# Toward protein imprinting with polymer brushes

Bogdan Zdyrko[a)]
*School of Materials Science and Engineering, Clemson University, 161 Sirrine Hall, Clemson, South Carolina 29634*

Olha Hoy and Igor Luzinov[b)]
*School of Materials Science and Engineering, Clemson University, 161 Sirrine Hall, Clemson, South Carolina 29634 and Center for Optical Materials Science and Engineering Technologies, Clemson University, Clemson, South Carolina 29634*

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The authors described an original approach for a surface protein imprinting employing grafting of polymer brushes. Protein molecules were first chemically bound to an ultrathin (1–3 nm) poly(glycidyl methacrylate) reactive polymer layer and later removed by protease treatment. Residual amino acids became grafted to the surface and to a certain extent imitated the surface chemical composition and shape of the template molecule on a nanolevel. The space surrounding the adsorbed biomolecules was modified with grafted poly(ethylene glycol) layer. This led to the formation of islands of spatial nanosized pockets complementary to the protein shape. The adsorbing protein recognized the surfaces imprinted and was anchored to the substrate. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3101907]

## I. INTRODUCTION

The objective of the present study is to describe an approach to fabrication of a synthetic material capable to recognize a specific protein. In general, we have been aiming at generation of a substrate possessing properties of antibodies that are widely used as analytical reagents in clinical and research laboratories.[1] Typical applications include immunoassays and separation, but there is an increased interest in biosensor and biomedical material applications as well. The critical common step in these applications is a selective recognition of the analyte by a substrate since substrate recognition and selectivity are distinctive and vital features of biological systems. To this end, design and synthesis of artificial antibodies with properties similar to the natural ones are important from both scientific and industrial points of view. Important component of the effort is targeting the synthetic analogs that exhibit chemical and thermal stability superior to notoriously unstable natural antibodies.

Recognition mechanism offered by an antibody is based on the correct chemistry arrayed in the correct geometry.[2] Thus, in analogy with nature, the synthetic materials should also possess recognition sites with correct size, functionalities, and stereochemistry. One way of generating synthetic antibodies is via three-dimensional (3D) molecular imprinting introduced by Wulff *et al.*[3] This is a process where functional monomers form a complex with a template molecule with subsequent cross-linking. Removal of the template leaves binding sites complementary to the imprinted molecule.[1] The 3D approach widely explored has been shown to be effective for the construction of synthetic receptors for small molecular structures.[4]

Although creating a molecularly imprinted polymer against small molecules is straightforward now,[5] imprinting of large, labile structures, such as proteins and other biomacromolecules, is still a challenge.[6] Small molecules are stable, and because they are quite mobile, they can easily diffuse inside the polymer matrix and reach the recognition sites. However, a number of inherent properties make protein imprinting problematic. These include, but not limited to, poor diffusivity of the proteins,[7] permanent entrapment,[8] and conformational flexibility.[9] Another factor limiting utilization of the imprinting methods designed for the small molecules is protein solubility.[6,10] The majority of the imprinting procedures are conducted in apolar, organic solvents aiming to maximize electrostatic interactions. However, poor protein solubility in apolar organic solvents makes typical imprinting procedures inappropriate toward protein imprinting. Because of those obstacles, special procedures to address the specific properties of proteins, such as large molecule size, low diffusivity, and a tendency to denaturize, should be applied for the preparation of protein molecule imprints. Prepared recognition sites should be designed for maximum accessibility for template molecules.

The most successful strategies implemented for the large molecules imprinting employ two-dimensional (2D) approaches.[11] These 2D approaches typically rely on the initial interactions between surface and template functionalities. For instance, Shi *et al.*[12] reported a technique to surface imprint proteins in a plasma deposited polymer film. Cavities were created with the orientations and dimensions of the proteins. The beds of the cavities were covered with polysaccharide molecules, capable to form hydrogen bonding with the protein. These polysaccharides showed a distinct selectivity toward imprinted biomolecules through formation of hydrogen bonds. Here, we report another approach for the 2D pro-

[a)]Electronic mail: bogdanz@clemson.edu
[b)]Electronic mail: luzinov@clemson.edu

tein imprinting through a reactive "reading" of the protein shape, which involves two stage surface grafting of (bio) macromolecules.

