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Protein responsive synthetic materials for biomedical applications

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ABSTRACT

The production of synthetic materials with molecular recognition abilities towards biomacromolecules has been widely researched because of its potential applications in separations, sensing, therapeutics, etc. While molecular imprinting has become a commercially viable method for the production of synthetic materials with molecular recognition abilities towards small molecules, little success has been recorded towards protein targets. Proteins are difficult to imprint as they present a number of epitopes for imprinting, are difficult to remove from the completed material, and are incompatible with the densely crosslinked structures required. As a result, a novel surface molecular imprinting technique was developed to synthesize polymeric materials with molecular recognition abilities towards proteins. The recognitive surface of these materials was formed through the use of a templating mask where the target macromolecules, immunoglobulin G (IgG) and protein A in these studies, were immobilized. As a result, the surfaces of the molecularly imprinted materials were chemically and sterically complementary to the epitopes presented by the template molecules. The molecularly imprinted polymer (MIP) materials formed by this surface imprinting technique were able to recognize and rebind the template molecule, even when competing with structural analogs. This newly developed surface imprinting technique demonstrates potential to become a substitute for ELISA (Enzyme-Linked Immunosorbent Assay) or as a sensing element in microdevices formed through standard photolithographic processes.

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1. INTRODUCTION

1.1. MOLECULAR IMPRINTING

Antibodies, biological receptors and enzymes have been widely used as reagents in the areas of biochemical assays, biosensor technology, biological separation and purification due to their ability to selectively recognize and bind target molecules [1-11]. However, these biological systems are unable to work in extreme environments such as the presence of high temperatures, large shifts in pH, or exposure to organic solvents, acids and bases [12-15]. In addition, the production and purification of these biological systems is usually time-consuming and expensive [16]. Therefore, the design and synthesis of materials as substitutes of biological receptors is of obvious importance [17].

One technique, molecular imprinting, has been increasingly adopted because of its ability to produce biomimetic receptor systems similar to their natural counterparts [18-19]. The materials made from this technique are so-called molecularly imprinted polymers (MIPs). The advantages of molecularly imprinted polymers are obvious when compared with their natural counterparts. MIPs are generally easy to prepare, inexpensive, robust and stable in extreme environments.

To date, MIPs have been utilized primarily in conjunction with small molecules such as organic compounds, amino acids and their derivatives [20-22]. MIPs using relatively small molecules as template has been found very promising when applied as recognitive elements of biomimetic sensors [23-25], affinity chromatography [26-28], solid phase extraction [29-36], and (immuno)assays [37-39]. However, the imprinting of macromolecules like proteins has seen only limited successful cases [40-44]. Two negative effects limited the successful imprinting of macromolecules - steric hindrance

and thermodynamic effect [45]. The imprinting process, when done in bulk, requires diffusion of large protein molecules through densely cross-linked polymer networks in order to reach the binding sites, a process that is slow and limits response, giving rise to the so-called steric hindrance. Furthermore, proteins lack a rigid shape, which makes it difficult to obtain well-tailored recognition sites, giving rise to the so-called thermodynamic effect. Moreover, proteins, due to their large size present a large number of surfaces for imprinting, which leads to binding site heterogeneity and cross-reactivity with chemically similar molecules. In addition, there are practical concerns with traditional methods for the imprinting of protein templates. In traditional imprinting processes, the templating protein, which is often expensive or of limited quantity, is usually denatured or destroyed during its removal from the imprinted matrix, making current techniques impractical for large scale production of MIPs.

1.2. SCOPE OF THIS WORK

Surface imprinted materials providing a large number of complementary recognition sites at polymer surfaces appear to be a suitable means for the recognition and rebinding functional groups on the surface of a protein molecule. The production of molecular imprinting sites on polymer surfaces could not only solve the problems of mass transfer and accessibility, but also make it possible to control many important parameters including binding sites orientation and local solvation state [46]. There are many different surface imprinting techniques developed to imprint proteins which have been introduced later in this dissertation. The aim of this dissertation was to present a surface imprinting technique developed in our laboratory, which was promising to replace traditional ELISA as an efficient and inexpensive immunoassay method. This

newly developed surface imprinting technique allowed large scale production of surface imprinted polymeric discs for the recognition of protein template such as monoclonal anti-c-myc IgG. The analysis conditions were optimized so as to enhance the rebinding efficiency of the surface imprinted materials. Furthermore, the templating mask using epoxy activated microscope slide immobilized with protein molecules demonstrated excellent reusability. Many factors such as functional monomer(s) and solvent, which may influence the recognition ability of the surface imprinted polymer, were discussed. The materials were characterized via atomic force microscope (AFM) and attenuated total reflection fourier transform infrared (ATR-FTIR).

This dissertation is organized as follows:

Paper I of this dissertation provides the background and theory of this newly developed surface imprinting technique using monoclonal anti-c-myc IgG covered 96-well plate as templating mask, along with a discussion of the analysis conditions that may affect the recognition ability of MIPs. Furthermore, the materials and templating mask were characterized via ATR-FTIR.

Paper II focuses on analysis of the main factors of synthesizing MIPs and reusability of the protein templating mask. In order to reuse the templating mask, epoxy-activated glass substrates were applied to replace 96-well microplates as the templating masks. Monoclonal anti-c-myc IgG was again selected as the template molecule. Two most commonly used functional monomers were selected to analyze the recognition properties of MIPs. Moreover, two kinds of solvents were also tried in this study in order to optimize the performance of MIPs. Finally, the selectivity of MIPs was investigated using a structural analog of monoclonal anti-c-myc IgG.

Paper III studied the efficiency of this surface imprinting technique using two different proteins as template molecules. These two proteins are protein A and monoclonal anti-c-myc IgG. The amount of cross-linker in the prepolymerization mixture was discussed in this paper. In addition, the influence of IgG orientation on the recognition abilities of anti-c-myc IgG MIPs was analyzed. Finally, the surface morphology of the polymeric discs was characterized via AFM.

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2. BACKGROUND

2.1. MOLECULAR IMPRINTING

Molecular recognition refers to the specific interactions between two or more molecules through noncovalent binding such as hydrogen bonds, van der Waals forces, hydrophobic forces, and electrostatic effects, etc. [1]. Molecular recognition is essential in biological systems and the examples of molecular recognition include protein-receptor interactions, enzyme-substrate binding, and sugar-lectin binding, etc. [2]. A technique, called molecular imprinting, has been widely used to synthesize materials mimicking natural biomolecules with recognition ability [3-6]. Three reasons make molecular imprinting an attractive technique: (i) molecularly imprinted polymers (MIPs) made with the molecular imprinting technique demonstrate good recognition and binding ability toward their target molecules; (ii) MIPs are much more stable in harsh environment than those of natural receptors; (iii) MIPs are simple and cheap to prepare [7-10].

The procedure of molecular imprinting includes the polymerization of functional and cross-linking monomers in the presence of a template molecule. After the removal of the template molecule, the molecular cavity left inside the polymer networks thus possesses a structure complementary both sterically and chemically to the template molecule. As a result, the imprinted polymer is capable of recognizing and rebinding the template molecule. The procedure is outlined in Figure 2.1. Functional monomer, crosslinker and photoinitiator are mixed with the template molecule before polymerization reaction. Later, the template molecule is removed from the synthetic polymer networks, forming a MIP with recognition ability.

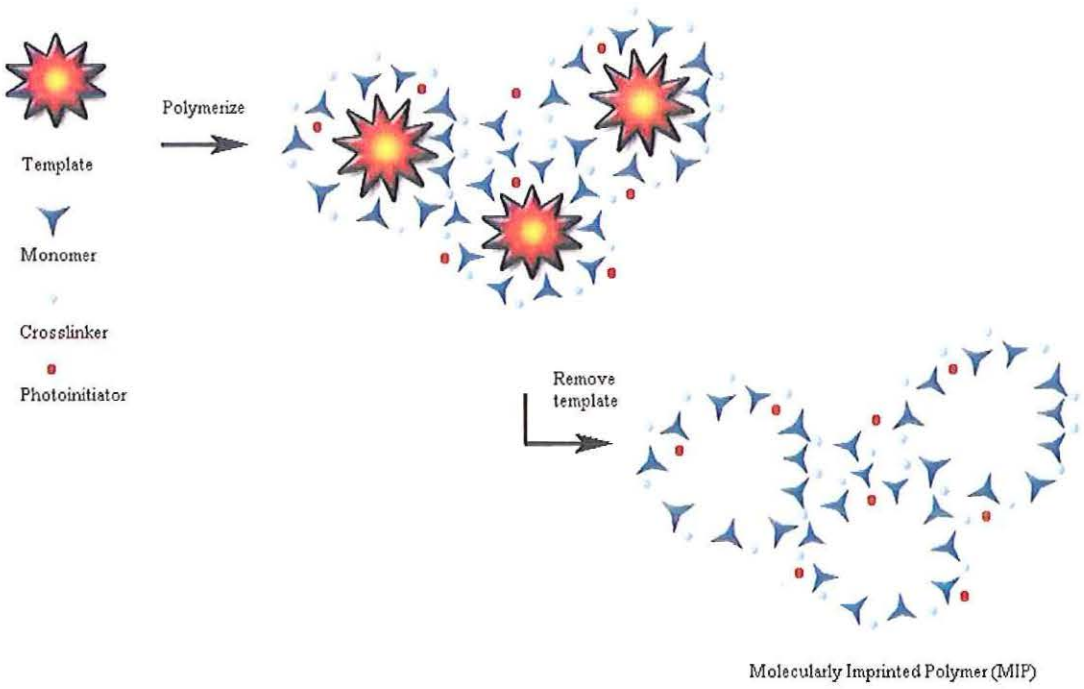


Figure 2.1: Scheme of the molecular imprinting procedure.

2.2. PREVIOUS WORK ON MOLECULAR IMPRINTING

Molecularly imprinted polymers (MIPs) have been applied in various fields including affinity separation, antibody binding mimics, enzyme mimics and bio-mimetic sensors [11]. In recent years, MIPs have been developed as stationary phases of high performance liquid chromatography (HPLC) to separate amino acids and their derivatives (chiral separation) [12-14], and separate biochemicals from their enantiomers [15-16], etc. MIPs can also be employed in immunoassays such as radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISA), in which MIPs are mimicking antibodies to recognize and bind the radio labeled template molecule out of a host of

competitive species [17-20]. MIPs have also been made to mimick enzymes to catalyze organic reactions such as aldol condensations and ester hydrolyses, etc. [11, 21]. In addition, MIPs are very promising elements that might be applied in bio-sensors such as QCM sensors and fluorescence-based sensing devices [22-24].

To date, many strategies have been applied for the production of MIPs capable of specifically binding macromolecules [14, 25]. One approach was developed by Shi and Ratner [26]. As presented in Figure 2.2, the template protein was adsorbed on a mica surface and covered by disaccharide. Later, a thin polymer film was formed on top of the disaccharide overlayer. Therefore, the recognition sites had been formed on the surface of MIPs. Another protein imprinting method studied was the so-called epitope approach (Figure 2.3) [27]. Rachkov et al. chose a short peptide as the template molecule to represent the whole protein molecule. After the removal of the template molecule, the recognition sites in MIP networks were proved to be able to recognize not only the short peptide but also the proteins possessing the same structural fragment as that of the template. Recently, Lin et al. reported a microcontact imprinting technique in which a protein stamp was prepared using protein templates to be immobilized on a piece of microscope cover glass pretreated with hexamethyldisilazane [28]. In the study, lysozyme, ribonuclease A, and myoglobin were selected as template molecules. As presented in Figure 2.4, the cover glass was removed after the polymerization reaction and the protein molecules were extracted from the surface imprinted polymer thin film. The surface imprinted film demonstrated recognition abilities toward their own template molecules. Shiomi et al. developed a new molecular imprinting technique in which hemoglobin (Hb) was immobilized covalently on glutaraldehyde-treated aminopropyl

silica as illustrated in Figure 2.5 [29]. Then, propyltrimethoxysilane and 3-aminopropyltrimethoxysilane were used to form a polymer layer on the Hb immobilized silica surface. After the polymerization reaction, Hb was removed from the silica surface using oxalic acid. As a result, the imprinted silica demonstrated selective binding ability for its template molecule. In addition, the selective binding ability is better than that of the material using free Hb as template.

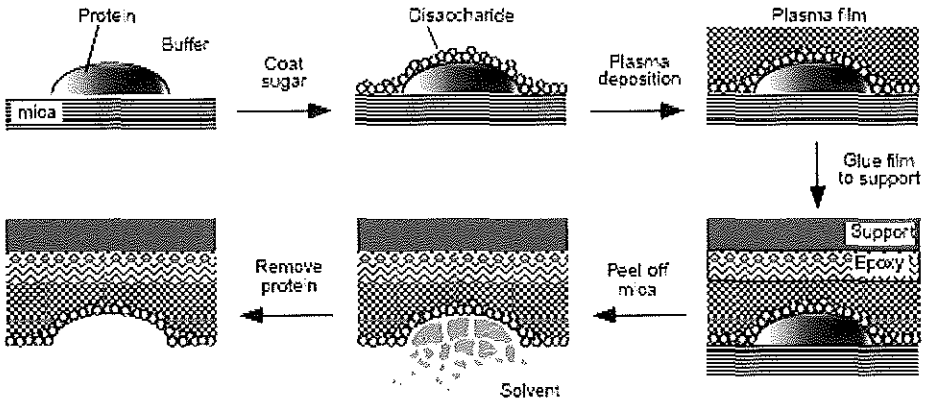


Figure 2.2: The scheme of surface molecular imprinting developed by Shi and Ratner [26].

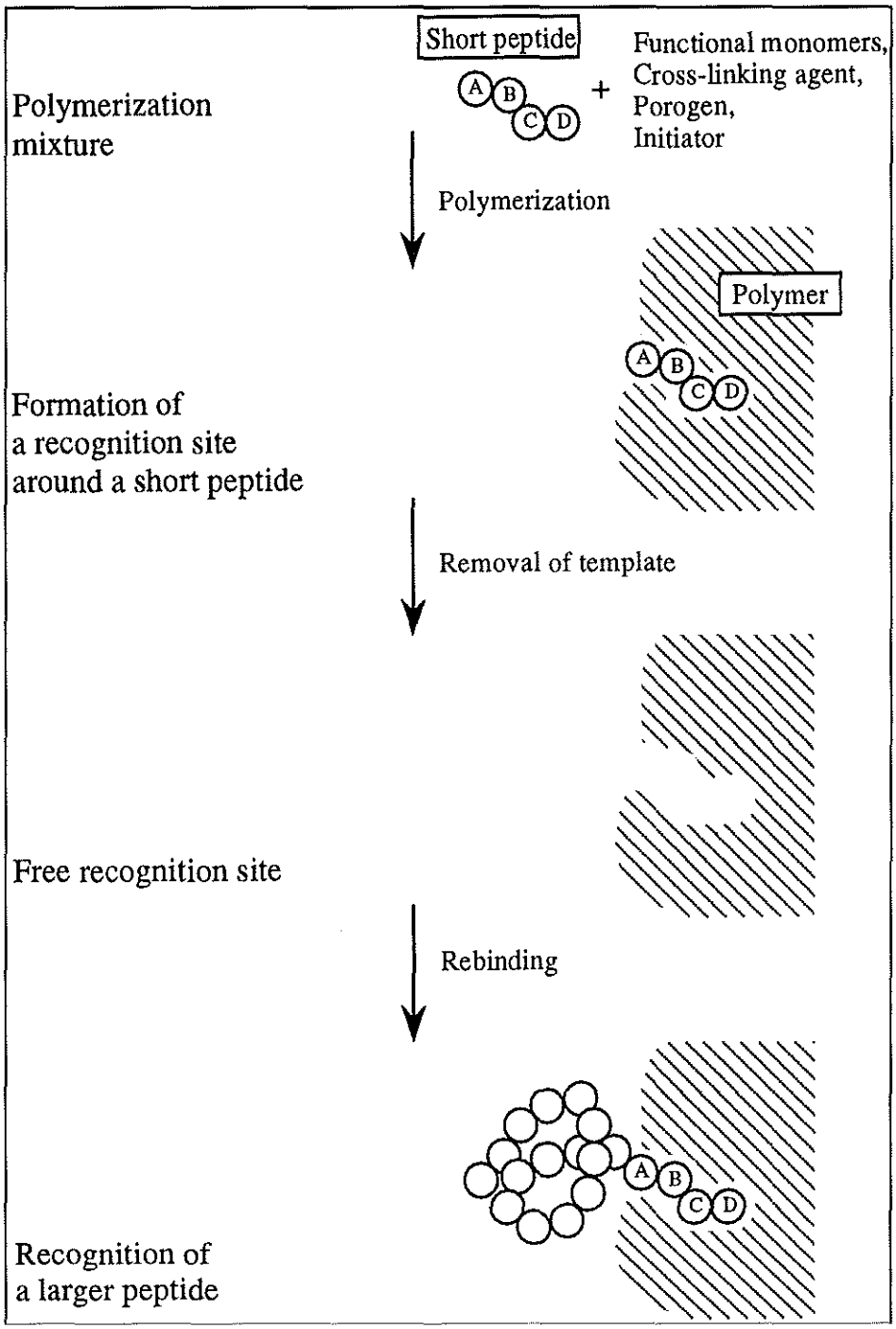


Figure 2.3: The scheme of epitope approach proposed by Rachkov et al. [27].

