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Two-Layer Architecture Using Atom Transfer Radical Polymerization for Enhanced Sensing and Detection in Complex Media

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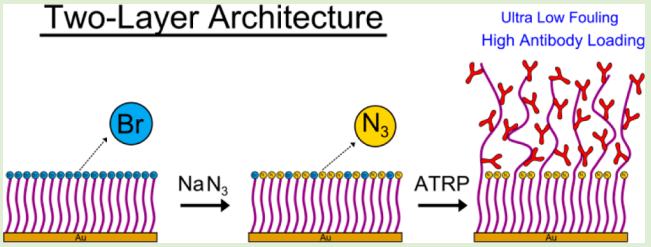
Supporting Information

ABSTRACT: A novel, two-layer hierarchical architecture based on surface-initiated atom transfer radical polymerization was investigated. It combines a thin and highly dense first layer, for nonfouling properties, with a loose second layer for high immobilization levels of active biomolecules. Sodium azide treatment, to reduce the concentration of macroinitiators on the first layer for reinitiation, and by controlling the polydispersity allowed one to achieve three polymer architectures with low, moderate, or high azide substitution. Moderate substitution enabled the highest immobilization levels with a nonfouling background. Integration with dual-functional zwitterionic poly(carboxybetaine) made this platform suitable for applications in undiluted complex media such as blood. It was demonstrated via a surface plasmon resonance biosensor that antigen accessibility and antibody loading were greatly improved. These results indicate the two-layer strategy as a generic concept suitable for applications from diagnostics to medical coatings in order to maximize and minimize specific and nonspecific responses, respectively.



INTRODUCTION

The development and implementation of new point-of-care diagnostic devices, drug delivery platforms, and therapeutics has the potential to offer significant benefits to the health of society.[1−3] However, the realization of this concept relies on both the discovery of biomarkers, which allow a given disease to be identified and monitored, as well as the development of assays, which can be used for actual detection. Due to the ease of accessibility as well as the ability to represent dynamic physiological and abnormal processes, human plasma and serum are the most common sources for biomarker analysis.[4] However, the lack of a means to sensitively detect these target analytes at or below nanogram/milliliter quantities buried in the comprehensive proteome of human blood (thousands of core proteins which span more than 10 orders of magnitude in concentration), has severely hindered the biomarker development process due to the inability to fully verify the analyte as being clinically relevant.[5−7]

The two major shortcomings that limit biomarker validation include high rates of false positives and a lack of sensitivity.[8,9] False positives are typically a result of the inability to prevent nonspecific protein adsorption thereby resulting in a high background noise leading to an incorrect diagnosis.[10] The lack of sensitivity arises from the limited immobilization capacity of molecular recognition elements (e.g., antibodies), which can bind the target of interest. The combined result of these two factors is the inability to achieve a high signal-to-noise ratio (S/N), which is necessary for detection. While protein-resistant materials have been utilized to aid in the reduction of background noise, attempts to circumvent the lack of sensitivity have almost solely relied on signal amplification.[11,12] However, this methodology requires the use of two antibodies or ligands (e.g., ELISA) per target analyte. The expense and difficulty of manufacturing large amounts of high affinity and specific antibodies (or ligands), in addition to their limited stability, make this a nonviable approach for the detection of analytes.[9]

Surface-grafted polymer films composed of zwitterionic carboxybetaine (CB) have been shown to be highly attractive materials for protein-resistant applications.[11] These films exhibit ultra low fouling properties to undiluted human serum and plasma (i.e., <5 ng/cm^2 of nonspecific protein adsorption) and possess abundant functionalizable groups that enable antibody immobilization via amino-coupling chemistry. Following deactivation, poly(CB) (pCB) returns to its original zwitterionic state allowing for the sensitive detection of target analytes from undiluted complex media.[2,13] However, such two-dimensional (2D) films have the limitation of a low ligand immobilization capacity (i.e., only an effective monolayer of protein (~250 ng/cm^2) can be achieved).[13,14] Substrates based on carboxymethylated-dextran commonly used on Biacore sensor chips have been shown to enable more than 1000 ng/cm^2 of antibody immobilization due to a "loose" three-dimensional (3D) polymer structure.[15] However, such a loose architecture is naturally susceptible to large amounts of nonspecific adsorption from undiluted complex media, such as human plasma and serum, thereby severely limiting its use in real-world applications.[16]

Received: August 25, 2012
Revised: October 25, 2012

   dx.doi.org/10.1021/bm301335r | Biomacromolecules XXXX, XXX, XXX−XXX

An additional approach for improving antibody grafting mobility, density, and activity utilized a living radical photopolymerization surface chemistry with acrylated whole antibodies. In these works, substrates were first photopolymerized from a mixture of urethane and triethylene glycol diacrylates, initiator, and deactivator and then reinitiated in the presence of acrylated antibody and a poly(ethylene glycol) (PEG) monoacrylate, which was necessary to resist nonspecific protein adsorption. The mobility and surface coverage of the grafted antibody could be controlled by adjusting the concentration of reinitiating species on the substrate or the antibody polymerization solution composition. This platform was shown to increase accessibility of antigen binding sites and enhance detection. However, this approach achieved only low levels of covalently attached antibody (1−2 ng/cm^2) and could only undergo detection using diluted complex media at 20% (v/v).[17,18]