## II. EXPERIMENT

Highly polished single-crystal silicon wafers of {100} orientation (Semiconductor Processing Co) were used as a substrate. The wafers were first cleaned in an ultrasonic bath for 30 min, placed in a hot piranha solution (3:1 concentrated sulfuric acid/30% hydrogen peroxide) for 1 h, and then rinsed several times with high purity water (18 MΩ, Nanopure).

Glycidyl methacrylate (Aldrich) was polymerized radically to give poly(glycidyl methacrylate) (PGMA), $M_n$ =58 000 g/mol, PDI=1.57. The polymerization was carried out in methylethyl ketone (MEK) (VWR) at 60 °C. AIBN (Aldrich) was used as an initiator. The obtained polymer was purified by multiple precipitations from MEK solution in diethyl ether. PGMA was dissolved in MEK at 0.05% w/v and thin films (~2 nm) were deposited on the substrate by dip coating and annealed for 15 min at 110 °C.

Poly(ethylene glycol) (PEG) monomethyl ether obtained from Polymer Source, Inc. (22 600 g/mol) and Aldrich (5000 g/mol) was modified by succinic anhydride (Aldrich) to obtain carboxy end group derivative (PEGs). Acylation was carried out by refluxing with large excess (approximately 20) of succinic anhydride in tetrahydrofuran (THF). PEG was purified by multiple precipitations from THF solution in diethyl ether.

Bovine serum fibrinogen and bovine serum albumin (BSA) (Sigma) were dissolved in phosphate buffer with *p*H=7.4. Adsorption of the proteins was done from 2 μg/ml for 20 s with the purpose of obtaining separate molecules and from 1 mg/ml for 1 h to get a monolayer of the proteins. Adsorption was done at room temperature (22–25 °C). For the chemical attachment of the protein, samples were placed into the sealed tubes with 100 mg of water. Tubes were kept at 36 °C for 14 h.

Thick layer (approximately 30 nm) of carboxy-terminated PEG was deposited on top of the PGMA layer covered with adsorbed and chemically attached protein and placed in sealed tubes with cyclohexane vapor for grafting. Grafting was done for 20–24 h at 36 °C. After the grafting, wafers were rinsed multiple times with MEK, sonicated for 10 min, to remove ungrafted polymer chains.

To hydrolyze protein on the surface, samples were treated with subtilisin A (EC 3.4.21.62) 1 mg/ml at room temperature of 22–25 °C for 1 h. After rinsing with water, samples were placed in MEK and sonicated to remove any attached enzyme.

Second protein adsorption was done from 1 mg/ml phosphate buffer solution during 1 h at room temperature of 22–25 °C. After adsorption, the wafers were rinsed with water and dried in a clean room.

Ellipsometry was performed with a COMPEL automatic ellipsometer (InOmTech, Inc.) at an angle of incidence of 70°. Original silicon wafers from the same batch and silicon wafers with PGMA layer were tested independently and used as reference samples for the analysis of grafted polymer layers. Ellipsometry and atomic force microscopy (AFM) were done after each stage of the experiments.

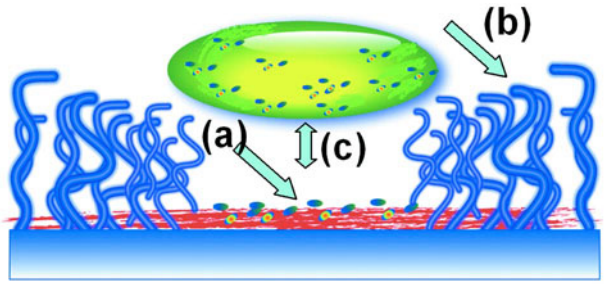


FIG. 1. (Color online) Concept of the artificial antibody. Polymer brush (b) forming cavities (a) complementary to the protein shape. Chemistry at the cavities bottom (c) can selectively recognize imprinted protein.