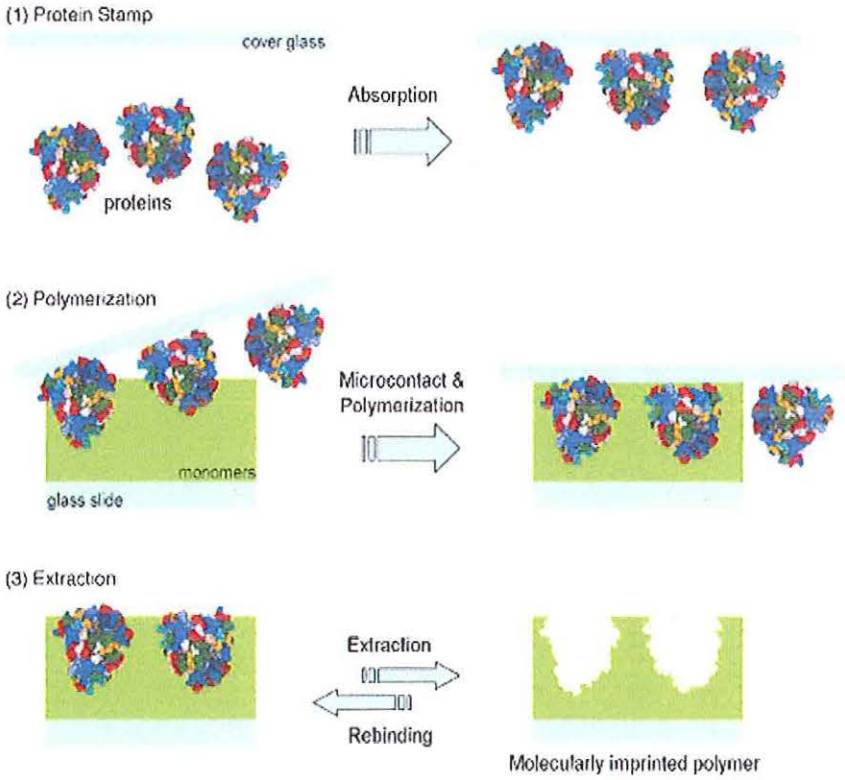


Figure 2.4: Outline of microcontact imprinting technique to form surface imprinted polymer film [28].

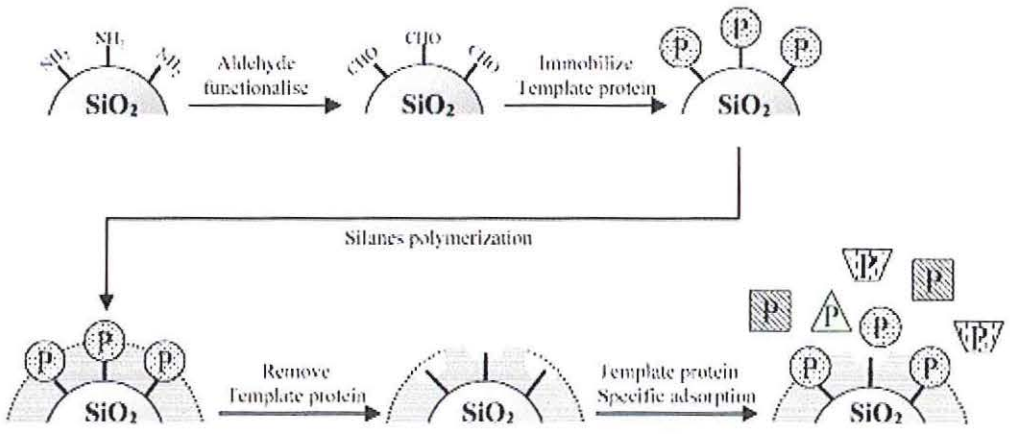


Figure 2.5: Illustration of the protein imprinting technique using covalently immobilized template [29].

2.3. FREE RADICAL CHAIN POLYMERIZATION

Polymerization reactions are typically classified into two types – condensation reaction and addition polymerization [30]. In addition polymerization, the polymer is always made from monomers with reactive double bonds [31]. One of the addition polymerization reactions most widely applied in industry is free radical chain polymerization [32]. The procedure of free radical chain polymerization includes initiation, propagation and termination. The procedure is presented below [30]:

Step 1: Generation of free radicals

$$I \longrightarrow 2R\cdot \qquad \text{eq. (1)}$$

In the equation, I represents the initiator which will decompose to two free radicals R·.

Step 2: Propagation

$$R\cdot + M \longrightarrow P_1\cdot \qquad \text{eq. (2)}$$

In the equation, M represents monomer and $P_1\cdot$ stands for an activated repeating unit of the polymer.

$$P_1\cdot + M \longrightarrow P_2\cdot \qquad \text{eq. (3)}$$

$$P_2\cdot + M \longrightarrow P_3\cdot \qquad \text{eq. (4)}$$

$$\vdots$$

$$P_{(x-1)}\cdot + M \longrightarrow P_x\cdot \qquad \text{eq. (5)}$$

The polymer chain is growing longer and longer by the addition of more and more monomers.

Step 3: Termination

A growing polymer chain will be terminated by one of two means. One is called combination and the other is disproportionation. In combination, there are (x+y) repeating units in a single polymer chain (one of length x, one of length y). While there are two different polymers generated in disproportionation. In most of the free radical reactions, one of these two means will be dominant which depends on the polymers formed by the reaction and the reaction temperature.

$$P_x \cdot + P_y \cdot \longrightarrow P_{(x+y)} \text{ (combination)} \qquad \text{eq. (6)}$$

$$P_x \cdot + P_y \cdot \longrightarrow P_x + P_y \text{ (disproportionation)} \qquad \text{eq. (7)}$$

2.4. ANTIBODY

Antibodies (also known as immunoglobulin) are the best understood molecules involved in specific immune recognition [33-34]. Immunoglobulin (Ig) is generally produced by B lymphocyte when the individual is exposed to some antigens and each Ig is only responding to one specific antigen [35]. In the human body, there are a large number of different lymphocytes, each of whom has their own specificity to a single antigen. Ig usually works as an antigen receptor bound on the membrane of B lymphocyte (Figure 2.6). When one particular antigen invades human body, the Ig specific to this antigen will recognize and bind the antigen, therefore, the B lymphocyte is activated and it will divide into tons of identical cells that will also interact with the specific antigen (Figure 2.7). As a result, the specific antigen bound by the receptors on the B lymphocytes will be killed and removed out of the body by phagocytes [36].

In general, there are five classes of Ig: immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin E (IgE) [36]. Among them, IgG is the most abundant isotope in plasma. As shown in Figure 2.8, IgG is a Y-shaped molecule with a molecular weight of roughly 150 kDa. IgG molecule is composed of four polypeptide chains: two heavy chains and two light chains. Each heavy chain is approximately 50 kDa and each light chain is about 25 kDa. These polypeptide chains are connected with each other by disulfide bonds. The antigen-binding sites are concentrated at the two tips of the IgG molecule and they are varied between IgG molecules. Therefore, they are named variable region or antigen binding fragment (Fab fragment). The variety of Fab fragments allows the IgG to recognize and bind only one specific antigen. The rest of IgG molecule is similar to all the IgGs, so it is called constant fragment (Fc).

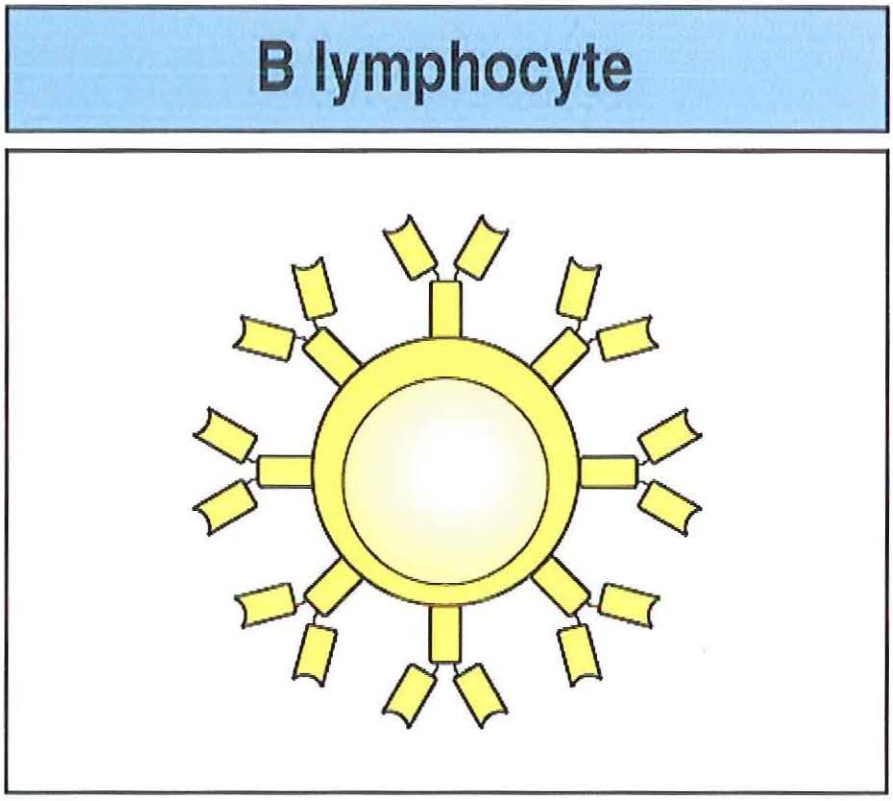


Figure 2.6: The scheme of B lymphocyte [36]. The Ig molecules working as antigen receptors are bound to the membrane of the B lymphocyte.

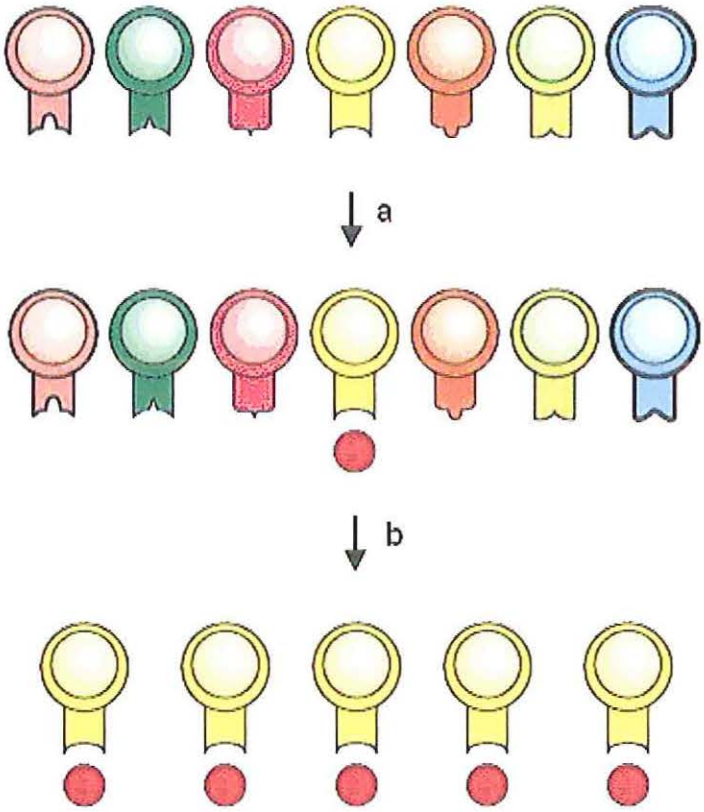
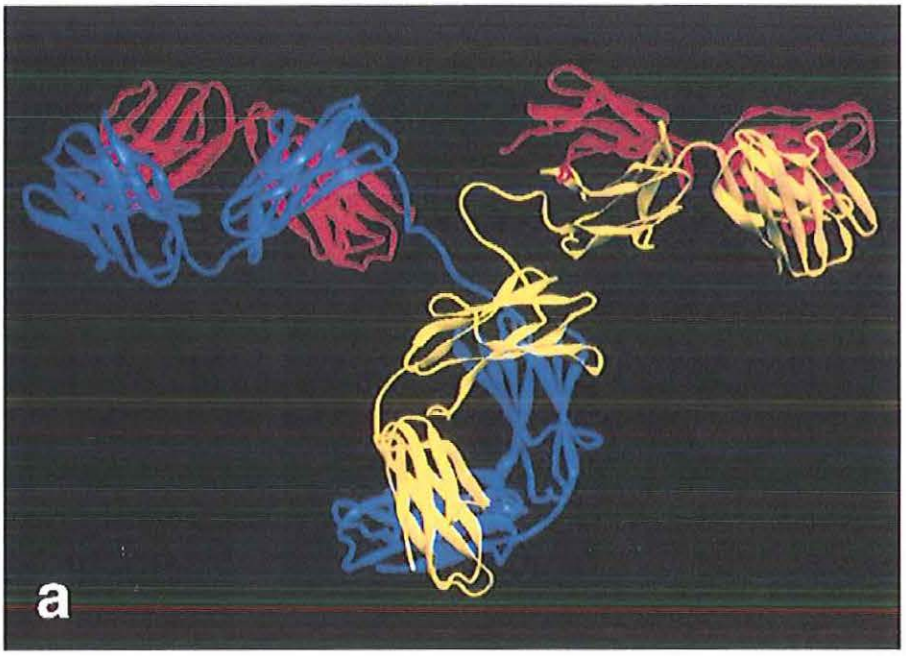


Figure 2.7: The simplified scheme of B lymphocyte and antigen interaction [36].
a) When the individual is exposed to a particular antigen, the B lymphocyte specific to this antigen will recognize and bind to the antigen. b) A clone of identical B cells will be generated to bind the foreign antigen, as a result, the immune defense system of human body has been activated.