We have recently demonstrated a novel two-layer architecture with pCB.[19] This hierarchical architecture involves the combination of a thin and highly dense first layer to provide ultra low fouling properties from undiluted human plasma with a loose and controlled second layer to enable high levels of antibody immobilization (i.e., multiple monolayers) and improved antigen detection. This integration of the 2D with the 3D to create a novel yet generic approach to biosensing can be used to achieve high sensitivity without the need for signal amplification. These initial experiments utilized two controlled living radical polymerizations: surface-initiated atom transfer radical polymerization (SI-ATRP) and photoiniferter-mediated polymerization (SI-PIMP). For proof-of-concept experiments, only one single condition was chosen for each to illustrate the two-layer concept.

Here, a systematic investigation of the individual parameters associated with the SI-ATRP approach was performed. As this method relies on azide treatment of the bottom layer for controlling the second layer graft density, this work investigated the effect of azide treatment (i.e., looseness of the top layer), the second layer polymerization rate (i.e., the polydispersity or polymer density), as well as alternative conditions for growing the initial bottom layer, as a function of the antibody immobilization capacity, antigen binding, and nonfouling properties to undiluted human serum using a surface plasmon resonance (SPR) biosensor. While SPR with pCB was chosen here, due to the ideal dual-functional properties of pCB for sensing and detection in complex media, the conclusions of this work can be readily applied to many other platforms and surface chemistries.

EXPERIMENTAL SECTION

Materials. Copper(I) bromide (99.999%), 2,2′-bipyridine (bpy, 99%), tetrahydrofuran (THF), methanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffered saline (PBS, 0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4), anhydrous acetone, and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol (200 Proof) was purchased from Decon Laboratories (King of Prussia, PA). Sodium carbonate anhydrous and sodium azide were purchased from EMD Chemicals (Darmstadt, Germany). Sodium chloride (NaCl) and ether were purchased from J.T. Baker (Phillipsburg, NJ). Sodium acetate anhydrous was purchased from Fluka (subsidiary of Sigma Aldrich, St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Acros Organics (Geel, Belgium). *β*-Propiolactone was purchased from Alfa Aesar (Ward Hill, MA). *N*-[3-(Dimethylamino)propyl] acrylamide (DMAPA, 98%) was purchased from TCI America (Portland, OR). Pooled human serum was purchased from Biochemed Services (Winchester, VA). Water was purified using a Millipore water purification system with a minimum resistivity of 18.2 MΩ cm. Mercaptoundecyl bromoisobutyrate (SI-ATRP initiator) was synthesized as described previously.[20]

Monoclonal antibody to human thyroid stimulating hormone (anti-TSH) and recombinant human TSH antigen were purchased from ThermoFisher Scientific (Waltham, MA). According to the manufacturer, anti-TSH is an IgG antibody produced in mice whose specificity was verified using ELISA, a radio-immuno-assay, and an immuno-precipitation assay while also showing reactivity with human samples. The concentration of TSH in normal human serum is ∼0.1 ng/mL.[21]

Methods. *Synthesis of Carboxybetaine Acrylamide Monomer.* (3-Acryloylamino-propyl)-(2-carboxy-ethyl)-dimethyl-ammonium (CB) was synthesized by reacting 48 mL of DMAPA with 25 g of *β*-propiolactone in 400 mL of anhydrous acetone at 0 °C under nitrogen. After removing the ice bath at 20 min, the solution was allowed to warm up to room temperature. After 6 h, the product was filtered, washed with ether, and dried under vacuum. The rough product was redissolved in a 30% (v/v) triethylamine in methanol solution and stirred overnight. After concentrating the solution, the CB was precipitated with acetone and filtered. The white solids were suspended in acetone and ether, for 1 h each, filtered, dried under vacuum, and stored at 4 °C. Yield: 61%. ^{1}H NMR (Bruker 500 MHz, DMSO-d_6): 8.61 (t, 1H, N−H), 6.28 (t, 1H, CHH=C*H*), 6.13 (t, 1H, C*H*H=CH), 5.61 (t, 1H, C*H*H=CH), 3.44 (t, 2H, N−C*H*$_2$-CH$_2$−COO), 3.21 (m, 4H, NH−C*H*$_2$-CH$_2$-C*H*$_2$), 2.97 (s, 6H, N-(C*H*$_3$)2), 2.25 (t, 2H, C*H*$_2$-COO), 1.87 (t, 2H, NH-CH$_2$-C*H*$_2$- CH$_2$).

Preparation of Two-Layer pCB Films. SPR chips coated with initiator self-assembled monolayers were prepared by soaking the gold-coated substrates in 0.1 mM mercaptoundecyl bromoisobutyrate in pure ethanol for overnight. The chips were then removed, rinsed with ethanol, THF, and ethanol, and blown dry using filtered compressed air and placed into a custom glass tube reactor. In a separate glass tube, 8.86 mg CuBr, 57.87 mg bpy, and 600 mg of CB were added. Both tubes were then placed under nitrogen protection. Nitrogen-purged methanol (4 mL) was then added to the solids. Once everything was completely dissolved (∼ 15 min), the mixture was transferred to the reactor and allowed to react for 24 h at 25 °C in a shaker. The reaction was removed and quenched with a solution containing methanol (4 mL) and $CuBr_2$ (275.87 mg). The chips were then rinsed with methanol and water and submerged in PBS.