AFM studies were performed on a Dimension 3100 and MultiMode (Digital Instruments, Inc.) microscopes. We used the tapping mode to study the surface morphology in ambient air. Silicon tips with spring constants of 50 N/m were used. Imaging was done at scan rates in the 1–2 Hz range.

X-ray photoelectron spectroscopy (XPS) (Kratos AXIS 165) was performed in survey regime with Al as a reference. Pass energy of 80 eV was used to obtain element composition of the layers.

XPS was performed at angle of incidence of −90°. This assures sufficient penetration depth for the layers. In fact, all surveys contained Si 2*p* peaks in spectrum originated from Si wafer. This indicates that penetration depth was enough to pass through the whole layer.

## III. RESULTS AND DISCUSSION

Natural antibodies are proteins capable of selectively binding corresponding antigens. Binding event takes place between hypervariable region of the antibody and corresponding part of the antigen called epitope. Epitope binds with antibody in a highly specific interaction that allows antibodies to identify and bind only their unique antigen. A synthetic material, performing similar to an antibody, should also possess regions resembling shape of the template with surface chemistry complementary to the latter. In our study, we built structures imitating ideas utilized by nature for the selective antigen recognition (Fig. 1). Specifically, we create pockets in the polymer brush with low nonspecific protein adsorption. The size and shape of the pockets mimic the size and shape of the template molecule. The bottoms of the pockets are decorated with covalently attached amino acids originated from the protein used as the template (see detailed explanation of the imprinting procedure below). The spatial position of the amino acids corresponds to their position on a template protein surface and might be responsible for the template recognition via hydrogen bonding.

The structures outlined in Fig. 1 were fabricated via several consecutive steps presented in Fig. 2. First, protein molecules were adsorbed on the reactive surface able to chemi-

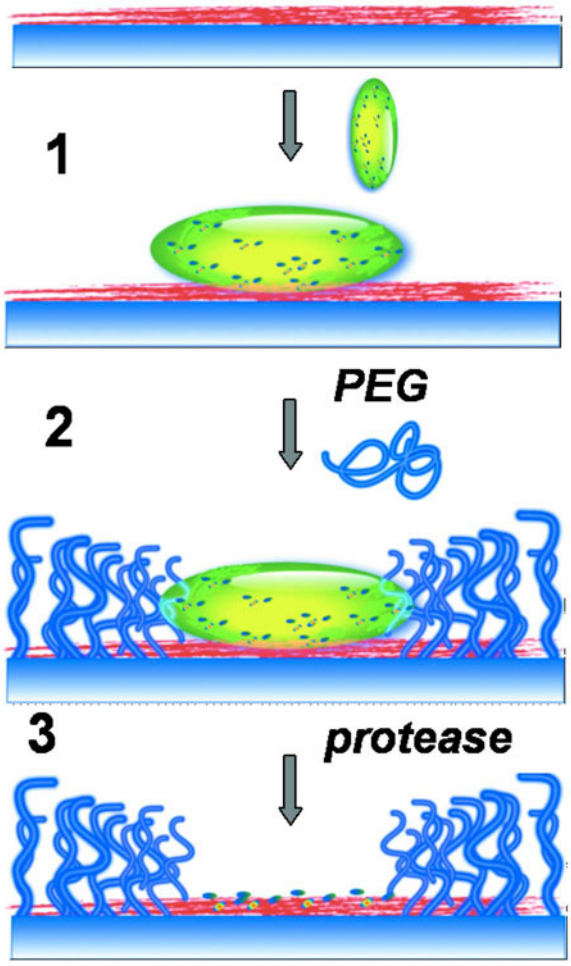


FIG. 2. (Color online) Procedure utilized for the preparation of the artificial antibody. (1) Protein (antigen) adsorption and grafting to the reactive surface. (2) Polymer grafting to the residual surface functional groups around the previously attached protein. (3) Proteolytical treatment with enzyme results in creation of the cavities in polymer film, which are geometrically and chemically complementary to the protein used in stage (1).