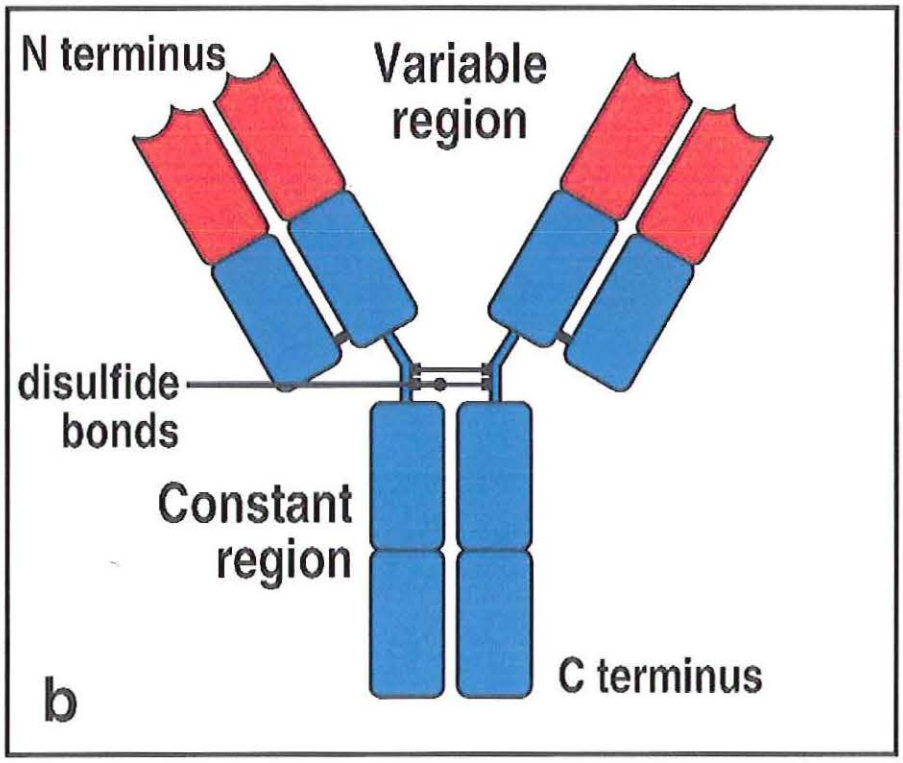


Figure 2.8: Illustration of IgG structure [36].
(a): IgG structure obtained by X-ray crystallography, (b): A simplified scheme of IgG structure. The two antigen-binding domains are at the tops of the arms (variable region).

2.5. ELISA

Enzyme-Linked Immunosorbent Assay (ELISA), a gold standard of immunoassay, has been widely used in the detection of viral proteins such as HIV test, cancer and West Nile Virus diagnostics, etc. [35]. There are two major types of ELISA: one is indirect ELISA and the other is sandwich ELISA (Figure 2.9) [37-38].

In indirect ELISA, the antigen is immobilized on the bottom of a microplate well and then antibodies from a patient are added into the well. If the sample contains the antibody specific to the coated antigen, that antibody will bind the antigen in the well. All the unbound antibodies are washed away and then a secondary antibody solution is added into the well. The secondary antibody is an enzyme-antibody complex which is able to bind the detection antibody on the bottom. Later, removal of the unbound secondary antibody from the well and addition of a substrate which will react with the enzyme leads to the change of a colorless solution to a colored solution. Finally, the colored solution is tested by a spectrophotometer or spectrofluorometer.

In sandwich ELISA, a monoclonal antibody is coated on the bottom of the microtiter plate well and then blood or urine from a patient is added into the well. If the blood or urine contains the antigen specific to the antibody coated in the well, the antigen will recognize and bind the antibody. All of the unbound antibodies are removed and an enzyme-linked antibody is added which can recognize and bind the specific antigen. The rest of the analysis procedure is similar to that of indirect ELISA.

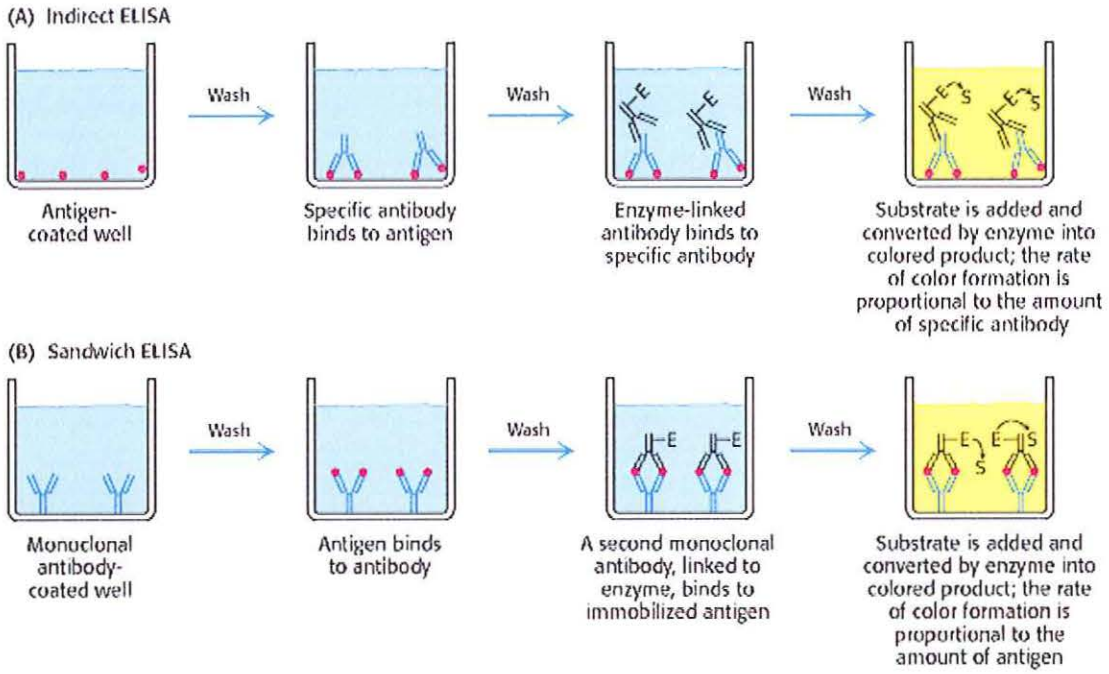


Figure 2.9: Two means of ELISA [37-38].

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PAPER

I. SURFACE MOLECULARLY IMPRINTED POLYMERS FOR THE RECOGNITION OF IMMUNOGLOBULIN G

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ABSTRACT

In order to prepare synthetic materials which mimic the recognition abilities of natural biomolecules, a surface imprinting technique using a templating mask of an immobilized target protein was developed. This technique was used to produce polymeric materials which were responsive to an immunoglobulin G test molecule (monoclonal anti-c-myc IgG). The recognitive ability of these materials was then compared to control materials which were of identical chemical composition but fabricated in the absence of the templating mask. The surface imprinted material made from our method exhibited significant molecular recognition towards the target IgG molecule, binding more than five times the amount of IgG as the control material. Furthermore, the recognitive material showed no preference for competing IgG molecules when compared to the control. The addition of a competing immunoglobulin G into the incubation mixture was found to be an effective strategy to maximize the

excess selective binding percentage between recognitive and control materials. It is postulated that this competing molecule vies for non-specific adsorption sites.

Keywords: Surface molecular imprinting technique; Molecularly imprinted polymer; MIP; Immunoglobulin G; Template mask

1. INTRODUCTION

Antibodies, biological receptors, and enzymes have been widely used as reagents in the areas of biochemical assays, biosensor technology, biological separation and purification due to their ability to specifically recognize and bind the target molecules [1-10]. However, these biological systems are usually expensive and time-consuming to prepare [11]. In addition, they are often extremely sensitive to the environment. Therefore, it is of obvious importance to design and synthesize chemically and mechanically stable materials as substitutes of biological receptors. One technique, molecular imprinting, has been increasingly adopted to produce biomimetic receptor systems similar to their natural counterparts [12-13]. The materials made from this technique are frequently termed molecularly imprinted polymers (MIPs). The procedure of molecular imprinting includes the polymerization of functional and cross-linking monomers in the presence of a template molecule. After removal of the template molecule, the molecular cavity left inside the polymer networks thus possesses a structure complementary both sterically and chemically to the template molecule. As a result, the MIP is capable of recognizing and rebinding the template molecule.

To date, MIPs have been utilized primarily in conjunction with low molecular weight compounds such as drugs, sugars, amino acids and their derivatives [14-16]. However, the imprinting of macromolecules like proteins has seen only limited successful cases [17-24]. There are mainly two negative effects inhibit the successful imprinting of macromolecules - steric hindrance and the thermodynamic effect [25-27]. Owing to the large size of macromolecules, it is difficult for the large molecule to move in or out of the cross-linked polymer network, the so-called steric hindrance. Another

negative effect – the thermodynamic effect – is caused because proteins lack a rigid shape, making it difficult to obtain well-tailored recognition sites.

In order to overcome these two difficulties, we propose an approach to synthesize MIPs with surface binding sites through the use of a template mask of immobilized target protein. Monoclonal IgG has been used as the template molecule because of its unique constant/variable structure [28]. In this work, the Fc domain of the Y-shaped IgG molecule was immobilized on a template mask (96-well microplate) [29]. As a result, both Fab and Fc domains of the IgG molecules were imprinted on the polymer surfaces after the polymerization reaction. The use of 96-well microtiter plates as template mask substrates allows rapid and efficient evaluation of material formation. In addition, this method opens the possibility of reusing the template mask in later material formation. The selective recognition ability of MIPs toward their template molecules were analyzed as well. The rebinding ability of the MIPs toward the template molecules were optimized through variation of washing temperature and the addition of another IgG as blocking protein. In this surface imprinting technique, a moderately swollen hydrogel, poly(HEMA-co-EGDMA), was chosen. In this work, a high mole percentage of the cross-linker (80 mole %) was selected so as to provide control over the polymer chain conformations and to "lock in" template binding sites [30-31]. All research techniques in this work have been chosen to mimic ELISA-type assay, as outlined in Scheme 1 and 2.

2. EXPERIMENTAL

2.1. Materials

Rabbit anti-pig IgG (whole molecule) alkaline phosphatase conjugate (RAP IgG-AP), anti-pig IgG (whole molecule) developed in rabbit affinity isolated antigen (RAP IgG), monoclonal anti-c-myc purified mouse IgG (AcM IgG), monoclonal anti-c-myc alkaline phosphatase conjugate (AcM IgG-AP), anti-mouse IgG (Fab specific)-FITC antibody produced in goat, ethylene glycol dimethacrylate (EGDMA), bovine serum album (BSA) and 1-hydroxycyclohexyl phenyl ketone (HCPK) were purchased from Sigma-Aldrich (St. Louis, MO.). Sodium-meta-periodate ($NaIO_4$) and 2-hydroxyethylmethacrylate (HEMA) were from ACROS Organics. The functional monomer HEMA was purified by vacuum distillation prior to use. Blank polystyrene microplates (Costar 3370), carbohydrate binding surface 96-well microplates (Costar 2507) and non-binding surface microplates (Corning 3990) were used. The enzymatic substrate 1-step PNPP (p-nitrophenyl phosphate, disodium salt) and serum-derived IgG from a rabbit source (Rabbit IgG whole molecule) were obtained from Pierce Biotechnology, Inc. (Rockford, IL.).

2.2. Templating mask preparation

The IgG coated template mask was prepared as follows. AcM IgG was first dialyzed overnight at 4 °C in the coupling buffer (sodium acetate buffer, 0.1 M, pH 5.5) using Slide-A-Lyzer MINI Dialysis Unit (7K MWCO, Pierce Biotechnology, Inc., Rockford, IL) to remove NaN_3 in the template molecule solution. Next, a solution of $NaIO_4$ (10 mM) in the coupling buffer was used to oxidize (4 °C in darkness for 30 min)

the carbohydrate side chains on the Fc domain of AcM IgG to produce reactive aldehyde groups. The oxidation reaction was stopped by adding glycerol to a final concentration of 15 mM and allowing it to react with the oxidized IgG for 5 min at 4 °C in darkness. The solution of functionalized IgG was dialyzed again in the coupling buffer overnight using Pierce Mini Dialysis Unit to remove unreacted $NaIO_4$ and glycerol. Aldehyde functionalized IgG was then added to a carbohydrate binding 96-well microplate (Costar 2507) where it reacted with surface hydrazide groups (one hour at room temperature) to immobilize the IgG molecules on the microplate surface. The concentration of functionalized AcM IgG placed in each microwell was 1 μg/ml. After the reaction, PBS-Tween 20 (pH 7.4) was used as washing buffer to remove unbound antibody from the microwells. A PBS-Tween 20 buffer solution (pH 7.4) containing 1 wt. % BSA was used as blocking agent to mask any remaining hydrazide sites on the microplate surface not covered by AcM IgG template molecule. The templating mask was treated in the blocking agent for 15 min at room temperature. The templating mask was then washed three times with PBS-Tween 20 buffer (pH 7.4), with each washing step taking 5 minutes. The RAP IgG immobilized templating mask was made in the same way and the concentration of the functionalized RAP IgG coated in each microwell was also 1μg/ml.

BSA coated templating mask was prepared using the following procedure: 100 μl of a 1 wt. % BSA in PBS-Tween 20 buffer (pH 7.4) solution was placed in an unmodified polystyrene microplate for 15 minutes at room temperature. The microplate was washed with PBS-Tween 20 buffer (pH 7.4) three times, with each washing step taking 5 minutes.

In order to verify that the Fab domain of the immobilized target molecule was still readily accessible, a secondary antibody, Fab specific anti-mouse IgG-FITC conjugate, was used to detect the primary antibody. A 100 µl aliquot of the secondary antibody in Tris buffered saline (protein concentration of 4 µg/ml) was added to test microwells of the AcM IgG templating mask, polystyrene templating mask, and BSA templating mask were allowed to incubate for 1 hour at room temperature. The microplate wells were then washed using PBS-Tween 20 buffer (pH 7.4) three times, with each washing step taking 5 minutes. Next, 100 µl of Tris buffered saline was added into each microwell and the fluorescence intensity was measured by using a FLUOstar OPTIMA multifunction microplate reader (BMG LABTECH, Durham, NC). This method was also employed to determine antibody presence after the imprinting reactions.

2.3. Preparation of MIP and control polymer

Recognitive and control materials were formed by polymerizing reactive mixtures in the templating microplate wells. Monomer solution was polymerized in 96-well microplates as shown in Scheme 1. A 45 µl aliquot of the prepolymerization mixture, consisting of HEMA (20 mole %) as functional monomer, EGDMA (80 mole %) as crosslinker, and HCPK (2% w/w of the final composition) as the photoinitiator, was mixed with 30 µl DMSO and pipetted into each microwell. Polymerization was initiated by UV light irradiation (375 nm, 300 mW/cm^2, UV Shark LED, Optotech, Inc. Wheeling, IL) at 20 °C for 3 min in nitrogen environment. The distance from the UV light source to the microwell was 3 cm. The MIPs were synthesized in contact with the protein templating mask, while non-MIPs were produced in unmodified polystyrene microplates.

In order to remove (peel out) the polymeric discs from the microwells, 1.5 cm high aluminum sleeves were used to limit contact between the monomer and side wall of the microwell. As presented in Figure 1, these sleeves were made from 0.16 mm thick aluminum sheets (Fisherbrand* disposable weighing pans). This ring also acted as a frame for polymer disc removal. The bottom diameter of each microwell was 6.4 mm and the thickness of the aluminum sheet was 0.16 mm, as a result, the diameter of the synthesized polymer samples was 6.08 mm. After polymerization, tweezers were used to remove the sidewall sleeve along with the polymerized disc.

