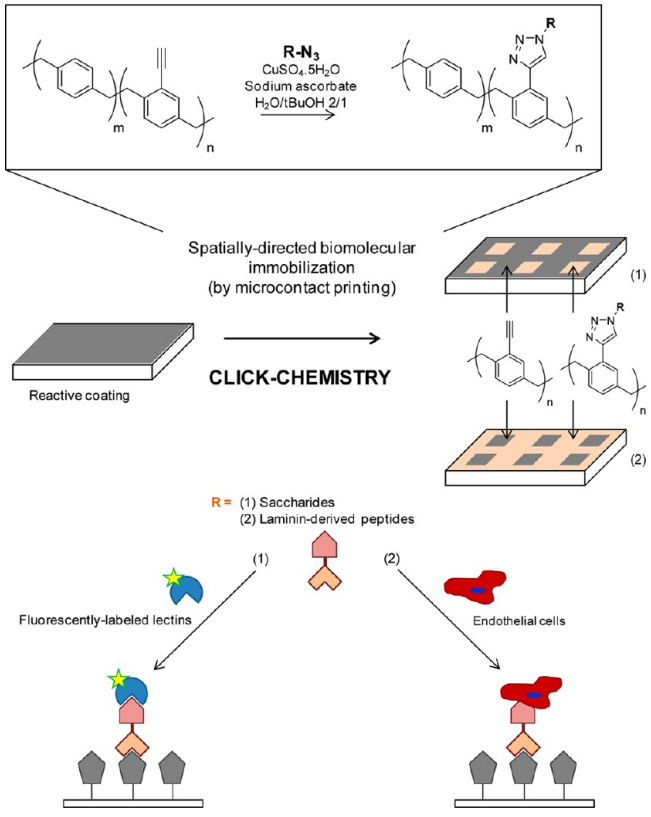


Figure 1. CVD polymerization process for the poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) preparation: (a) illustration of the coating process and (b) polymer film thickness as a function of the feeding amount of precursor.

Scheme 2. Schematic Illustration of the Strategy Used for Spatially-Directed Biomolecular Immobilization by Microcontact Printing via Click Chemistry



conditions, copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition was expected to occur on the substrates and bind biomolecules onto the surface through the formation of five-membered heterocyclic triazoles. For this purpose, a poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) coating was exposed to azido-functionalized saccharides (1-azido-1-deoxy-β-D-glucopyranoside and 1-azido-1-deoxy-β-D-galactopyranoside) or azido-functionalized peptides (YIGSR-N_3) solutions. The spatial selectivity of the Huisgen cycloaddition was ensured by microcontact printing of a Cu(I) catalyst. A square-patterned PDMS stamp was inked with copper sulfate and kept in contact with the substrates for several hours. Saccharide-pattering was then evidenced by incubation of the substrates with fluorescently labeled lectins and subsequent imaging by fluorescence microscopy. As shown in Figure 2a, fluorescein-conjugated concanavalin A (FITC-Con A), a β-glucose-binding lectin, was successfully immobilized on β-glucose-modified square islands patterned on the substrate, because fluorescence intensity is higher in the regions where saccharides were previously immobilized. Similarly, Figure 2b confirms the immobilization of β-galactose on the square islands of the patterned substrate, because rhodamine-labeled peanut agglutinin (TRITC-PNA), a specific lectin for β-galactose, was primarily visualized. The slight fluorescence observed outside the square islands probably originates from free residual fluorescently labeled lectin that was not removed by rinsing.

As previously described, patterned peptide-immobilization was also performed by microcontact printing of a laminin-derived peptide (YIGSR) on poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene) coatings. Ellipsometry images shown in Figure 2c reveal a slightly higher thickness of the bioactive coating outside the square islands, which demonstrates a successful immobilization of YIGSR. A thickness difference of about 1 nm in ellipsometry can correspond to a peptide monolayer immobilized on a substrate.[23] This result has been confirmed by the selective adhesion of human endothelial cells (HUVECs). As depicted in Figure 2d, the presence of HUVECs was observed, where the cell-adhesive peptide (YIGSR) was immobilized. These findings confirm an efficient patterning by microcontact printing and good selectivity of the click reaction for spatially directed immobilization of peptide. To avoid uncontrolled cell adhesion, when proteineaceous serum is used, wae also immobilized azido-PEG on the remaining alkyne-terminated areas by microcontact printing prior to cell culture experiments being conducted. As expected, minimal cell attachment was observed on PEG-modified regions.

The microstructuring of different functional groups that can engage in orthogonal click reactions on the CVD substrate was attempted. For this purpose, two successive CVD steps were performed to generate a pattern with multiple functional parylenes through the VAMPIR technique.[40] The CVD coatings were selected to subsequently undergo different click chemistry reactions. First, the sample was homogeneously coated with poly(4-formyl-*p*-xylylene-*co*-*p*-xylylene). The second CVD step then creates the functional pattern by polymerization of poly(4-ethynyl-*p*-xylylene-*co*-*p*-xylylene). To confirm the presence and the reactivity of both functional groups that can undergo subsequent click chemistry reactions, we immobilized two different sugars, mannobiose and azido-terminated galactose, on the microstructured CVD coating, as depicted in Scheme 3.