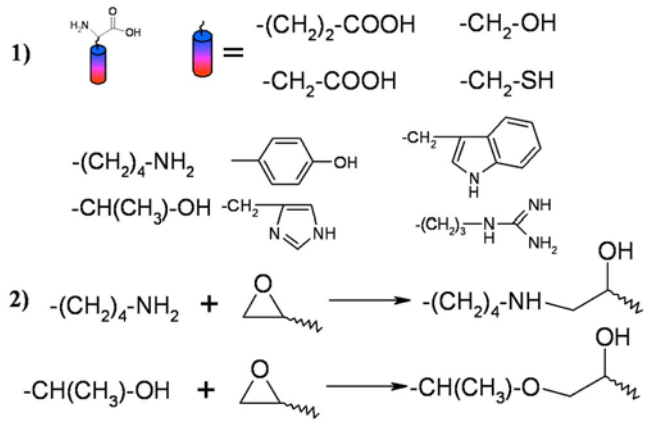


FIG. 3. (Color online) (1) Chemical structure of the 10 amino acids residuals, among the 20 comprising natural proteins, bearing active groups capable of reacting with the epoxy functionalities. (2) Example reaction of the epoxy group with lysine (Lys) and threonine (Thr) residuals.

cally react with functional groups of amino acids present on the exterior of the protein template (Fig. 2, step 1). Next, the protein was grafted to the surface via reaction between the amino acids and complementary surface functionalities. Among the 20 amino acids comprising natural proteins, there are 10 acids bearing groups capable of reacting with the epoxy functionalities (Fig. 3). Reaction of the protein peripheral functional groups with the surface secured conformation and ensured spatial stability of the imprinted molecule. The vital requirement for this step of the imprinting was a minimal distortion of the natural conformation of the protein[13] during the adsorption and following polymer grafting. Protein adsorption was done from phosphate buffer solution during 1 h at room temperature (22–25 °C, *p*H=7.4). Grafting of the protein to the surface was performed at 36 °C in the air saturated with water vapor. Residual reactive groups of the surface (not involved in the protein grafting) were used to attach polymer chains around the secured protein molecules (Fig. 2, step 2). The grafting of the polymer had to be done with a minimum influence on the protein geometry, i.e., at low temperatures and avoiding aggressive chemicals. The last step was an enzymatic cleavage of the template protein, which created cavities geometrically complementary to the adsorbed biomolecule (Fig. 2, step 3). The enzymatic treatment left residual amino acids, grafted to the reactive surface in the positions correlated with those in the protein template.

To create the imprinted structures, deposition of thin (2–3 nm) film of PGMA was used for the initial surface functionalization. The polymer contains epoxy functional groups, which are highly active in various chemical reactions (with carboxyl, hydroxyl, amino, epoxy, thiol, and anhydride functional groups) at relatively mild conditions. Consequently, the imprinting method described in the present study is virtually universal toward both surface and reactive species being attached to the substrate. (In our previous investigations, we successfully conducted grafting of different (bio)polymers to various surfaces employing PGMA as a primary anchoring layer.[14]) When deposited on a substrate, the primary ultrathin PGMA layer first reacts with the surface through formation of covalent bonds. The reactive units located in the "loop" and "tail" sections of the attached macromolecules are not connected to the surface.[15] These free groups offer a synthetic potential for the further chemical modification reactions and serve as reactive sites for the subsequent attachment of proteins and end-functionalized macromolecules. Thus, the epoxy groups of the primary polymer layer served triple purpose: to attach PGMA to the surface,[16] to anchor amino acids of the template protein, and to offer reactive sites for the subsequent grafting of end-functionalized polymers.