In order to determine if there was any template molecules adsorbed on the polymer surfaces during the removal process, the prepared materials – control polymer, AcM IgG MIP, and BSA MIP, were first washed by Tris buffered saline for an hour at room temperature and then treated by a 200 µl aliquot of anti-mouse IgG-FITC conjugate in Tris buffered saline (protein concentration of 4 µg/ml) for an hour at room temperature. The polymeric discs were then rinsed three times by PBS-Tween 20 buffer, with each washing step taking 5 minutes. Next, 200 µl of Tris buffered saline was added into each microwell and the fluorescence intensity was measured by using a FLUOstar OPTIMA multifunction microplate reader (BMG LABTECH, Durham, NC).

2.4. Selective binding analysis

After the polymerization reaction, the newly formed polymeric discs were peeled away from the microplate wells and placed into Corning non-binding surface microplates with the imprinted or control surface facing upwards. Next, Tris buffered saline (pH 7.5) was added in 200 µl aliquots to the microplate wells for 60 min at a designated

temperature (4, 20, and 37 °C) in order to wash away any unreacted monomers or residual solvent from the polymer networks. A 200 µl aliquot of enzyme-IgG conjugate dissolved in Tris buffered saline was added to microplate wells containing the polymer samples and incubated for 40 min at a designated temperature (4, 20, and 37 °C). The polymeric discs were then washed with PBS-Tween 20 (pH 7.4) to remove any unbound protein. The quantity of bound protein was measured as using a colorimetric assay based on the activity of the conjugated alkaline phosphatase enzyme. A 100 µl aliquot of 1-step PNPP was placed in each microwell containing the polymeric materials and allowed to react with any remaining alkaline phosphatase of the enzyme-IgG complex for 20 minutes at room temperature in darkness. Later, the substrate solution was diluted with 100 µl fresh de-ionized (DI) water after reaction and 70 µl of the solution was withdrawn from the microwell to be measured at 405 nm using a Multimode Detector (Beckman Coulter, DTX 880, Fullerton, CA) to determine the optical density of the solution. The analysis procedure is shown in Scheme 2. The increased binding of analyte by the imprinted materials was expressed as an excess selective binding percentage, as calculated using the following equation:

Excess Selective Binding % = 100 % × [[MIP absorbance] – [NIP absorbance]]/[NIP absorbance]

All experiments in this study were done in triplicate, and error bars in the following figures represent ± one standard deviation of the data.

2.5. Temperature effects on rebinding ability of MIP

To investigate the temperature effect on the interaction of the template molecules and the polymeric discs, both AcM IgG MIP and control material were washed and incubated at room temperature, 4 °C, and 37 °C. The protein incubation solution was 1.6 μg/ml AcM IgG-AP dissolved in Tris buffered saline. The rebinding analysis was conducted as described earlier.

2.6. Binding affinity analysis in the presence of a competing protein

A competitive species, serum IgG derived from rabbit (Rabbit IgG), was introduced in the incubation solution as a blocking/competing protein. In this set of experiments, Rabbit IgG was added at various concentrations (0.0 μg/ml, 1.1 μg/ml, 2.2 μg/ml, and 5.5 μg/ml) alongside the target AcM IgG-AP molecule (held fixed at 1.6 μg/ml). The rebinding analysis followed the earlier section and the temperature to rinse and incubate the polymeric disks was 4 °C. In this study, four independent experiments were conducted, triplicates for each examination group. The absorbance data were exhibited as mean ± standard deviation.

2.7. Selective rebinding examination based on different templating masks

Polymeric discs were prepared on four types of masks: pristine polystyrene microplates or microplates with immobilized BSA, RAP IgG, or AcM IgG. The formation of the polymeric discs and their rebinding analysis followed the earlier description, while the temperature to wash and incubate these polymeric discs was kept at 4 ° C. The solution used to incubate the polymeric disks in the rebinding analysis was

either 1.6 μg/ml AcM IgG-AP or RAP IgG-AP mixed with rabbit IgG (2.2 μg/ml) in Tris buffered saline. The experiments were carried out under exactly the same conditions in order to keep data consistency.

2.8. Material characterization

Samples were evaluated using attenuated total reflectance FTIR. In the test, the blank polystyrene microplates, polymeric discs, newly prepared templating masks, and used templating mask were studied using FTIR spectrophotometer (Nicolet 6700 FTIR, Thermo Electron, Madison, WI) equipped with a single bounce attenuated total reflectance (ATR) attachment (Smart Performer, Thermo Electron, Madison, WI). A Ge crystal was used as the internal reflection element.

3. RESULTS AND DISCUSSION

The main objective of this work was to investigate the selective recognition ability of the surface imprinted materials produced using an immobilized protein template mask. Besides this proof of concept, optimization of the effects of temperature and the binding of other competing species were also investigated in order to optimize this newly developed surface molecular imprinting technique.

3.1. Temperature effects on MIP recognition ability

Temperature is one of the most important factors in the design of the molecular imprinting experiments since it will have significant impact on the interaction between

template molecules and the MIP. As a result, three different rinse and incubation temperatures (4, 20, and 37 °C) were selected to study the optimal working temperature for the MIP. As illustrated in Figure 2, the binding of protein by recognitive and control materials, as measured by an absorbance change of enzyme substrate, was nearly identical when the rinse and incubation temperatures were 37 °C. In comparison, when the rinse and incubation temperature was lowered to 20 °C, the Excess Selective Binding% increased to 108%, and when the rinse and incubation temperature decreased further to 4 °C, the Excess Selective Binding% rose to 189%.

There are two factors that may influence the rebinding ability when temperature changes – hydrogel surface morphology and affinity for protein adsorption. PHEMA is a stable hydrogel whose water content is not easily affected by the change of pH value or surrounding temperature. In addition, more highly crosslinked PHEMA demonstrates a lower degree of equilibrium swelling [32]. It has been shown that greater amounts of protein are deposited on PHEMA with increasing temperature [33], which in these experiments would manifest as increased adsorption by both recognitive and control polymeric materials. As a result, the overall Excess Selective Binding% is decreasing with increasing protein incubation temperature. Therefore, the rinse and incubation temperature was set to 4 °C in all of the following experiments.

3.2. Competing protein effect on MIP rebinding ability

A competitive species, serum IgG derived from rabbit, was introduced in the AcM IgG-AP solution as a competing protein. It was proposed that the addition of a competing, but "silent" (i.e. not enzyme conjugated) molecule would have two effects.

First, competition between the competing protein and the template molecule for binding sites – either specific or non-specific – would further elucidate binding site affinity. In addition, the competing protein, when added at significantly higher concentrations than the target molecule, will exhaust the non-specific binding sites and create greater contrast between the imprinted and non-imprinted materials. At lower concentrations of competing IgG, target protein adsorption was slightly enhanced when compared to the control experiment with no competing IgG as illustrated in Figure 3. While this effect was not deemed to be statistically significant (p value of 0.23 as calculated by the two-tailed unpaired t test, GraphPad Prism version 5.0), such a phenomenon would not be entirely unexpected. It is well established that addition of a competitive protein may increase the adsorption of the primary protein. Baszkin and Boissonnade reported the adsorption of fibrinogen on a polyethylene surface is enhanced due to the presence of albumin [34]. As Baszkin and Boissonnade observed, when 0.2 mg/ml albumin was added into the solution, fibrinogen adsorption rose more than twofold when compared to experiments with fibrinogen only. In addition, according to Vroman et al.'s study on many series of binary protein systems, relative concentration of one protein in the binary protein solution decided which protein was adsorbed most on the surface [35-36]. However, there are still few experimental measuring methods currently to analyze the nature of competitive protein adsorption at interfaces to help fully understand the mechanism of protein adsorption in a binary protein system.

Addition of competitive species to concentrations higher than that of the target molecule led to the desired effect. When the concentration of the competing protein was raised to 2.2 μg /ml, the Excess Selective Binding% was increased to 406%. This

appeared to be an optimal value, as further addition of the competing protein lowered the Excess Selective Binding%, mainly due to excess binding on the NIP. As a result, all remaining experiments were conducted in the presence of 2.2 μg /ml serum IgG.

3.3. Analysis of imprinting site effectiveness

During the production of the templating masks, BSA was used as a blocking protein to cover residual area not occupied by immobilized template molecules. It was therefore instructive to investigate the surface imprinting of BSA itself, as imprinted materials are formed in contact with a surface coated with a mosaic of IgG and BSA molecules and their respective epitopes. In addition, there exists the possibility that the non-covalently bound BSA may become incorporated into the polymeric disks during the imprinting reaction, resulting in disks with BSA covered surfaces. To understand any effect the BSA epitopes had on the imprinting procedure, templating masks coated with BSA only were produced for the creation of BSA imprinted materials. RAP IgG immobilized microplates were also used in this series of experiments to synthesize RAP IgG imprinted materials in order to investigate the selective binding ability of the materials toward AcM IgG and RAP IgG. These materials were then tested with AcM IgG-AP and the structural analog RAP IgG-AP using the optimized conditions from previous experiments (temperature of 4 °C, rabbit IgG competing protein at 2.2 μg /ml). As presented in Figure 4, the binding of different IgG molecules on the polymeric discs formed on blank polystyrene and BSA coated polystyrene templating masks were not significantly different, indicating no preference for either IgG. Only materials formed using the AcM IgG templating mask show recognition ability towards AcM IgG-AP,

with the previously determined Excess Selective Binding% of 406%. Moreover, similar levels of binding on NIP and BSA MIP materials were obtained when tested with AcM IgG-AP, indicating that BSA binding on the polymer surface may not affect the adsorption of AcM IgG-AP in the experiments.

RAP IgG imprinted materials were treated with AcM IgG-AP and the Excess Selective Binding% was similar to those of BSA MIP and NIP materials, showing no preference for the monoclonal specie. As expected, when the RAP IgG imprinted materials were tested with its own target molecule conjugated with enzyme, RAP IgG-AP, the MIP demonstrated higher recognition ability than that of the NIP, as evidenced by an Excess Selective Binding% of 206%. The experimental results indicate the imprinting sites on the AcM IgG MIP surface were made by the imprinting of AcM IgG but not BSA. In addition, when the AcM IgG MIP and NIP were tested with RAP IgG-AP solution, binding of both recognitive and control materials was similar, demonstrating the excellent selectivity of the MIP at the optimized working conditions. More importantly, the RAP IgG targeted MIP illustrates no significant binding affinity towards AcM IgG-AP, providing further evidence that the AcM IgG MIP synthesized using an AcM IgG templating mask has excellent recognition ability and the optimal working conditions are very efficient to improve the Excess Selective Binding% for the AcM IgG MIP. It is worth noting that binding of RAP IgG by the RAP IgG-imprinted material was not as strong as binding of AcM IgG by the AcM IgG-imprinted materials. During the initial stages of this work, substantial effort was expended to find an optimal polymerization recipe for maximum binding of AcM IgG. This optimization step was not undertaken for the RAP IgG targeted materials so as to use identical reaction conditions

for this comparison test. Future work is indicated to identify the level of optimization needed for the production of synthetic materials for recognition of multiple IgG targets, for example in synthetic protein microarrays.

3.4. Fluorescence intensity study of templating masks and recognitive materials

In order to investigate the fate of the immobilized template molecules after imprinting and evaluate potential for these template masks to be reused, fluorescence intensity measurements were conducted using AcM IgG coated template masks, BSA coated microplates, blank polystyrene microplates, and their corresponding imprinted materials. To understand why imprinting effectiveness decreased upon further imprinting, two sets of experiments were conducted. First, experiments were designed to quantify the amount of template IgG exposed and bioactive both before and after the imprinting procedure. Secondly, the completed MIP materials themselves were evaluated using a secondary IgG molecule to detect whether template was transferred from mask to MIP.

As illustrated in Figure 5, both new and used AcM IgG templating masks bound significantly more fluorescent conjugate, anti-mouse IgG – FITC, than that of the blank polystyrene or BSA coated microplates. According to Figure 5, we see the fluorescence intensity of all the used masks decreased following polymerization and MIP removal, indicating that less AcM IgG was available on the mask surface for interaction. There are several possible reasons for this decrease in IgG availability. First, some polymer may remain on the template mask despite repeated washings, covering the protein.

Additionally, some protein may be transferred from the template mask to the completed MIP.

A second test was then conducted where the secondary IgG, anti-mouse IgG – FITC conjugate, was used to probe whether immobilized antibody was transferred from the templating surface to the imprinted material. According to the fluorescence intensity results, there was no significant fluorescence difference between binding of the secondary IgG on the AcM IgG templated MIP, BSA templated MIP, and NIP (Table 1), demonstrating that no significant transfer of template molecule was detected through use of the secondary IgG technique.

3.5. Material characterization

ATR-FTIR measurements were then used to characterize the surfaces of a new templating mask, a templating mask used once, a control polymeric disk, and a polystyrene surface used as a control. The spectra of these surfaces are presented in Figure 6. The AcM IgG coated microplate and control materials were scanned as references. By comparing the spectra of the used templating masks with the reference spectra of the polystyrene substrate and polymeric discs, it becomes clear that polymeric material remains on the templating mask following the surface imprinting procedure.

4. CONCLUSIONS

A new surface imprinting method for the production of synthetic materials with recognitive properties towards proteins was presented. The IgG class of molecules was

chosen as model proteins due to their conserved/variable structure. Templating masks were prepared using controlled bioconjugation methods that immobilized the Fc domain to the microplate surface. Experimental data demonstrates that MIPs made from this surface imprinting technique were able to specifically recognize and rebind the template molecule. The surface imprinted polymeric disks were prepared with a mixture of the functional monomer, cross-linker, photoinitiator, and solvent using AcM IgG coated microplates as the templating masks. The AcM IgG imprinted polymeric disks present selective rebinding ability towards their template molecules at various temperatures (4 and 20°C, pH 7.4-7.5) and the best performance of the MIPs was obtained when treated at 4 °C. In addition, the binding ability could be further enhanced by adding a competing protein (rabbit IgG) which competed with the analyte for binding on non-specific sites. Under optimized conditions, the recognitive surfaces bound five times more target molecule than control materials without any significant increase in analog binding. A combination of ATR-FTIR analysis and use of a secondary antibody technique illustrated that the template mask loses some imprinting ability after the initial procedure, and that polymer is deposited on the template mask surface following reaction.