To adjust the concentration of bromine groups and replace them with nonreactive azide moieties, the chips were submerged in an aqueous solution of sodium azide (6.5 mg/mL) and mixed at room temperature. After a specific time, the chips were removed, dipped in PBS, rinsed with water, dried, and then placed into the glass reactor for reinitiating ATRP. The second layer was then grown by repeating the above protocol and adjusting only the solvent ratio between nitrogen-purged methanol and water. For example, a water content of 20% consisted of 0.8 mL of water and 3.2 mL of methanol. The reinitiated polymerization was allowed to react for 3 h at 25 °C in a shaker. Following the reaction, the chips were rinsed with copious amounts of water and submerged overnight in PBS.

SPR Sensor, Chips, and Calibration of the Surface Sensitivity. A laboratory SPR sensor developed at the Institute of Photonics and Electronics, Prague, Czech Republic, was used as described previously.[14] This custom-built SPR is based on the attenuated total reflection method and wavelength modulation. It is equipped with a four-channel flow cell, temperature control, and uses a peristaltic pump for delivering samples. SPR sensor chips were made of a glass slide coated with titanium film (∼2 nm) followed by a gold film (∼48 nm) using an electron beam evaporator. As the SPR sensitivity depends on the distance of the binding event from the SPR active gold surface, the sensor response due to the polymer films had to be calibrated. This was done using previously described methods.[13]

Determination of Polymer Film Thickness, Refractive Index, and Polymer Density. Film thicknesses and refractive index measurements

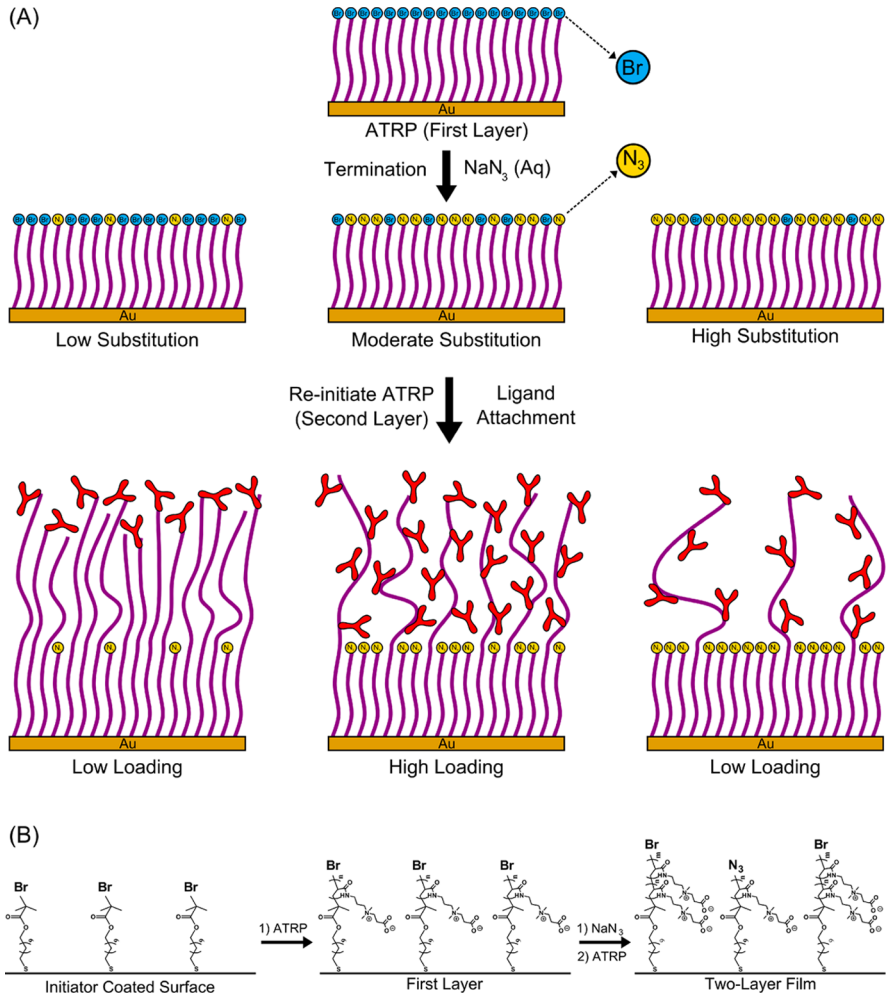


Figure 1. (A) A novel strategy for improving sensing and detection from undiluted complex media using a unique "two-layer" architecture integrated with zwitterionic dual-functional pCB via SI-ATRP. (B) The polymerization scheme for the "two-layer" pCB films.

were determined using a multiwavelength ellipsometer (J. A. Woollam Co., Inc., Model α-SE). A liquid cell with a volume of 0.5 mL supplied by the manufacturer was used for obtaining the liquid measurements. The data was analyzed via fitting a Cauchy model for a bare SPR substrate which enabled the film thickness and refractive index to be simultaneously determined. The wet and dry refractive index measurements were then used with the effective medium approximation for calculating the polymer volume fraction (i.e., the polymer density) as described previously.[22] The data presented are the average values and one standard deviation of three independent measurements.