In our experiments adsorption of the protein molecules on the PGMA film from buffer solution was conducted in regimes where complete monolayers of adsorbed proteins were not formed (Fig. 2, step 1). The remaining space between the adsorbed protein macromolecules was grafted with the end-functionalized PEG, a protein-repelling polymer (Fig. 2, step 2). The grafting was conducted at 36 °C in the presence of the vapor of poor solvent (cyclohexane) for the polymer to

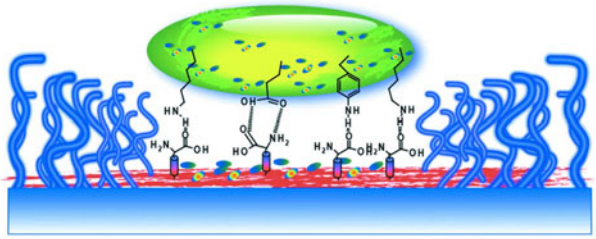

FIG. 4. (Color online) Schematics of possible template recognition by imprint via hydrogen bonding.

be grafted.[17] Specifically, a relatively thick film of PEGs was deposited on top of PGMA/protein layer and the sample was placed for 24 h in a sealed tube containing a small droplet of the solvent.

The solvent vapor treatment has two major effects on the polymer film:[18] (i) plasticization effect, which leads to drop off glass transition/melting temperature, (ii) dilution effect, which leads to polymer layer viscosity decrease. The presence of the small fraction of cyclohexane in the polymer film increased mobility of the polymer chain and allowed grafting the relatively thick (3–5 nm) polymer layer at mild (36 °C) temperatures to prevent protein denaturation. [In a control experiment, where grafting was attempted in the same conditions but without the solvent, only minute amount (less than 0.8 nm) of the grafted polymer was detected.] On the other hand, employment of good and not poor solvent caused dewetting of the deposited polymer film during the grafting. After the polymer anchoring was completed, the ungrafted macromolecules were removed by rinsing the sample with a good solvent for the polymer.

At the last stage (of the imprinted structure formation) treatment of the anchored protein/grafted polymer layer with nonselective protease was conducted (Fig. 2, step 3). The treatment destroyed the polypeptide chains and left bound amino acids placed in virtually the same positions as in the protein employed as a template. These amino acid sites might be able to form hydrogen bonds that recognize the protein used as a template (Fig. 4).

To study enzymatic cleavage of the protein chains, monolayer of the adsorbed fibrinogen was obtained. While chemically attached to the surface, proteins were treated with protease. Ellipsometry measurements revealed a decrease in the protein layer thickness from 4–5 to 1.5–2 nm and XPS confirmed the presence of nitrogen in the residual layers (Table I). In a separate experiment, it was found for the grafted PEG

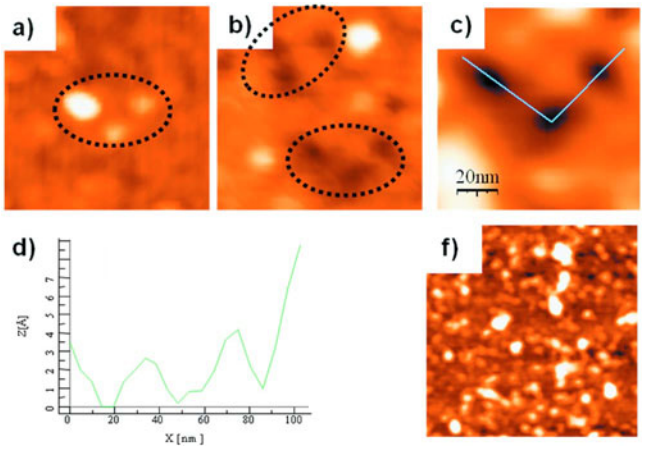


FIG. 5. (Color online) AFM images of the (a) separate fibrinogen molecules grafted to the reactive surface; [(b) and (c)] fibrinogen imprints in PEG matrix; (d) cross section of fibrinogen imprint (c); and (f) fibrinogen adsorbed onto imprinted surface. Scan area [(a) and (b)] $150\times150$ nm$^2$; (c) $100\times100$ nm$^2$; and (f) $500\times500$ nm$^2$. Vertical scale: [(a), c), and (f)] 5 nm and (b) 3 nm.

layer that the PEG macromolecules were not removed by the protease action.