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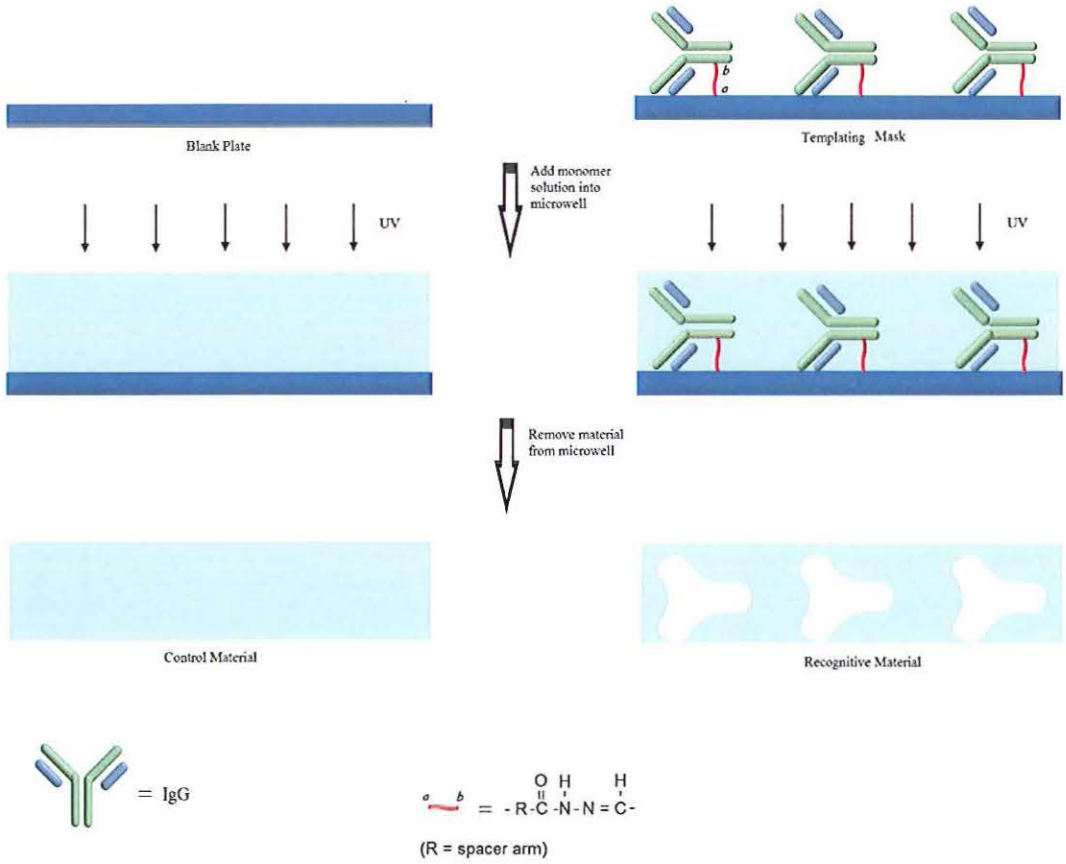
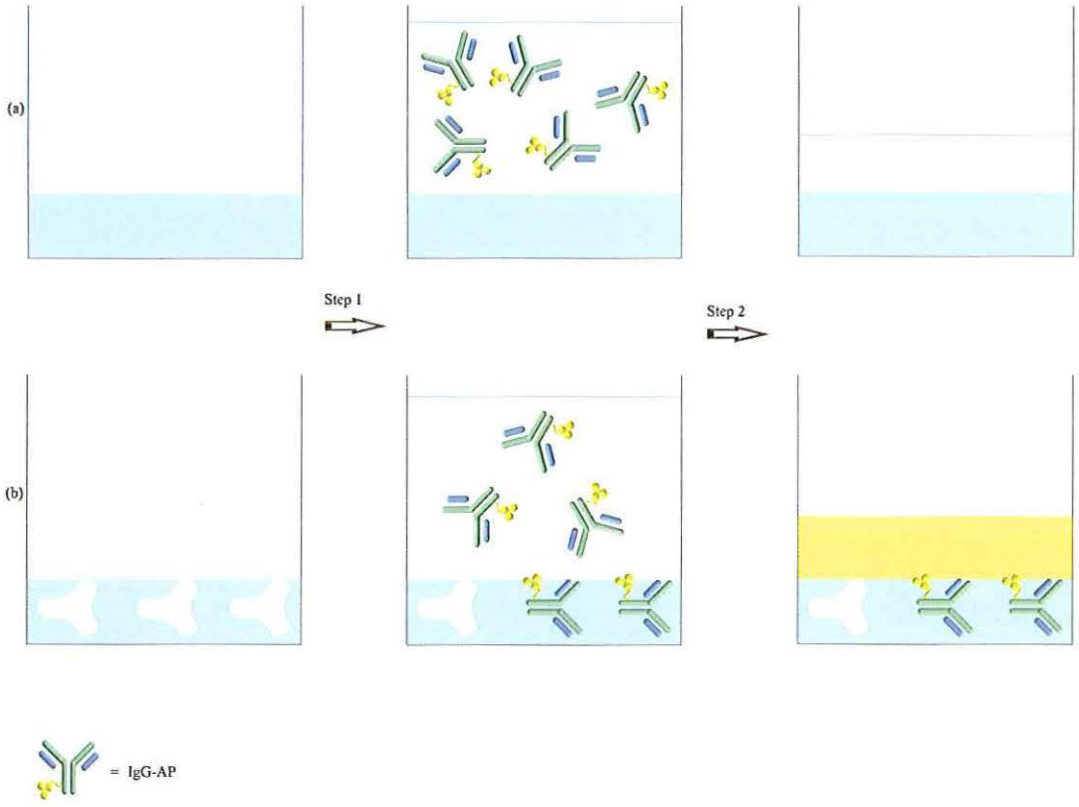
Table 1: Adsorption of FITC tagged anti-mouse IgG on various materials to study possible transfer of template molecule to the finished MIP after material removal.

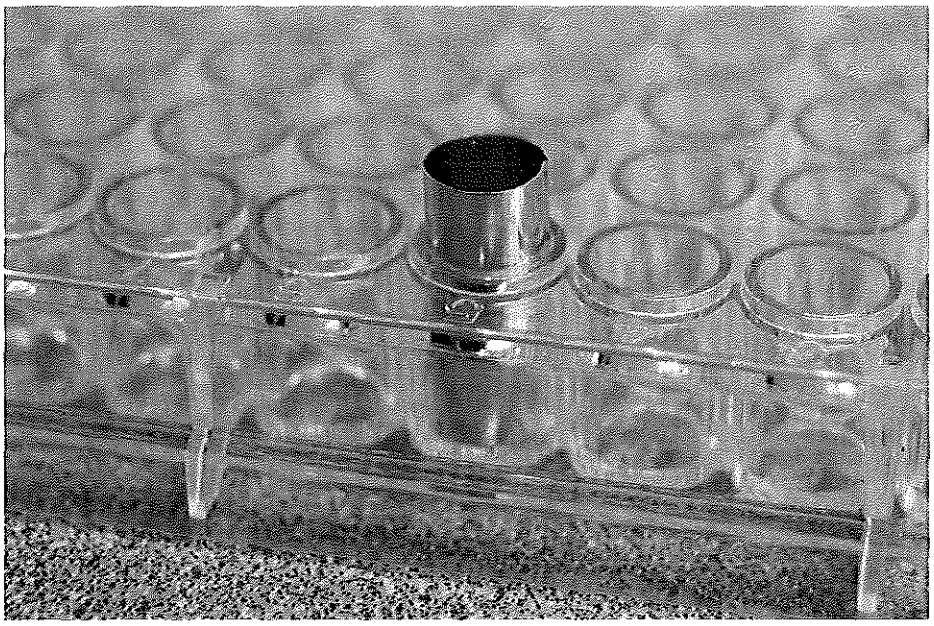
	AcM IgG MIP	**BSA MIP**	**NIP**
Fluorescence Intensity	**3818±196**	**4173±233**	**4084±124**



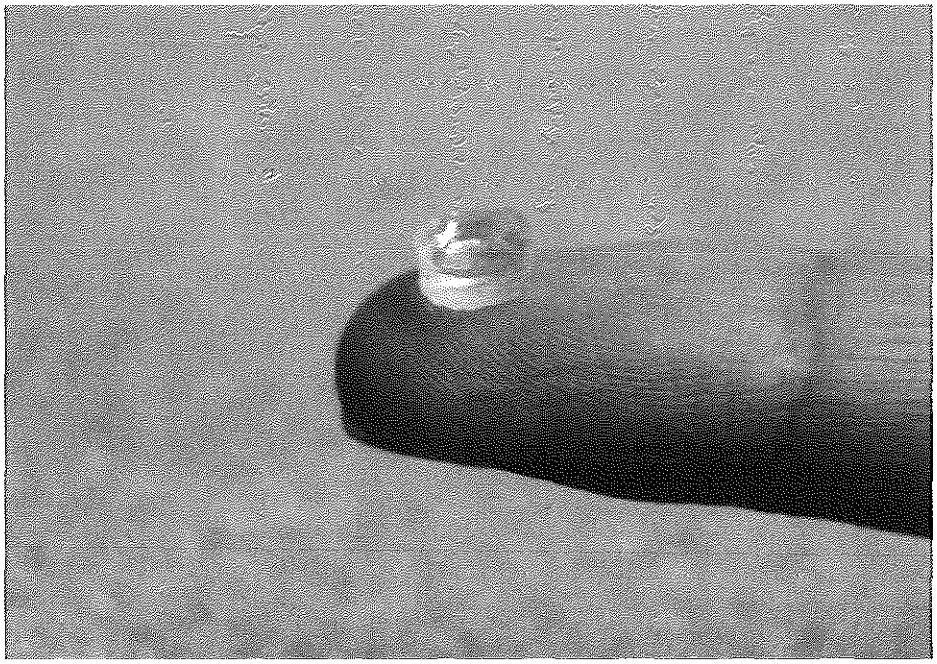
Scheme 1: Formation of recognitive and control materials by the surface imprinting technique. The target molecule is immobilized on a templating mask. Monomer is brought in contact with the templating mask to produce a recognitive material or a pristine surface to produce a control.



Scheme 2: Selective rebinding analysis of the recognitive and control materials.
(a) control material, (b) recognitive material.
Step 1: incubate in IgG-AP solution,
Step 2: rinse with PBS-Tween 20 buffer. Add enzyme substrate solution and measure absorbance.



(a)



(b)

Figure 1: Pictures of the aluminum sleeve and polymeric material.
(a) aluminum sleeve inserted in a microplate well,
(b) polymeric disc removed from the microplate well after the polymerization reaction.

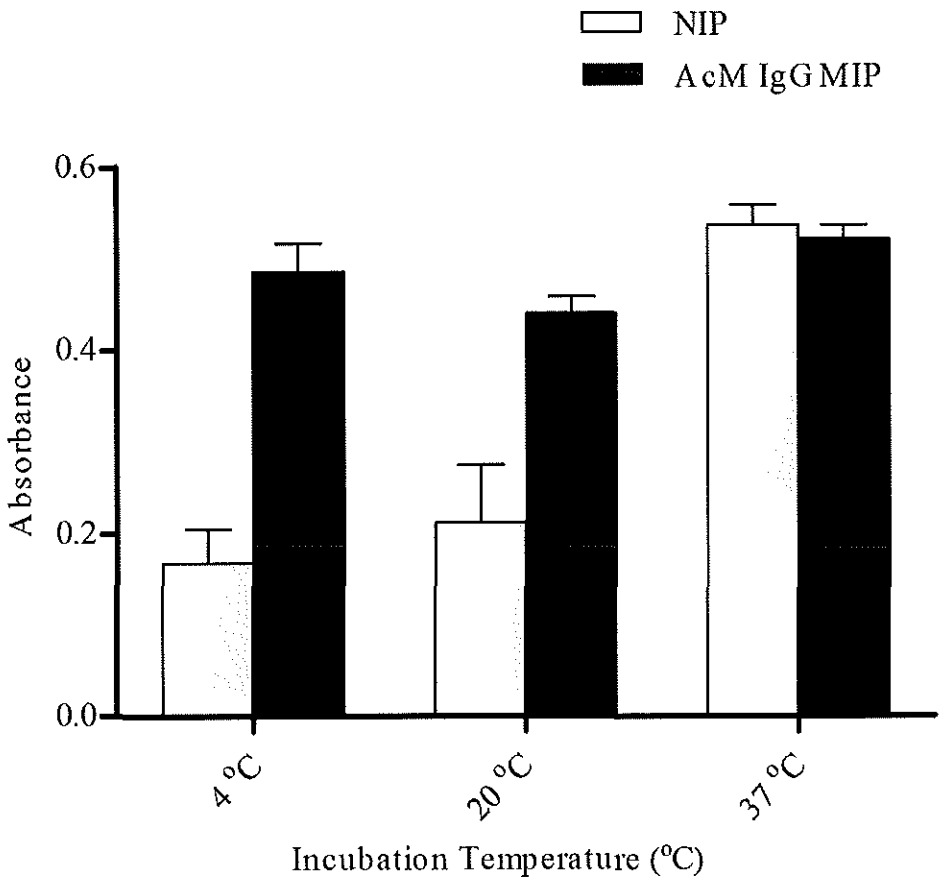


Figure 2: Temperature effect on recognition and rebinding ability.

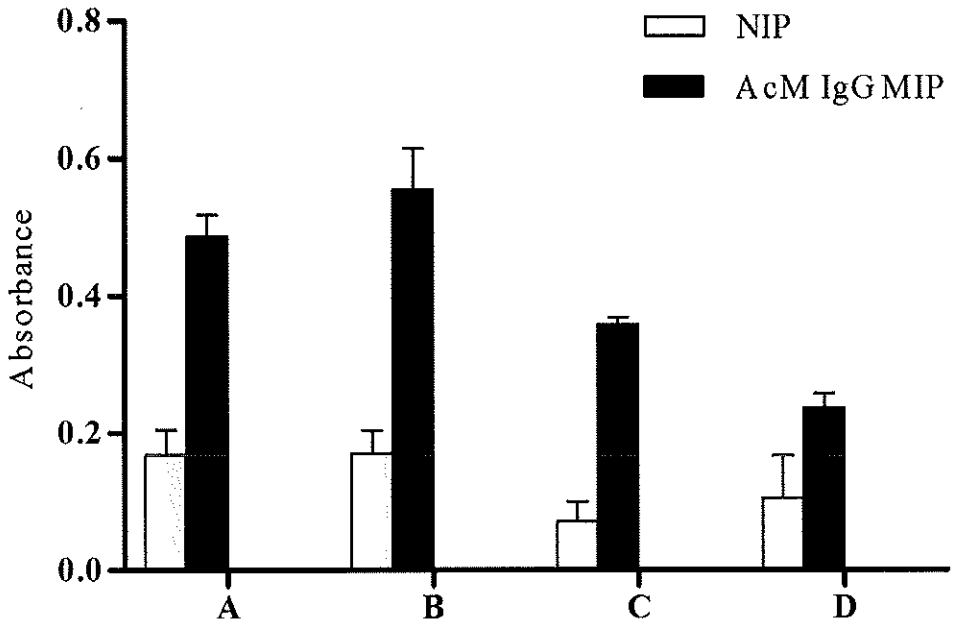


Figure 3: Binding affinity analysis in the presence of a competing protein.
A: materials evaluated solely with 1.6 µg/ml AcM IgG-AP solution, B: materials incubated in a solution containing 1.6 µg/ml AcM IgG-AP and 1.1 µg/ml competing protein, C: materials incubated in a solution with 1.6 µg/ml AcM IgG-AP and 2.2 µg/ml competing protein, and D: materials incubated in a solution with 1.6 µg/ml AcM IgG-AP and 5.5 µg/ml competing protein.