Measurements of Nonspecific Protein Adsorption. The nonspecific protein adsorption of the pCB films was determined with a SPR biosensor using a flow rate of 50 μL/min at 25 °C. After first establishing a baseline using PBS, undiluted human serum was flowed for 10 min, followed by buffer to re-establish the baseline. Protein adsorption was quantified as the difference between buffer baselines and converted to a surface coverage using the appropriate sensitivity factor.

In Situ Functionalization of pCB Polymer Surfaces. The functionalization procedure was monitored step-by-step in real time using an SPR sensor at 25 °C. Sodium acetate buffer (10 mM) at pH 5.0 (SA) was first injected at 30 μL/min to obtain a stable baseline. Carboxylate groups on the polymer were then activated by flowing a solution of 0.05 M NHS and 0.2 M EDC in water for 7 min. Followed by a brief injection of SA buffer, anti-TSH (50 μg/mL) in 10 mM HEPES buffer (pH 7.5) was flowed for 20 min at 20 μL/min. Subsequent washing for 10 min with 10 mM sodium carbonate (pH 10) containing 0.3 M NaCl (SC) at 30 μL/min removed noncovalently bound ligands and deactivated residual NHS-esters. SA buffer was then used to re-establish a stable baseline. The amount of immobilized antibody was determined as the difference between the SA injection following EDC/NHS activation and the final baseline. The data representing antibody surface coverage as a function of the azide exposure time shows the average values and one standard deviation of three independent measurements.

Measurements of Post-Functionalized Nonfouling and Specific Protein Activity. Following antibody immobilization, the pCB surface was washed with PBS until a steady baseline was established at 50 μL/min and 25 °C. For post-functionalized nonfouling, undiluted human serum was injected for 10 min followed by PBS. The net adsorption was calculated as the difference between buffer baselines and converted to a surface coverage. Antigen detection for each polymer film was compared by measuring the maximum sensor response achieved for a fixed injection time. After establishing an initial buffer baseline, PBS spiked with TSH at 1000 ng/mL was flowed through the sensor for 10 min, followed by buffer. The antigen binding was calculated as the difference between the original buffer baseline and the maximum signal observed, which was then converted to a surface coverage using the appropriate sensitivity factor. The data representing antigen detection as a function of the azide exposure time shows the average values and one standard deviation of three independent measurements.

RESULTS AND DISCUSSION

The strategy for developing the novel two-layer architecture via SI-ATRP for sensing and detection in complex media and the

dx.doi.org/10.1021/bm301335r | *Biomacromolecules* XXXX, XXX, XXX–XXX

corresponding polymerization scheme is shown in Figure 1. SI-ATRP was used due to its ability to provide excellent control over polymer growth. Additionally, the "living" characteristic enabled by the reversible equilibrium between active and dormant species allows for the formation of block copolymers due to the presence of initiating species which cap the chain ends upon stopping the polymerization.[23] The use of this hierarchical architecture enables one to achieve a thin and highly dense first layer to provide ultra low fouling properties with a loose second layer for high levels of antibody immobilization. The combined result of these two factors provides the high S/N necessary for enhanced detection sensitivity.

As shown in Figure 1A, the concentration of reinitiating groups (i.e., bromines) on the surface is vital for controlling the second layer graft density. The bromine end groups can be reduced by reacting with an aqueous sodium azide solution to elicit a dramatic effect on the architecture of the subsequent two-layer film as well as its biosensing performance. This termination forms a stable azide group which remains dormant and nonreactive as the second polymerization takes place.[24,25] Both the concentration and reaction time of sodium azide can affect the degree of termination resulting in surfaces which are low, moderately, or highly substituted with azide moieties. A low degree of substitution will result in most chains being reinitiated, thus maintaining a relatively high polymer density and allowing for only monolayer antibody immobilization. For polymer chains to grow vertically, they require a certain graft density to stretch away from the surface. However, if they are too dilute, then the chains will preferentially grow laterally in a mushroom configuration or along the top of the film.[26] In this highly substituted and very dilute case, the second layer will be thin, consisting of a small number of long chains with minimal surface area available for antibody immobilization, also resulting in a low loading capacity. Therefore, to achieve high antibody loading a moderate amount of substitution is desired. In this scenario, the chains will be close enough to be obliged to grow vertically but dilute enough so as to maximize the polymer surface area for achieving high protein functionalization and enable sufficient diffusion of the ligand to reactive NHS-esters. It is also important to note that a second layer grown from a moderately substituted bottom layer can grow longer (i.e., thicker) than from a low azide substituted film. This is due to a reduction in bimolecular termination as a result of more space between the growing polymer chains in addition to the polymerization rate.[27]

In this work, a systematic investigation of the individual parameters associated with the two-layer SI-ATRP approach was performed. In order to achieve the best control over the second layer graft density, conditions for growing the bottom layer which maximized the number of reinitiating sites and enabled ultra low fouling polymer brushes were first investigated. Next, the effect of azide treatment (i.e., looseness of the top layer) and the second layer polymerization rate (i.e., the polydispersity or polymer density) were studied as a function of the antibody immobilization capacity, antigen binding, and nonfouling properties to undiluted human serum using a SPR biosensor.