Mannobiose was first immobilized outside the square islands by reacting it to the aldehyde groups on the coating via aldehyde-hydrazide coupling. This aldehyde-hydrazide coupling was made possible with the help of dihydrazide linkers. We chose adipic acid dihydrazide as the linker because we hypothesized that it has an intermediate chain length that allows just one of the hydrazide moieties to bind to the aldehyde groups present in the CVD coating and the other

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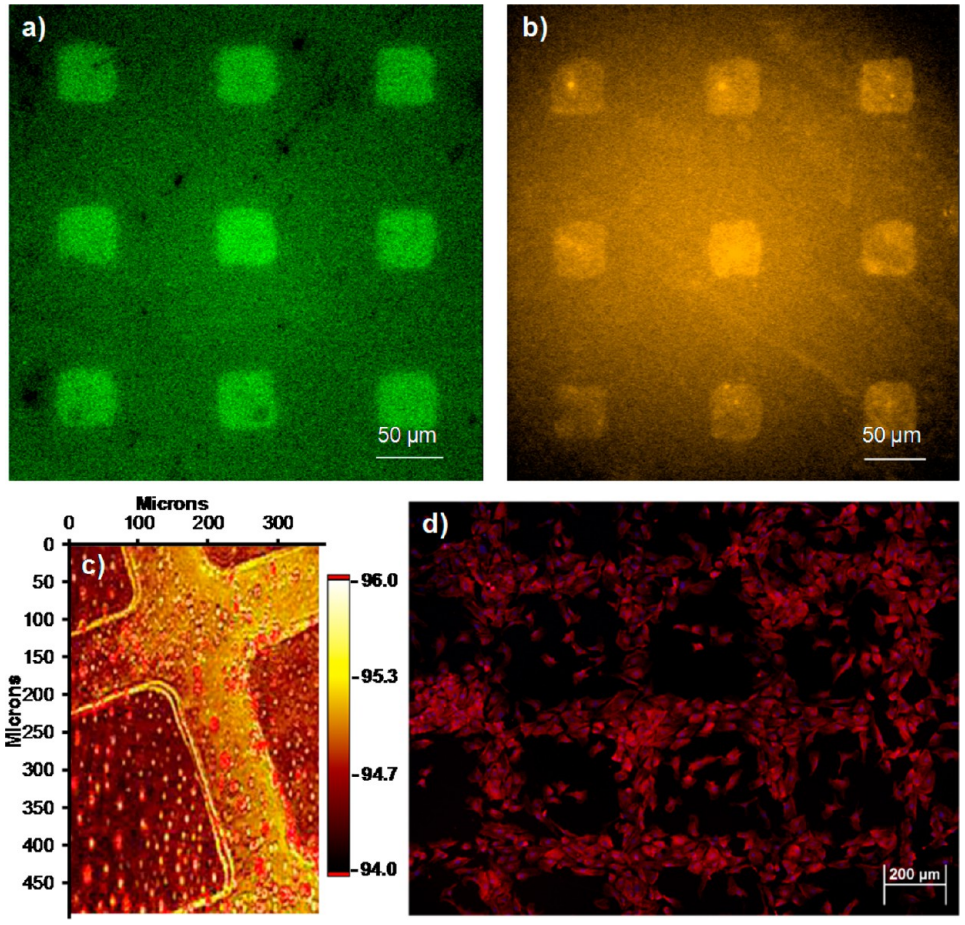
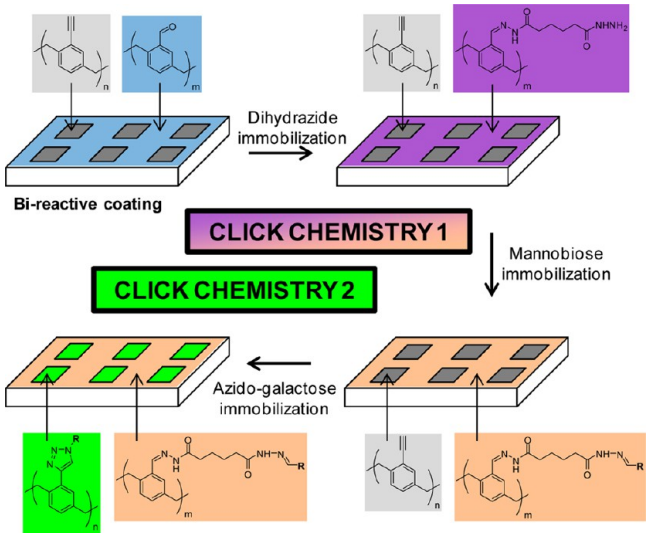


Figure 2. (a, b) Fluorescence micrographs of samples prepared by immobilization of saccharides and fluorescently labeled lectins on the square islands; (c) ellipsometry imaging of samples prepared by immobilization of peptides outside the square islands and (d) fluorescence micrographs of subsequent adhesion of HUVECs. Immunostaining with rhodamine-conjugated phalloidin (for actin) and DAPI (for nucleus) was carried out.