Figure 5 shows experimental results of the fibrinogen imprinting according to the method described. Carboxy-terminated PEG with molecular weight of 5000 g/mol was used for the imprinting. The grafted layer consisting of PEG has to diminish nonspecific protein adsorption to the imprinted surface.[19] Specifically, AFM topography images of the surface templated with the fibrinogen molecules are presented. The footprints geometrically corresponding to fibrinogen size[20] and shape can be clearly observed (Fig. 5, dotted ovals). Cross sections of the AFM image revealed that the depth of the pockets is in the range of 3–5 nm, which corresponds to the size of the fibrinogen reported in the literature. Notably, that during the fibrinogen rebinding experiments significant amount of the attached fibrinogen was absorbed into the pockets on the imprint surface [Fig. 5(f)]. In a control experiment there was practically no fibrinogen adsorption onto the complete (nonimprinted) PEG grafted layer.

Polymer brush grafted around imprinted protein molecule serves dual role. First, after the template molecule removal, the walls of the polymer brush will form pocket which geometrically corresponds to the template. Second, polymer brush itself, in the space between the pockets should prevent nonspecific protein adsorption, thus enhancing selectivity of

TABLE I. XPS analysis of protein monolayers before and after the enzyme treatment.

| PGMA layer | | | Fibrinogen grafted and enzymatically treated surface | | | BSA grafted and enzymatically treated surface | | |
|---|---|---|---|---|---|---|---|---|
| C (%) | N (%) | O (%) | C (%) | N (%) | O (%) | C (%) | N (%) | O (%) |
| 54 | ··· | 46 | 48.5 | 9.1 | 42.4 | 54.5 | 7.6 | 37.9 |

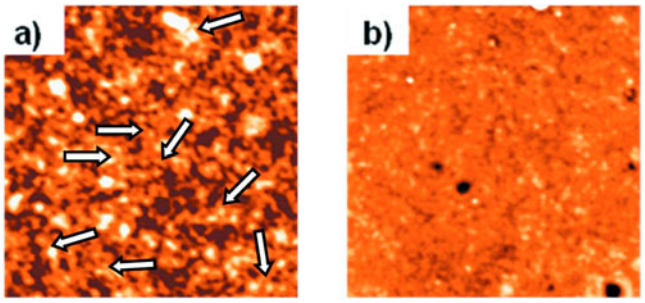


FIG. 6. (Color online) SPM topography images ($1 \times 1$ $\mu$m) of the fibrinogen adsorbed (a) to fibrinogen imprint and (b) to imprint of smaller than fibrinogen protein (BSA). On (a) some adsorbed fibrinogen molecules are indicated with arrows. Vertical scale of both images, 10 nm. Imprints were prepared with PEG $M_w$=22 600 g/mol.

the whole structure. Because of the abovementioned factors, polymer brush grafting density, as well as molecular weight will play significant role in the performance of the imprint. To explore this subject we imprinted fibrinogen using the carboxy-terminated PEG possessing higher molecular weight of 22 600 g/mol. Once the imprint was subjected to the fibrinogen solution, the fibrinogen molecules were still readily adsorbed to the substrate imprinted with the protein [Fig. 6(a)]. Although when smaller than fibrinogen protein was used for the imprinting (such as bovine serum albumin), no fibrinogen adsorption to the surface was observed [Fig. 6(b)].

## IV. CONCLUSIONS

In summary, we have proved feasibility of a new approach toward fabrication of a synthetic material capable to recognize a specific protein. The developed experimental procedures (employing approach designed and proved by nature) could potentially be used for the synthesis of artificial antibodies via precise complementary positioning of amino acids on a surface in conjunction with solvent assisted polymer grafting. All synthetic manipulations were conducted at low temperatures without utilization of aggressive chemical reagents. The implementation of the developed imprinting protocol led to the formation of oriented pockets on the surface with protein related residuals at the depressions beds, surrounded by a grafted polymer layer. The parent protein is absorbed into the pockets of the imprint; however, when the size of the pockets is smaller than the protein dimensions, no protein adsorption was observed in our experiments.

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