It was previously shown that thin (~10 nm) and highly dense pCB films made via SI-ATRP from pure methanol as the solvent can maintain ultra low fouling properties to undiluted human serum.[22] The ability to minimize the final overall thickness of the two-layer film is also crucial for SPR detection among other sensing platforms. For SPR, this is due to a rapid decrease in sensitivity as the measured biomolecular interaction gets further from the SPR active gold surface.[28] Hence, pure methanol, due to its ability to obtain ultra low fouling thin films while minimizing chain−chain termination events, was also used in this work. This, combined with the use of quenching (i.e., spiking the reaction solution with a high concentration of deactivator (e.g., $CuBr_2$)) to stop the polymerization, was vital for providing the maximum number of reinitiating sites and controlling the second layer graft density.[29]

The effect of polymerization times (0.5−24 h) for the bottom layer was first investigated. It was found that 24 h resulted in the thickest second layer (made using a 50% water content), the highest antibody immobilization, and excellent functionalized and nonfunctionalized protein resistance to undiluted human serum (Supporting Information, Table S1 and Figure S1). The response to antigen was similar for all first layer polymerizations greater than or equal to 1 h, with bioactivity ratios (i.e., the molar ratio of antigen to antibody) of 0.98, 0.92, and 0.82 for reaction times of 1, 2, and 24 h, respectively. The similar antigen responses combined with the 24 h result having the lowest bioactivity ratio, even though it had the highest antibody loading, illustrates that both antibody immobilization as well as antigen accessibility are important for achieving the high S/N necessary for sensitive detection. The 24 h reaction enabled the same level of antibody immobilization on the two-layer film as that achieved from a single layer grown using a 50% water content (~290 ng/cm^2). A first layer made using a 50% water content was also explored (Supporting Information, Figure S2). However, this condition was very difficult to control even with short reaction times on the order of just a few min, resulting in the final two-layer polymer structure being mostly determined by the rapid polymerization of the bottom layer. Furthermore, simply spiking solutions grown from a first layer of either 50% water or pure methanol with a high concentration of monomer dissolved in pure nitrogen-purged water was also not found to be effective. Due to the ability to achieve ultra low fouling properties for the two-layer films with a 24 h first layer polymerization time, such conditions were then used throughout the remainder of this work.

As shown in Figure 1A, the use of azide substitution can result in three hierarchical architectures. The effect of azide treatment on the second layer polymer thickness and the corresponding antibody immobilization for two different second layer solvent conditions is shown in Table 1 and Figure 2, respectively. Due to the significant influence of water on the polymer density of SI-ATRP films,[22] two concentrations were used. A 10 and 50% water content was chosen for slower (more

Table 1. Effect of Azide Exposure Time on the Second Layer Polymer Thickness for Second Blocks Grown Using SI-ATRP Water Contents of 10 and 50% (v/v)

azide exposure time (min)	second layer thickness (nm; 10% water)[a]	second layer thickness (nm; 50% water)[a]
0	4.6 ± 1.7	5.6 ± 1.1
15	8.8 ± 1.5	3.4 ± 0.4
30	3.1 ± 0.5	6.8 ± 0.1
60	1.7 ± 0.1	13.8 ± 0.4
120	2.5 ± 0.5	9.9 ± 0.7

[a]The thickness of the first layer was ~6 nm.

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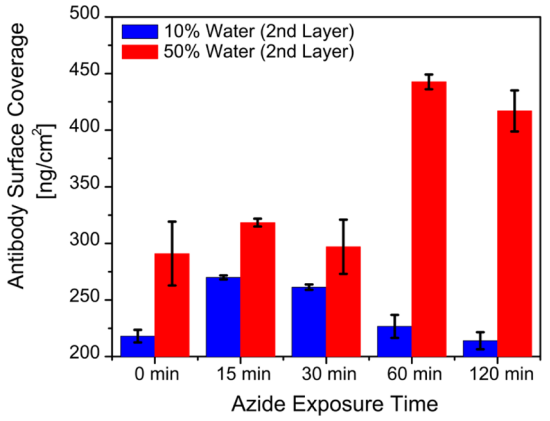

Figure 2. Antibody immobilization vs azide exposure time for two-layer pCB films grown using SI-ATRP water contents of 10 and 50%.