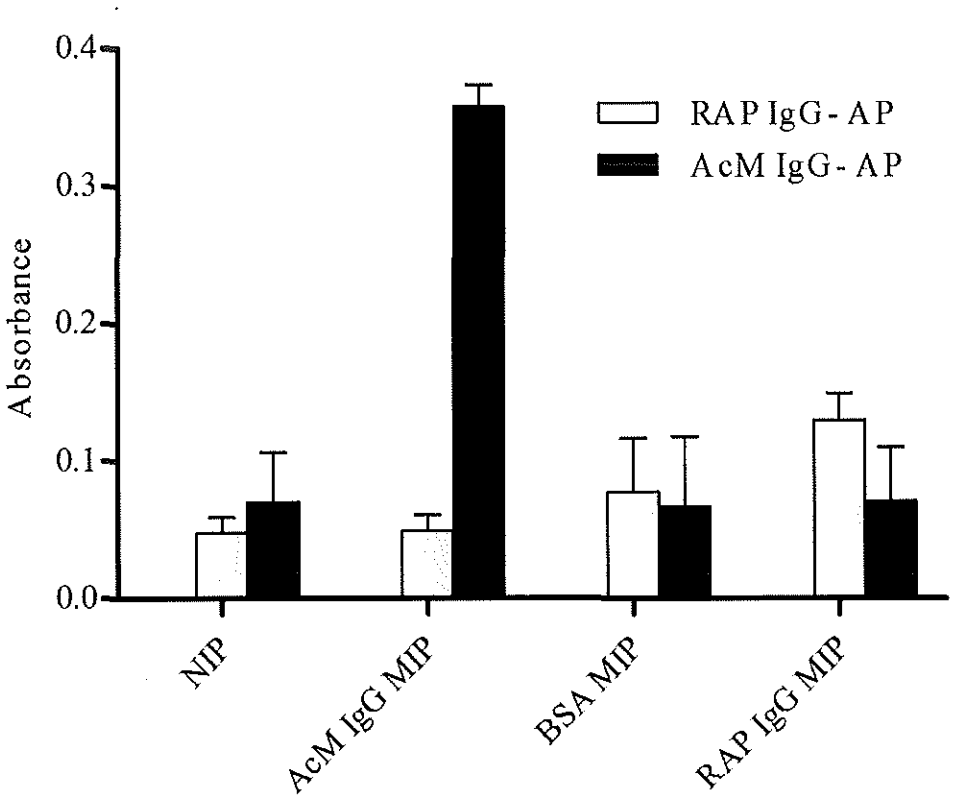


Figure 4: Analysis of the binding of different protein targets (RAP IgG-AP or AcM IgG-AP) on a series of materials formed with varying templating masks (polystyrene surface, surface with immobilized AcM IgG surface, with adsorbed BSA, and with immobilized RAP IgG).

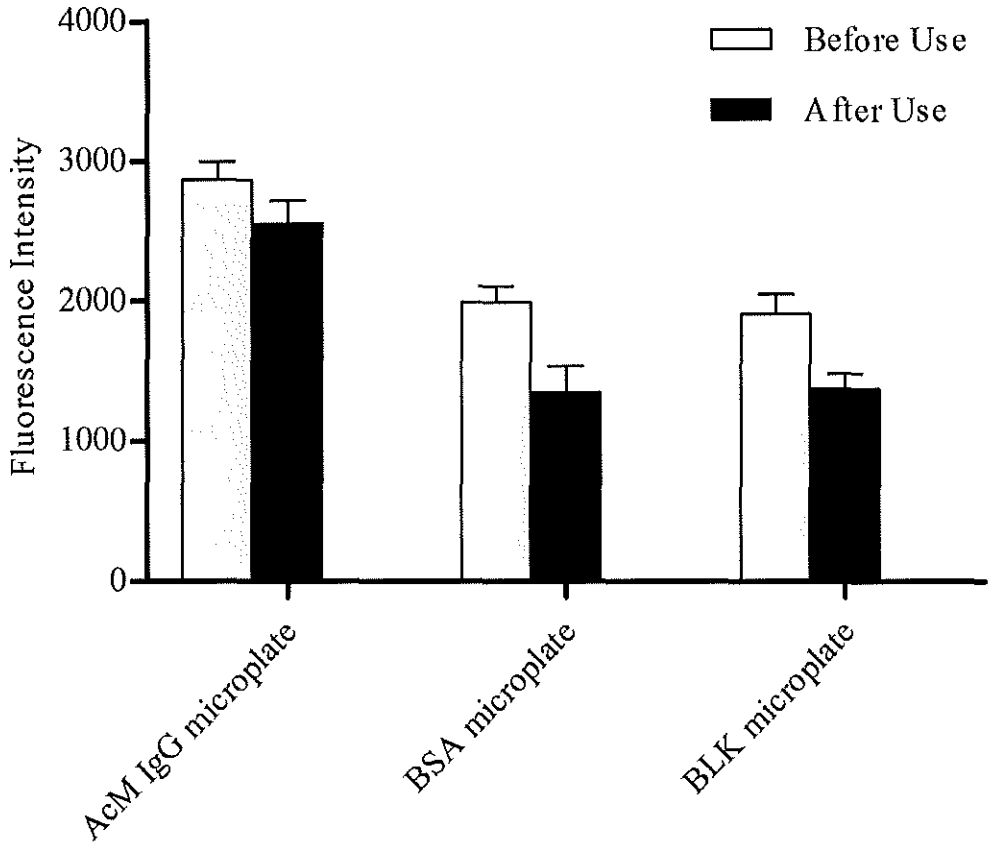


Figure 5: Quantification of surface bound AcM IgG using an FITC tagged anti-mouse IgG for evaluation of template mask reuse. Also shown are control experiments conducted with BSA coated and blank polystyrene microplates to establish the level of non-specific binding.

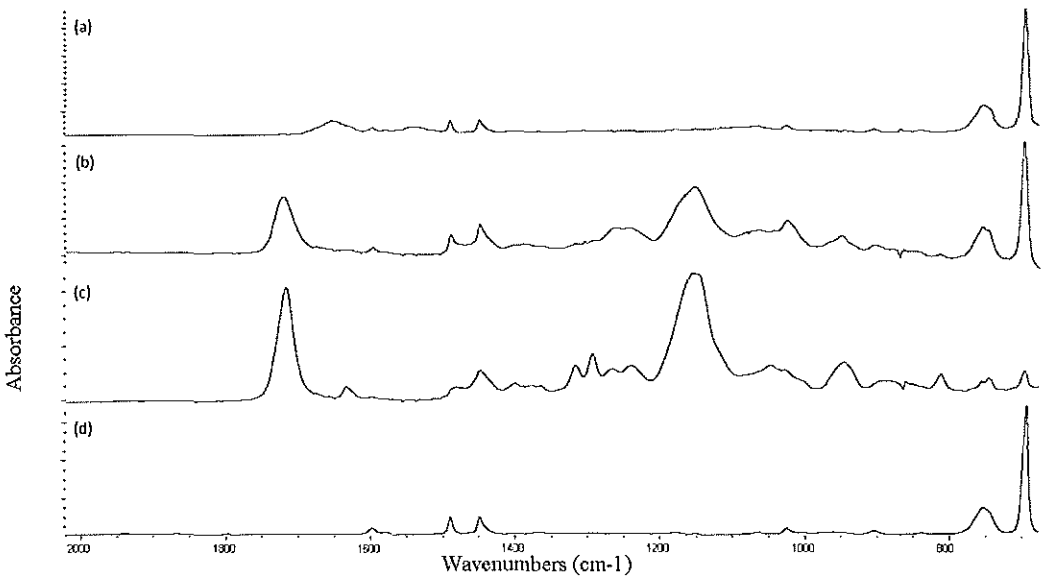


Figure 6: Material characterization via ATR-FTIR.
(a) spectrum of AcM IgG coated polystyrene microplate (templating mask),
(b) templating mask used once,
(c) reference spectrum of polymeric material made of 80 % EGDMA, 20 % HEMA and 2 wt. % HCPK,
(d) reference spectrum of polystyrene substrate material.

II. RECOGNITIVE IMPRINTED POLYMERS FORMED USING A PROTEIN STAMP TEMPLATE

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ABSTRACT

Molecularly imprinted polymers (MIPs) have attracted significant attention in the past few decades as synthetically produced elements for use in analytical instruments, drug delivery systems, and biomedical devices, etc. A new surface imprinting technique is reported in which a template mask, coated with immunoglobulin G (IgG) as a recognition target, was used to synthesize materials capable of antibody recognition. Various functional monomers and porogens were investigated in an effort to produce materials with substantial recognition for the protein target. As the template protein was linked to the imprinting mask, recovery of the template and reusability of the template mask was feasible and therefore examined.

In this work, monoclonal mouse anti-c-myc IgG was selected as the template molecule and was immobilized on a protein A functionalized glass substrate. Among the functional monomers and porogens used, recognitive materials formed using HEMA as the functional monomer, EGDMA as the cross-linker, and DMSO as the solvent are the

most effective. In addition, template mask reusability was studied, with up to five repeated uses possible before degradation of the mask and loss of recognitive abilities.

Keywords: Molecular imprinting, Protein, Immunoglobulin G

1. INTRODUCTION

Antibodies, due to their excellent recognitive abilities, are featured prominently in various immunoassays such as ELISA (enzyme-linked immunosorbent assay), RIA (radioimmunoassay), and FIA (fluoroimmunoassay) [1-3]. However, antibodies as a class of protein molecule tend to be expensive to produce and are easily denatured. As a result, the synthesis of artificial materials capable of mimicking the recognitive abilities of immunoglobulins has been an active research area. Of the various techniques employed in the production of synthetic recognitive materials, molecular imprinting has been found especially interesting. Molecular imprinting is a technique where functional and cross-linking monomers are copolymerized in the presence of a template molecule, producing a cross-linked material with memory for the template. The molecularly imprinted polymer (MIP) is thus complementary chemically and sterically to the template molecule [4,5]. After removal of the template molecule from the MIP, the polymer network possesses sites for recognition of the template molecule or structural analogs.

Imprinted materials have been seen as promising substitutes for certain biological molecules due to their ease of preparation, low cost, and their thermal and chemical stabilities [6-10]. Owing to these advantages, MIPs have many potential applications in the fields of sensing, purification, separation, and catalysis of reactions. However, MIPs produced for the recognition of proteins have seen only limited success due to flexible conformation of proteins, the abundance of interaction sites on the protein surface, and the accessibility of binding sites [11-15]. Traditional imprinting methods rely on addition of template into the bulk of the monomer solution. With large molecules such as proteins, diffusion into a densely crosslinked material would be extremely hindered and

impractically slow. In addition, removal of the template protein would be challenging and recovery of the valuable protein difficult.

As a result, there is growing interest in materials which are recognitive solely at the surface. This allows the bulk of the material to be rigid, restricting chain mobility and thereby retaining binding site conformation. Shi et al. [16] utilized mica as the protein adsorption substrate. Proteins were coated with disaccharide and a polymer film was formed around this "sugar shell". The synthesized imprinted material demonstrated selective recognition ability toward the template proteins. Lin et al. [17] used a protein stamp to photopolymerize surface imprinted materials via a microcontact imprinting technique.

In this work, a surface imprinting technique was developed which employed an immunoglobulin G template molecule. In this work, a templating mask was first prepared using oriented template protein. The template protein, monoclonal anti-c-myc IgG, was coated on an epoxy-activated microscope slide previously covered with a layer of protein A. In order to optimize this surface imprinting technique, serum IgG was used as a competitive protein in the ELISA-like analysis. Both the target protein (monoclonal anti-c-myc IgG) and its structural analog (rabbit anti-pig IgG) were selected as template molecules to form surface imprinted materials in the investigation of the specific recognition ability of the MIP. Several functional monomers and porogens were compared in an effort to optimize MIP performance. This method allows not only the efficient synthesis of the synthetic materials with protein recognition abilities but also opens the possibility of template molecule reuse.

2. EXPERIMENTAL

2.1. Chemicals and materials

Rabbit anti-pig IgG (whole molecule) alkaline phosphatase conjugate (RAP IgG-AP), anti-pig IgG (whole molecule) developed in rabbit affinity isolated antigen (RAP IgG), protein A from Staphylococcus aureus, monoclonal anti-c-myc purified mouse IgG (AcM IgG), monoclonal anti-c-myc alkaline phosphatase conjugate (AcM IgG-AP), ethylene glycol dimethacrylate (EGDMA), bovine serum album (BSA), acetonitrile, methacrylic acid (MAA), acrylamide and 1-hydroxycyclohexyl phenyl ketone (HCPK) were purchased from Sigma-Aldrich (St. Louis, MO.). 2-hydroxyethylmethacrylate (HEMA) was obtained from ACROS Organics. The functional monomers MAA and HEMA were purified by vacuum distillation prior to use. SuperEpoxy 2 slides were ordered from TeleChem International, Inc. (Sunnyvale, CA). Non-binding surface microplates (Corning 3990), and blank polystyrene microplates (Costar 3370) were used. The photomask was made of poly(ethylene terephthalate) and printed in black with transparent circles by an ECRM Scriptsetter ZRL36 system (Brakensiek Systemhaus, Dortmund Nordrhein-Westfallen, Germany). Teflon sheets were ordered from Ridout Plastics Company Inc. (San Diego, CA) to make spacers. The enzymatic substrate PNPP (p-nitrophenyl phosphate, disodium salt) and serum-derived IgG from a rabbit source (Rabbit IgG whole molecule) were obtained from Pierce Biotechnology, Inc. (Rockford, IL.). The chemical structures of the cross-linker, functional monomers, and solvents involved in this study are listed in Table 1.

2.2. Templating mask preparation

Material formation and analysis methods are shown graphically in Schemes 1, 2, and 3. First, a protein template mask was constructed for use in the preparation of the surface imprinted materials. The template mask was made of epoxy functionalized glass substrate coated with proteins (Scheme 1). Monoclonal anti-c-myc IgG was selected as the template molecule. The SuperEpoxy 2 slide was first treated with a protein A solution (0.5 mg/ml protein A in 1X phosphate buffered saline, 1X PBS, pH 7.4) at 4 °C for 24 hrs. Then the protein A coated substrate was rinsed by 1X PBS for 3 times (5 minutes each time). The protein A immobilized substrate was later incubated in a BSA solution (0.5 wt. % BSA in phosphate buffered saline-Tween 20, PBST, pH 7.4, 0.05% Tween 20 in PBS) for 15 minutes at room temperature to block the area without protein A. Later, the substrate was washed by PBST 3 times (5 minutes each step). The substrate was then incubated in an IgG solution (2 μg/ml monoclonal anti-c-myc IgG in tris buffered saline, TBS, pH 7.5) for 1.5 hours at room temperature. Finally, the substrate was washed with PBST three times (5 minutes each time) to remove any weakly bound template molecules from the substrate surface. The protein template mask was dried by nitrogen gas and stored at 4 °C prior to using within 24 hours. Anti-pig IgG template masks were formed in the analogous manner. To study the influence of the protein A and BSA molecules on the recognitive structures, materials were made under the same conditions and procedures but no IgG was added.

The templating mask for the production of control materials was made using the same SuperEpoxy 2 slides, which were then blocked using a BSA solution (0.5 wt. %

BSA in PBST) for 15 minutes at room temperature. Unbound BSA was removed by rinsing with PBST three times (5 minutes each time) and drying with nitrogen gas.

2.3. Preparation of molecularly imprinted or control polymeric discs

Molecularly imprinted polymers (MIPs) were synthesized by photopolymerizing reactive mixtures directly in contact with the protein template mask. The photoinitiator HCPK was mixed with the functional monomer(s) (MAA, HEMA, or the combination of MAA and HEMA), the cross-linker EGDMA, and solvent (DMSO or acetonitrile). The polymeric discs were synthesized as shown in Scheme 2: the prepolymerization mixture contained 20 mole% of the functional monomer(s), 80 mole% of the cross-linking monomer, and the photoinitiator HCPK (2% w/w of the final composition). Solvent (volume ratio of solvent to monomers was 3:10) was added to the mixture and mixed well before the solution was pipetted in between two clamped glass slides with a Teflon spacer of 0.76 mm. One of the glass slides was the template mask and the other was a piece of microscope slide attached with a photomask. The photomask was a black poly(ethylene terephthalate) film with 12 transparent circles of 5.08 mm diameter and a center-to-center distance of 8.64 mm. Polymerization was initiated by UV light irradiation (365 nm, 150 mw/cm^2, INTELLI-RAY 600, UVi Tron International, West Springfield, MA) and the reaction was done at room temperature for 25 seconds in a nitrogen environment. The control polymer (CP) was synthesized in a similar way except that the control materials were formed on the BSA coated mask instead of the IgG functionalized template mask.