Scheme 3. Schematic Illustration of the Strategy Used for Successive Immobilization of Two Saccharides on Microstructured CVD Coatings; Mannobiose Underwent Aldehyde–hydrazide Coupling (click chemistry 1) with a Dihydrazide Linker Previously Anchored on the Reactive Coating and Azido-Galactose Was Bound via Huisgen Cycloaddition (click chemistry 2)



hydrazide moiety can serve to immobilize mannobiose onto the substrate. The substrate was then immersed in an aqueous solution containing 1-azido-1-deoxy-β-D-galactopyranoside and copper catalyst. Under these conditions, Huisgen cycloaddition occurred with the alkyne groups present on the square islands of the microstructure.

The spatial distribution of the two saccharides was then investigated by immersing the substrate in a solution containing a fluorescently labeled lectin with selective affinity to one of the two sugars. Figure 3 depicts fluorescence micrographs after conjugation of FITC-PNA with galactose (Figure 3a) and of TRITC-Con A with mannobiose (Figure 3 b). The higher fluorescence of FITC-PNA in the squares, where galactose was previously immobilized, and of TRITC-Con A outside the squares, where mannobiose was bound, confirms the selectivity and efficiency of the click chemistry reactions.

CONCLUSIONS

CVD polymerization is a versatile process that can be used to tailor reactive polymer coatings, in terms of chemical functionalities as well as coating thicknesses. Stable, ultrathin reactive polymer coatings can be obtained providing desired functionality while preserving properties inherent to the bulk material. Copper-catalyzed Huisgen cycloaddition was performed on the reactive coatings by microcontact printing and saccharides and peptides alike can be immobilized. In addition, microstructured coatings provide a versatile route toward the

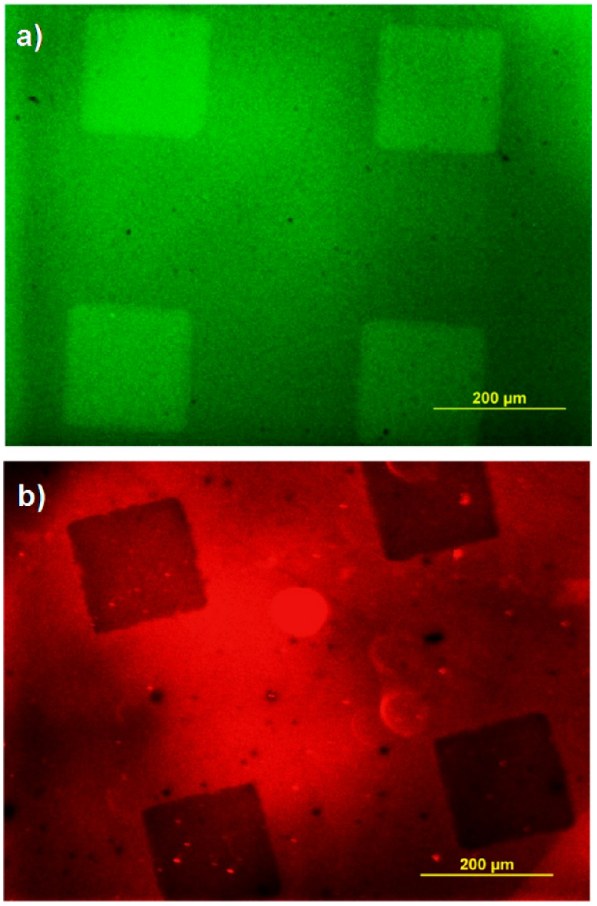


Figure 3. Fluorescence micrographs of microstructured CVD coatings after conjugation of (a) FITC-PNA with galactose and (b) TRITC-Con A with mannobiose; the saccharides being immobilized via click chemistry reactions.

immobilization of various saccharides through multiple click chemistry reactions. As an exemplary demonstration, substituted poly(*p*-xylylene) coatings in conjunction with multiple, orthogonal click reactions were successfully used for spatially directed surface modification and biomolecular immobilization. The strategy developed in this work for surface engineering is versatile enough to be adapted to other anchoring chemistries and also to other biomolecules. The combination of various orthogonal chemistries in a microstructured manner is also a step forward in the development of biomimetic interfaces. Numerous potential applications in biotechnology can be considered from the results of this study. For instance, synthetic substrates for cell coculture, structured microsensors or more generally platforms for biomolecular screening may benefit from selective and multiple immobilizations of biomolecules via orthogonal click reactions, as outlined in this work.

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Notes

The authors declare no competing financial interest.

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