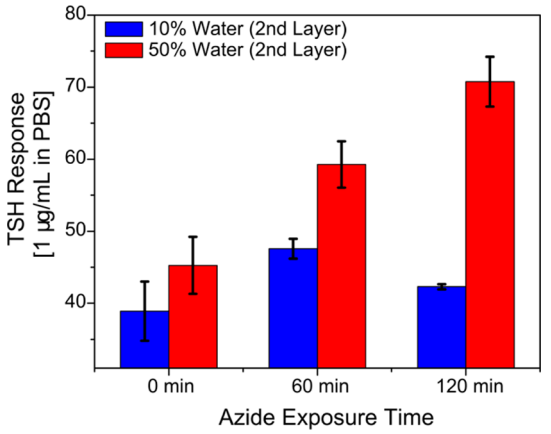

Figure 3. Comparison of the antigen responses versus azide exposure time for second layers made using 10 and 50% water contents. PBS spiked with antigen (1 μg/mL) was flowed for a fixed time of 10 min.

controlled) and faster (less controlled but more polydisperse) polymerizations, respectively.[22,30] As shown in Table 1 and Figure 2, both conditions resulted in the expected trends as discussed above for Figure 1A. A reduction in the concentration of reinitiating species generally lead to an increase in the film thickness which then decreased as the chains became more dilute. It was also observed that as the second layer thickness increased, the antibody immobilization also increased. As a moderately substituted second layer should result in the thickest film, this indicates that such films have more surface area (i.e., more accessible NHS-esters) available for protein attachment. Thus, azide treatment can be used to maximize antibody immobilization due to its effect on both the thickness (i.e., the chain length) and chain density of the second layer. However, as the chains became more dilute (i.e., more azide substitution), the available surface area decreased along with the immobilization. As shown in Figure 2, the highest level of immobilization achieved was ~440 ng/cm^2, which was significantly more than that achieved for a single layer film grown from pure methanol (~210 ng/cm^2) or a 50% water content (~290 ng/cm^2).[19] While the ability to achieve high levels of antibody loading is important, it must also be balanced with the need to allow for sufficient diffusion of the desired target analyte into the film to obtain a large sensor response, in addition to the ability to reduce background noise arising from nonspecific adsorption.

The antigen response for azide exposure times of 0, 60, and 120 min using films made with second layer water contents of 10 and 50% is shown in Figure 3. For second layers grown using a 10% water content, an effective monolayer of antibody was achieved for all exposure times leading to all films exhibiting similar antigen responses. The two-layer film made from a 50% water content and a 0 min azide exposure time exhibited the smallest level of immobilization and hence provided the smallest amount of antigen binding. Interestingly, the antigen binding for the 60 min exposure time was significantly less than that for 120 min despite the higher level of antibody loading for the former (i.e., a lower bioactivity ratio). For these experiments, PBS spiked with 1 μg/mL TSH was flowed over the surface for a fixed time of 10 min. For all experiments except the 60 min exposure time with a 50% second layer water content, the 10 min time period was sufficient to nearly saturate antigen binding. Thus, while the result for the 60 min case shown in Figure 3 does not represent the fully saturated value, which would be larger, it does imply that the polymer density of the second block is higher relative to the 120 min exposure time. This indicates that the ability of the antigen to diffuse through the film was more restricted compared to the 120 min film which would be expected as the azide substitution reaction time increased. As a reference, a single layer pCB film made via pure methanol with ~210 ng/cm^2 of antibody immobilization gave a response of ~39 ng/cm^2.[19]

Importantly, a comparison of Table 1 with Figures 2 and 3 reveal that, while the thickest film did enable the highest degree of antibody immobilization, it did not enable the best detection due to the inability of the target analyte to rapidly diffuse through the film. Hence, these results indicate that there must be a balance between azide treatment, second layer thickness (i.e., chain length), and antibody immobilization level in order to achieve the best detection. While antibody immobilization and antigen accessibility are important for achieving a high sensor signal, the amount of sensor noise or protein fouling can significantly influence the limit of detection. Thus, the nonfouling properties of the two-layer films were subsequently investigated.

The nonspecific protein adsorption from undiluted human serum as a function of azide exposure time is shown in Figure 4. All cases except the 60 min azide time with a second layer

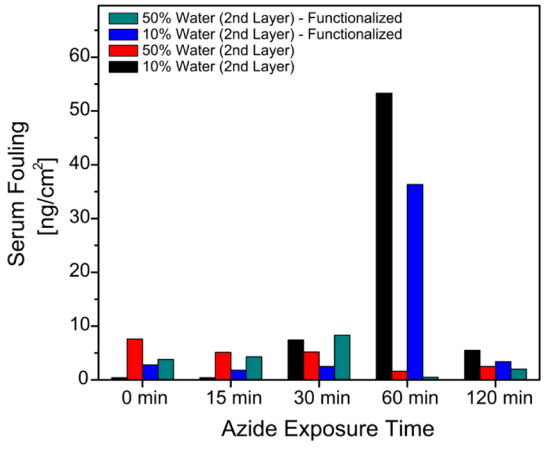

Figure 4. Fouling to undiluted human serum vs azide exposure time for second layer films made using 10 and 50% water content.