2.4. Recognition ability analysis of the MIP

After the polymerization reaction, the polymeric discs were carefully peeled off from the glass chip and then put into the microwell of a Corning non-binding 96-well microplate. An aliquot of 200 µl TBS was used to dialyze the polymeric discs in each microwell for an hour at 4 °C in order to remove any unreacted monomers or residual solvent from the polymer networks. The polymer discs were 0.76 mm in thickness and 5.08 mm in diameter. Next, an enzyme-linked immuno-sorbent assay (ELISA) was applied in order to analyze the recognition ability of the MIP. A 200 µl aliquot of IgG alkaline phosphatase conjugate solution (1.6 µg/ml IgG-AP in TBS) was added into the non-binding microplate wells containing the polymeric discs and incubated for 40 minutes at 4 °C. The polymer samples were then rinsed with PBST at room temperature to remove the weakly absorbed IgG molecules. The quantity of specifically absorbed IgG alkaline phosphatase conjugate was later measured through use of a colorimetric assay based on the activity of the conjugated alkaline phosphatase enzyme. A 100 µl aliquot of PNPP was placed in each microwell containing the polymeric discs and allowed to react for 40 minutes at room temperature in darkness. Later, the substrate solution was diluted with 100 µl fresh de-ionized (DI) water after reaction and 100 µl of the solution was withdrawn from the microwell to be measured at 405 nm using FLUOstar OPTIMA multifunction microplate reader (BMG LABTECH, Durham, NC) to determine the optical density of the solution. The analysis procedure is demonstrated in Scheme 3. The recognition ability was defined as Excess Selective Binding% (ESB%):

$$ESB\% = [[MIP\ absorbance] - [CP\ absorbance]]/[CP\ absorbance] \times 100\% \quad (1)$$

All of the experiments were done in triplicate under the same conditions.

2.5. Binding affinity analysis in the presence of a competing protein

A competitive specie to the monoclonal IgG, serum IgG derived from rabbit (Rabbit IgG), was introduced in the IgG-AP solution as a blocking/competing protein. In this study, Rabbit IgG was added at various concentrations (0.0, 0.9, 1.6, 2.1, and 2.5 μg/ml) alongside the target IgG-AP molecule (held fixed at 1.6 μg/ml). The rebinding analysis followed the previous section. In this study, five independent experiments were conducted, triplicates for each examination group.

2.6. Repeating use of the template mask

The template mask was repeatedly used in the preparation of the MIP. The preparation of MIP procedure was similar to that described in the section, Preparation of Molecularly Imprinted or Control Polymeric Discs. After each use, the template mask was thoroughly washed by de-ionized H_2O and dried by N_2 gas. The template mask was stored at 4 °C prior to use.

3. RESULTS AND DISCUSSION

3.1. Competing protein effect on MIP rebinding ability

A competitive species, serum IgG derived from rabbit, was introduced into the AcM IgG-AP solution in order to improve the rebinding efficiency of the MIP. There are two reasons to add a competitive IgG in the AcM IgG-AP solution. First, the addition of

a competitive protein in the secondary antibody system will enhance the amount of the target protein adsorbed on the polymer surface [18]. Second, the competing protein will compete with the template protein for either specific or non-specific binding sites, when added at significantly higher concentrations than the target protein, and the competitive IgG will exhaust the non-specific binding sites and create greater contrast between the imprinted and non-imprinted materials. In this set of experiments material composition was kept constant, adopting a formulation where the material was formed in DMSO using 20 mole% HEMA and 80 mole% EGDMA. This formulation was used previously by our group in our initial studies with IgG recognitive materials and serves as the starting point for this work. The competitive protein, Rabbit IgG, was added into the AcM IgG-AP solution at various concentrations; 0, 0.9, 1.6, 2.1, and 2.5 μg/ml. As presented in Figure 1, the ESB% of the MIP increased gradually when Rabbit IgG was added as a competitive species. However, the ESB% began to decrease when the concentration of Rabbit IgG grew larger than the concentration of the desired component. As a result, the MIP gave best performance when the concentration of competitive analyte was the same as that of the target protein in the binary protein system. As a result, 1.6 μg/ml was chosen as the concentration of both the target molecule (AcM IgG-AP) and the competitive specie (Rabbit IgG) in the remaining experiments.

3.2. Optimization of MIP composition

For non-covalent imprinting, it is believed that a pre-polymerization complex is generated between the template molecules and functional monomers. Later, the pre-polymerization complex will be incorporated into the polymeric network during the

polymerization reaction. Moreover, the selectivity and affinity of the polymerized materials are related to the initial strength and integrity of the functional monomer(s)-template complex [19]. Therefore, it is very important to select the most suitable functional monomer(s) and the porogenic solvent so as to optimize the MIP formulation. In this work, two well known monomers, HEMA and MAA, were selected as possible functional monomers. As presented in Table 2, the MIP made from 20 mole% HEMA, 80 mole% EGDMA, and DMSO demonstrated the highest ESB% (368%), which indicated a relatively good recognition ability of the MIP. ESB% of the MIP using the combination of MAA and HEMA as functional monomers is relatively lower than that of the materials using only MAA or HEMA. It is noticeable that the ESB% was decreased with the increase of MAA added in the monomer mixture, which may be due to the prepolymerization mixture of HEMA and MAA giving a negative effect to the template-functional monomers complex.

In addition to functional monomer, the inert solvent present during the reaction is of crucial importance. Solvent interaction with the monomer and crosslinker is capable of stabilizing or destabilizing template binding in noncovalent imprinting systems. It should be noted that water, while a good solvent for the protein, is a poor solvent for imprinting and no usable results were obtained. As a result, two different solvents, DMSO and acetonitrile, were evaluated in this series of experiments. DMSO is a polar aprotic solvent and acetonitrile is a polar solvent. As presented in Table 2, the ESB% values of the polymeric materials formed using DMSO as solvent are much higher than that of the materials using acetonitrile as solvent. DMSO is therefore more suitable for this imprinting system. As a result, the optimized composition -- 20 mole% of HEMA,

80 mole% of EGDMA, and DMSO as the solvent -- was used in the remaining experiments.

3.3. MIP recognition ability analysis

Immunoglobulin G is a protein with Y-shaped structure [20], in which the antigen binding domains are concentrated on the two ends of the fork. In order to analyze the selectivity of the resulting MIP, two different types of IgGs were used to make template masks. As detailed previously, materials for the recognition of AcM IgG were produced. In addition, its structural analog, RAP IgG, was also used as a template molecule. As shown in Figure 2, both of the imprinted materials showed recognition ability toward their own template molecules. Both materials also showed no significant recognition of the structural analog when compared to the control material. The ESB% of the AcM IgG templated MIP was 368% while that for the anti-pig IgG template MIP was 202%. These results illustrate the potential application of the surface imprinting technique in the production of materials for the recognition of IgG molecules. Moreover, the experimental results also reflect that the antigen binding domain of the IgG molecule was successfully imprinted on the MIP surface since the MIPs showed preference for their template molecules over structurally similar components.

3.4. Excess Selective Binding% comparison using various masks

In this study, polymeric materials were synthesized on different substrates including pristine glass, epoxy-activated glass, BSA coated on epoxy-activated glass, protein A and BSA coated on epoxy-activated glass, and the AcM IgG template mask

described previously. The purpose of this study was to clarify that the imprinting sites that showed recognition ability towards the IgG molecules were not artifacts of the BSA or protein A that are also present on the templating mask. The results of this study, shown in Figure 3, demonstrate the importance of the IgG template protein in the production of IgG recognitive binding sites. Only materials formed in the presence of the IgG template molecule showed recognition towards it. However, it should be noted that the presence of BSA or BSA/protein A on the template mask does lead to some apparent non-specific binding of IgG. The lowest IgG binding occurred for surfaces prepared with no protein contact. Two comments result. Firstly, since the values for ESB% are computed using the BSA coated surfaces as control materials, the values reported throughout this work are conservative and greater contrast (absorbance values measured using imprinted vs. non-imprinted materials) may be attained using pristine glass to produce the control. Secondly, in the production of surface imprinted protein microarrays, an obvious application of this technology, no surface would remain free of protein due to the use of a blocking step. The BSA imprinted material as control polymer was justified for this reason.

3.5. Repeated usage of the template mask

A promising aspect of this surface imprinting technique is the possibility to reuse the template mask so as to further lower the cost of this technique. Therefore, the AcM IgG template mask was repeatedly used to synthesize the molecularly imprinted materials. As presented in Figure 4, ESB% of the MIP using template mask from the 1st to 7th time is 368%, 190%, 179%, 107%, 71%, 52% and 0%, respectively. According to

Figure 4, when the template mask was repeatedly used, the imprinting sites became less and less effective with each cycle. However, it is still unclear whether this is a result of the protein being denatured, removed, or blocked from contact with the monomer mixture.

4. CONCLUSIONS

This surface imprinting technique provides an efficient method in the preparation of protein recognitive synthetic materials. The addition of a competitive protein was studied so as to improve the Excess Selective Binding% of the MIP. Different functional monomers and solvents were tried in this work to optimize the MIP compositions, and an optimal formulation was obtained. In order to analyze the specific recognition ability of the MIP, two different template masks were employed to synthesize MIPs and their affinities for the different targets were studied. The experimental results illustrated the surface imprinted materials formed on these two different template masks recognized only their template molecules and not the structural analog. The effect of the template mask substrate was also studied. Pristine glass serves as the best templating surface for control material formulation and that the presence of the traditional blocking protein, BSA, slightly increased non-specific adsorption.

In conclusion, the surface imprinting technique introduced in this study offers a convenient way to prepare synthetic materials with protein recognitive abilities. The materials showed affinity for their template molecule and exhibited good selectivity. The

technique could find possible applications in synthetic protein microarray chips, photopatterned sensor elements on microfluidic devices, or antibody sensitive areas in the construction of affinity-based separation devices.

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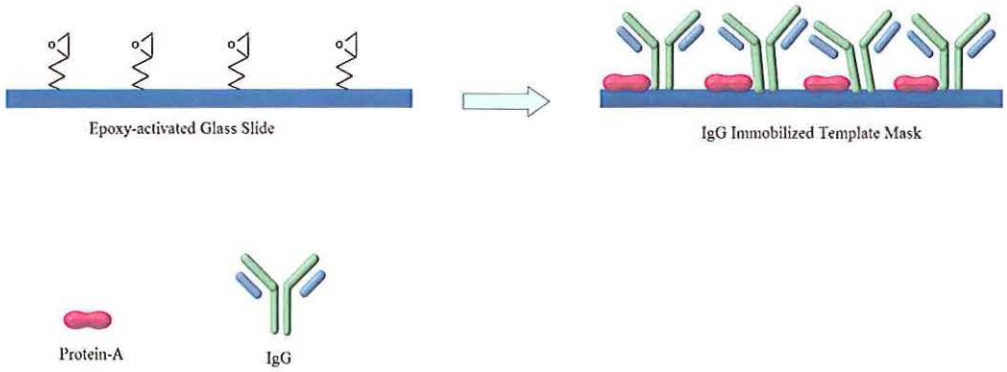
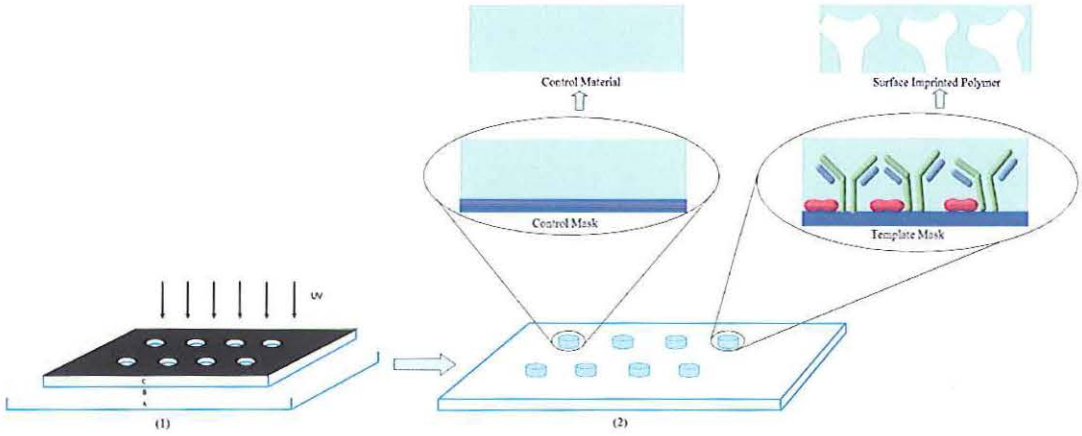
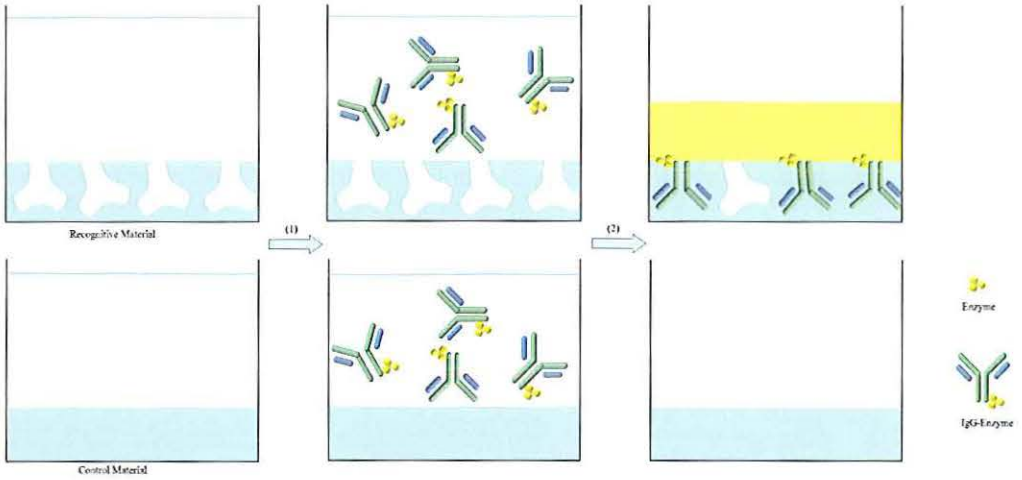


Scheme 1: Illustration of IgG immobilized template mask.



Scheme 2: Formation of molecularly imprinted and control materials.
A: IgG immobilized template mask or BSA coated substrate
B: Teflon spacer
C: Photomask attached to glass substrate



Scheme 3: ELISA-like analysis of the recognition ability of the MIPs and control materials.
Left: Materials are placed in a microwell with recognitive surface facing up
Center: Enzyme coupled IgG solution is added to the microwell and allowed to incubate. Solution is removed and the material washed to remove weakly bound protein
Right: Materials with bound protein are incubated in substrate solution and the corresponding color change quantified.

Table 1: Molecular weights and chemical structures of monomers and solvents used in this study.

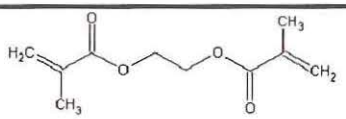
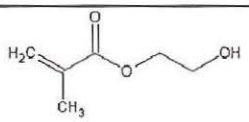
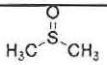
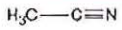
	Molecular Weight	Chemical Structure
EGDMA	198	
HEMA	130	
MAA	86	
DMSO	78	
Acetonitrile	41	

Table 2: List of MIP compositions evaluated and resultant target molecule recognition.