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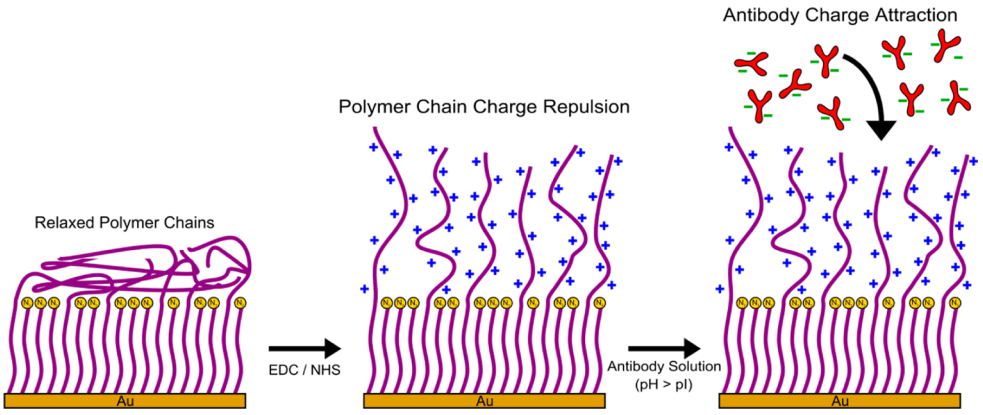

Figure 5. The influence of EDC/NHS coupling chemistry on "two-layer" pCB films. Activation of the carboxylate groups on the relaxed polymer chains into NHS-esters leads to a net positive charge, forcing them to extend due to charge repulsion. The addition of antibody at a pH greater than the isoelectric point of the protein permits rapid accumulation of the negatively charged antibody to the positively charged polymer chains thereby enabling immobilization.

grown from 10% water content resulted in excellent functionalized and nonfunctionalized protein-resistant properties, with most being at or below ultra low fouling levels. This also indicates that the azide moiety itself does not affect the protein-resistant properties of the film. Due to the natural concentration of TSH in human serum being very low, ~0.1 ng/mL,[21] the low post-functionalized serum fouling results (typically <5 ng/cm^2) shown in Figure 4 also demonstrate that the response to 1 μg/mL of TSH spiked into PBS (up to 75 ng/cm^2, Figure 3) was due to only specific binding with the immobilized antibody.

However, a relatively high fouling result achieved with a second layer water content of 10% following a 60 min azide treatment was observed. This can be simply explained as a highly azide substituted film with long and dilute second layer polymer chains. Evidence for this explanation is provided by the SPR sensor-grams (Supporting Information, Figure S3) that compare this film with a typical response (Figure S3, 50% water) which was observed for all but one other two-layer film in this work. As shown in Figure S3, the film made from 10% water content revealed both faster binding during the antibody immobilization step as well as a larger decrease in response upon the injection of deactivation buffer.

Zwitterionic pCB films take on a slight positive charge following EDC/NHS activation.[13] This forces the long and dilute chains for the 10% water condition to extend from the surface due to charge−charge repulsion. Using an immobilization buffer pH of 7.5, anti-TSH maintains a partial negative charge. Thus, the charge interaction between the surface and protein will result in a rapid accumulation of antibody near the surface due to easily accessible and dilute polymer chains. These two effects are illustrated in Figure 5. However, for the 10% water condition, the limited accessible polymer surface area of these chains prevents most of the protein from being covalently bound. Upon deactivation, the surface regains its zwitterionic backbone and as a result the proteins are washed away which can be seen as a large drop in the SPR response upon injection of the deactivation buffer (Figure S3). It is believed that this particular conformation of collapsed polymer chains may increase the hydrophobicity of the surface by exposing more of the polymer backbone and thereby lead to the observed increase in fouling.

It is important to note that due to the combination of abundant carboxylate groups in pCB films which become activated with NHS-esters and the presence of numerous primary amines on antibodies, there is the potential for cross-linking between polymer chains to occur via binding to multiple sites on the protein, if the chains are too close together (i.e., not "loose" enough). A significant amount of cross-linking would drastically reduce antibody immobilization levels and also worsen the ability to detect antigen, due to fewer antibody receptors being present as well as restricted diffusion throughout the film. This was resolved with the two-layer approach via reducing the density of the second layer so as to achieve far enough spacing between polymer chains which minimizes the potential for cross-linking while maximizing the surface area available for immobilization. Due to achieving high immobilization levels for both second layer films made using 50% water content for azide exposure times of 60 and 120 min, as mentioned above for Figure 3, it is unlikely that a significant amount of cross-linking occurred. Hence, the significant increase in the antigen response combined with ultra low fouling properties indicates that this novel architecture has the potential to dramatically enhance diagnostic device performance. The ability of the azide group to react with terminal bromines was important for controlling the "looseness" of the two-layer films. However, adjusting the second layer polymerization rate (i.e., the polydispersity or polymer density) also influenced the film properties.

Due to the ability of the water concentration to significantly affect the rate of the ATRP reaction and subsequent polymer density, the amount of water in the second layer polymerization was varied from 0−90% (v/v) using a 60 min azide treatment time as this condition enabled the highest antibody immobilization.[22] The functionalized and nonfunctionalized serum fouling, antibody immobilization, and antigen response are shown in Figure 6. A peak in the antibody immobilization was found for the 50% water condition. Further investigation of these films by calculating the polymer volume fraction (PVF) in PBS (i.e., the wet polymer density) found that this particular condition had an effective PVF of ~25%, whereas all other films except for those made using a 0% water content second layer had a PVF of ~43% (Supporting Information, Figure S4). A lower value for the PVF indicates a more dilute polymer film. Hence, based on PVF alone, the film made using a 50% water content would be expected to yield the highest level of immobilization due to having the most "loose" structure. All second block thicknesses for each condition are shown in the

dx.doi.org/10.1021/bm301335r | *Biomacromolecules* XXXX, XXX, XXX−XXX

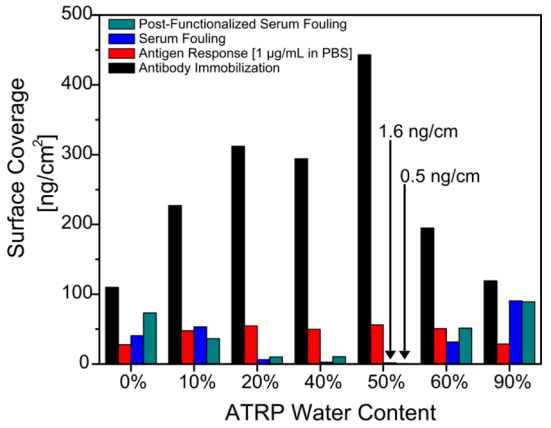

Figure 6. Effect of water concentration on the second layer polymerization for 60 min azide-treated films and the subsequent antibody immobilization level, antigen response, and nonfouling properties.

Supporting Information, Table S2. Note that the film made using a 0% water second layer condition resulted in a lower thickness in PBS than in air, which could be indicative of its slightly hydrophobic character and explain the correspondingly high fouling result.

The two-layer films made from 20−50% water all resulted in excellent nonfouling properties, had the highest level of immobilization, and also had the highest second layer thickness, again illustrating the trend from Figure 1A. It is believed that the two-layer films made from 0% water content grew a very small second layer due to the limited solubility of the highly dense first layer polymer brush in pure methanol, resulting in few polymer chains actually being reinitiated. Additionally, the significantly low level of immobilization (~100 ng/cm^2) indicates that very few functionalizable groups were accessible. This could be interpreted as laterally grown polymer chains covering the surface, interacting with the bottom layer via ion-pairing intermolecular interactions and resulting in the hydrophobic acrylamide backbone being exposed, leading to the high fouling observed.

Beyond the 50% water condition, the films appear to be much more affected by the balance between improved solubility at higher water concentrations, which improves the accessibility of the terminal ends of the bottom layer, with the loss of control in the ATRP reaction.[27] The 60% water condition resulted in a similar SPR response during immobilization as for the 10% water case and thus it is probable that a similar phenomenon, long and dilute chains, occurred as illustrated by its SPR sensor-gram (Supporting Information, Figure S3). However, increases in chain−chain termination reactions as a result of the loss of control are likely to have further reduced the effective area for immobilization. The 90% water result was attributed to a significant presence of radical recombination reactions with the same but more pronounced negative consequences as for the film made using 60% water. With the exception of the two extreme end points (films made using 0 and 90% water contents), the similar antigen response corresponds with the expected result for 2D surfaces (~39 ng/cm^2), with the 50% case simply having a low rate of diffusion as discussed above.

CONCLUSIONS

The two major shortcomings that severely limit biomarker validation and the advancement of personalized medicine include high occurrences of false positives, from nonspecific binding, and a lack of sensitivity, due to low ligand loading. In this work, a unique two-layer architecture made via SI-ATRP with zwitterionic dual-functional pCB was investigated. This strategy consists of a highly dense and ultra low fouling bottom layer with a loose second layer to enable high antibody immobilization. In this work it was found that the polymerization of the bottom layer had a significant effect on the final film structure and conditions to maximize the number of reinitiating terminal sites was vital for control over the second layer graft density. For SI-ATRP with pCB, this was accomplished with pure methanol as the solvent. Using azide treatment to adjust to the density of the second block resulted in three distinct conformations of the polymer architecture (i.e., with low, moderate, or high substitution). Moderate substitution enabled the highest antibody immobilization, twice that compared to single layer films, with excellent nonfouling properties to undiluted human serum. The results also indicated that antibody immobilization in addition to accessibility was vital for detecting the target analyte and hence for providing the highest possible S/N. The second layer film structure could be further modified by adjusting the water content in the SI-ATRP reaction, thus enabling more control over the film properties. The ideal qualities of pCB integrated with this novel architecture offer the ability to significantly improve detection abilities from undiluted complex media. In summary, these results indicate the two-layer strategy as a generic concept suitable for applications from diagnostics to medical coatings in order to maximize and minimize specific and nonspecific responses, respectively.

ASSOCIATED CONTENT

Supporting Information

The investigation of the first- and second-layer SI-ATRP reaction conditions, SPR sensor-grams, and the polymer volume fraction of the two-layer films. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Defense Threat Reduction Agency (HDTRA1-10-1-0074) and the Office of Naval Research (N000140910137).

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