Monomer(s)	Cross-linker	solvent	ESB (%)
20% HEMA	80% EGDMA	DMSO	368±46
15% HEMA+5% MAA	80% EGDMA	DMSO	67±27
10% HEMA+10% MAA	80% EGDMA	DMSO	26±21
5% HEMA+15% MAA	80% EGDMA	DMSO	24±12
20% MAA	80% EGDMA	DMSO	82±34
20% HEMA	80% EGDMA	Acetonitrile	NA
20% MAA	80% EGDMA	Acetonitrile	26±21

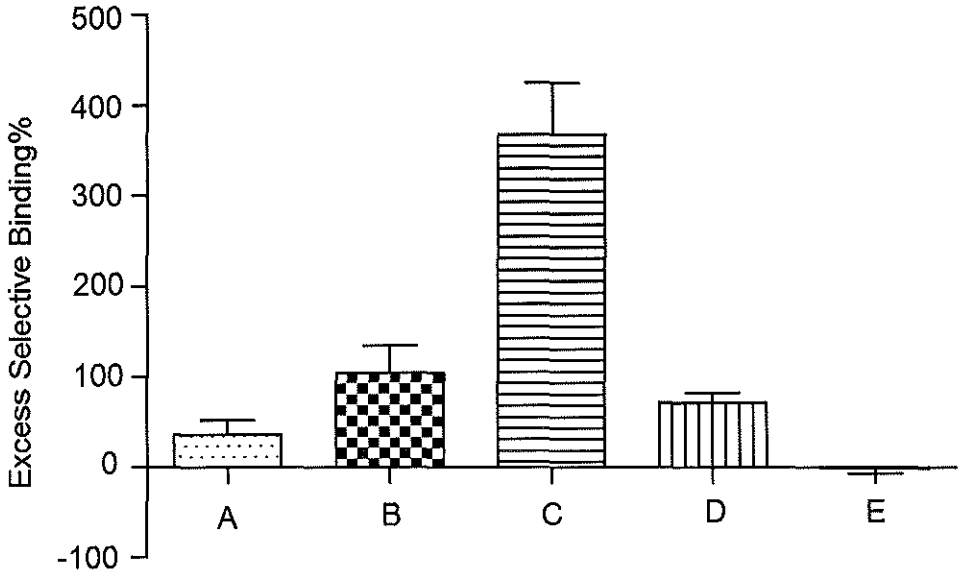


Figure 1: The effect of adding a competitive IgG on MIP rebinding ability.
A: MIP treated with a solution containing only 1.6 ug/ml AcM IgG-AP;
B: MIP treated with a solution containing 1.6 ug/ml AcM IgG-AP and 0.9 ug/ml Rabbit IgG;
C: Increased to 1.6 ug/ml Rabbit IgG;
D: Increased to 2.1 ug/ml Rabbit IgG;
E: Increased to 2.5 ug/ml Rabbit IgG.

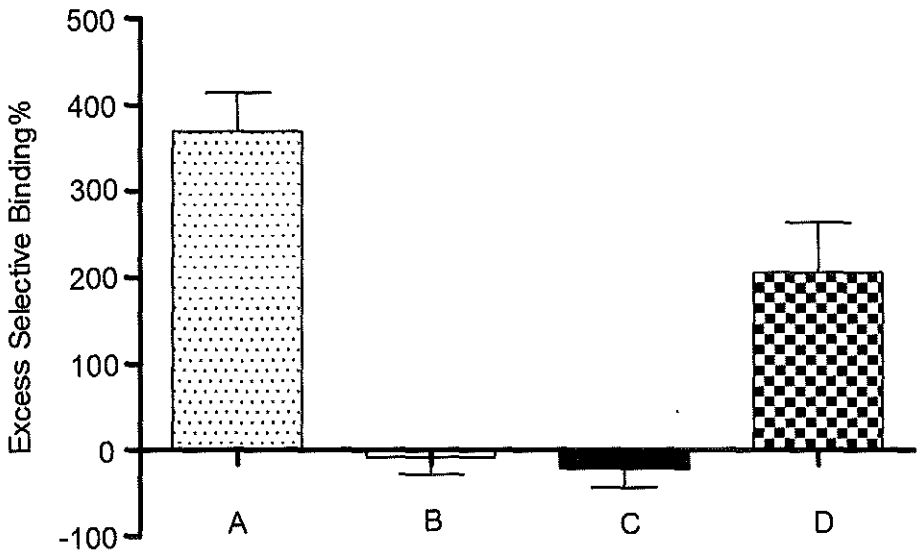


Figure 2: MIP recognition ability analysis using different template masks and incubation solutions.
A: AcM IgG MIP treated with AcM IgG-AP solution in the ELISA-like analysis;
B: AcM IgG MIP treated with RAP IgG-AP solution in the ELISA-like analysis;
C: RAP MIP treated with AcM IgG-AP solution in the ELISA-like analysis;
D: RAP IgG MIP treated with RAP IgG-AP solution in the ELISA-like analysis.

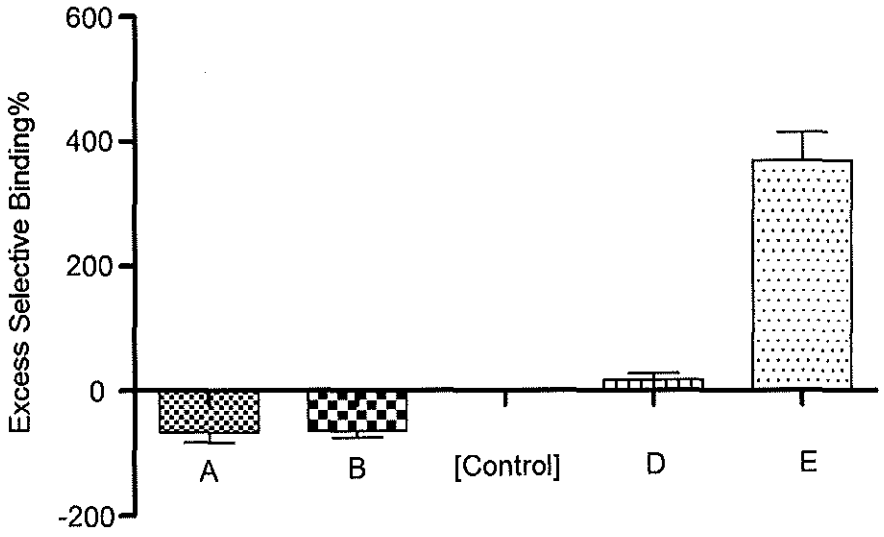


Figure 3: Excess Selective Binding% comparison of materials formed on different substrates using AcM IgG-AP test molecule.
A: Polymer synthesized in contact with pristine glass slide;
B: Polymer synthesized in contact with epoxy-activated glass surface;
[Control]: Polymer synthesized in contact with BSA coated epoxy-activated glass surface;
D: Polymer synthesized on BSA and Protein A immobilized surface;
E: Polymer synthesized on AcM IgG template mask.

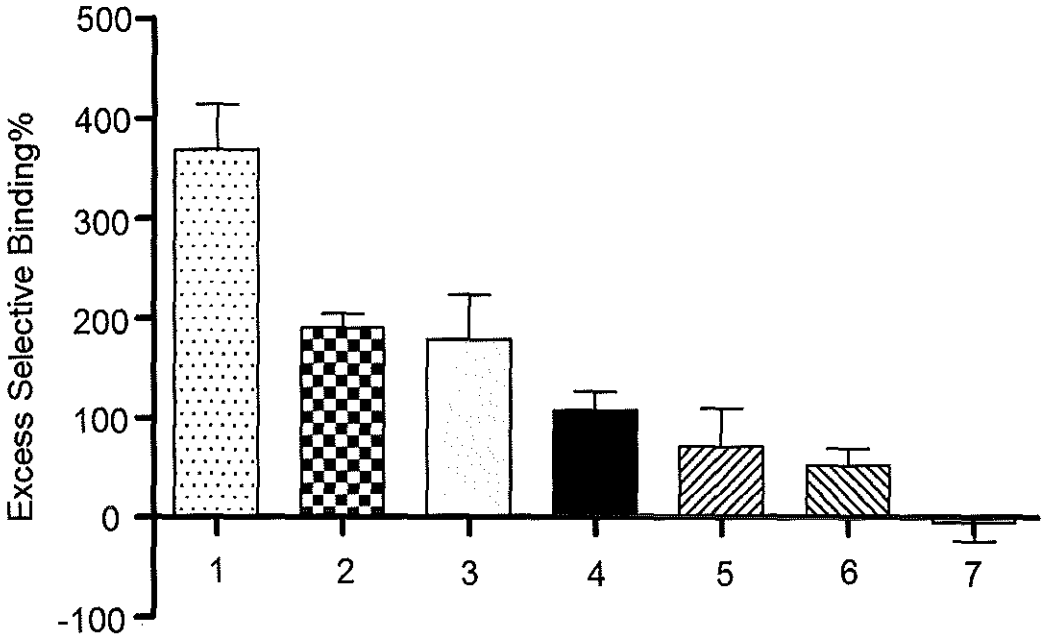


Figure 4: Analysis of template mask reusability. X-axis denotes the number of imprinting cycles the mask has undergone. Excess Selective Binding% is measured using the material formed with the mask.

III. RECOGNITION OF PROTEIN TARGETS BY SURFACE IMPRINTED POLYMERIC MATERIALS

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ABSTRACT

A surface imprinting technique using protein templating masks is described to creat artificial materials which mimic antibodies. In this study, two model antigens (protein A and monoclonal anti-c-myc immunoglobulin G) were selected as the template molecules. Protein A is employed not only to synthesize surface imprinted polymeric discs, but also to control the orientation of the immunoglobulin G molecules, due to its ability to bind strongly to the Fc domain of antibody. The importance of functional versus cross-linking monomers was analyzed so as to optimize the performance of the recognitive materials. It was found that controlling the orientation of the template molecule during the imprinting procedure resulted in recognitive materials with superior binding ability. Moreover, the surface imprinted polymers demonstrated much higher affinity towards their own template molecules when compared with other non-template proteins. The protein A coated templating mask and protein A imprinted materials were also characterized via atomic force microscope to help understand the effectiveness of this surface imprinting technique.

Keywords: molecular imprinting, surface imprinted polymer, protein A, IgG

1. INTRODUCTION

Biological molecules with recognition abilities have been widely applied in immunoassays, biosensor technology, and biological separation [1-10]. However, biomolecules such as proteins are usually expensive, difficult to work with, and sensitive to pH and temperature, etc. [11]. One technique, molecular imprinting, has drawn more and more attentions as a means to synthesize biomimetic materials with recognition abilities [12-13]. This technique allows for inexpensive, easy preparation of recognitive materials capable of working in harsh environments. The molecular imprinting process includes the polymerization of functional and cross-linking monomers in the presence of a template molecule to guide the production of binding sites. After removal of the templates, the molecular cavity left inside the polymer networks thus possesses a structure which is sterically and chemically complementary to the template molecule. As a result, the molecularly imprinted polymer (MIP) is capable of recognizing and rebinding the template molecules.

Until recently, molecular imprinting technique has been conducted using amino acids, polypeptides, and low molecular weight compounds as templates [14-16]. MIPs for recognition of small molecules have been found very promising applications in recognitive elements of biomimetic sensors [17-19], affinity chromatography [20-22], solid phase extraction [23-30], and (immuno)assays [31-33]. However, there are relatively few successful cases demonstrating the imprinting of biomacromolecules such as proteins. One major problem arises due to the large size of proteins – it is difficult for macromolecules to diffuse in or out of the cross-linked polymer network in a reasonable amount of time, the so-called steric hindrance [34]. Another negative effect which

inhibits the imprinting of proteins is due to the flexible nature of proteins, making it difficult to obtain well-tailored recognition sites [35-36]. In order to overcome these shortcomings, surface imprinting techniques have been applied by several research groups to synthesize protein recognitive materials. For example, Shi et al. synthesized surface imprinted materials using functionalized mica plates as protein templates [37]. In their study, template proteins were adsorbed on a mica surface and covered with disaccharide. Later, a thin polymer film was formed on top of the disaccharide overlayer, creating recognition sites on the surface of the materials. Lin et al. reported a microcontact imprinting technique which utilized a protein stamp prepared from a microscope cover glass pretreated with hexamethyldisilazane [38]. In the study, the cover glass was removed after the polymerization reaction and the protein molecules were extracted from the surface imprinted polymer thin film. The resulting surface imprinted film demonstrated affinity toward its own template molecules.

In this work, a new surface imprinting technique has been developed which employs epoxy-activated glass slides immobilized with proteins as the templating masks to prepare MIPs. Both protein A and monoclonal anti-c-myc IgG were selected as template molecules. In addition to the challenges noted previously, proteins are difficult targets for imprinting because the large number of potential surface interaction sites can lead to binding site heterogeneity. The pair of molecules used in this study, protein A and IgG, were chosen to study the effect of template molecule orientation during imprinting. It is known that IgG possesses a Y-shaped structure composed of Fab and Fc domains, with protein A capable of binding strongly to the Fc region of antibodies [39-40]. As a result, protein A was not only used as a template, but also to bind the Fc

domain of the IgG molecules and orient them with the antigen binding domains away from the mask. Different ratios of the functional monomer and cross-linker were also studied in order to improve the performance of the imprinted polymers. This surface imprinting technique allows for inexpensive and efficient analysis of medium-sized (protein A) and larger (monoclonal anti-c-myc IgG) proteins and it has potential use in a variety of possible applications.

2. EXPERIMENTAL

2.1. Chemicals and materials

Rabbit anti-pig IgG (whole molecule) alkaline phosphatase conjugate (RAP IgG-AP), protein A from Staphylococcus aureus (protein A), protein A-Alkaline Phosphatase (protein A-AP), monoclonal anti-c-myc purified mouse IgG (ACM IgG), monoclonal anti-c-myc alkaline phosphatase conjugate (ACM IgG-AP), ethylene glycol dimethacrylate (EGDMA), bovine serum album (BSA), (3-glycidoxypropyl) trimethoxysilane (3-GPTS) and 1-hydroxycyclohexyl phenyl ketone (HCPK) were purchased from Sigma-Aldrich (St. Louis, MO.). Absolute ethanol (200 proof), 2-hydroxyethylmethacrylate (HEMA), and plain glass microslides were obtained from Fisher Scientific Inc. (Houston, TX). HEMA was purified by vacuum distillation prior to use to remove polymerization inhibitor. Non-binding surface microplates (Corning 3990), and blank polystyrene microplates (Costar 3370) were used. Photomasks were made using poly(ethylene terephthalate) sheets and printed in black with 2×7 transparent circles using an ECRM Scriptsetter ZRL36 (Brakensiek Systemhaus, Dortmund Nordrhein-Westfallen, Germany) system. Teflon sheets were ordered from Ridout

Plastics Company Inc. (San Diego, CA) and used as chemically inert spacers. The enzymatic substrate PNPP (p-nitrophenyl phosphate, disodium salt) was obtained from Pierce Biotechnology, Inc. (Rockford, IL.). The multi-well Hybridization cassette was ordered from Arrayit Corporation (Sunnyvale, CA).

2.2. Preparation of epoxy-activated glass surfaces

Epoxy-activated glass substrates were prepared based on the protocol introduced by Nam et al. [41]. Microscope glass slides were first rinsed in fresh deionized (DI) water under ultrasonication for 10 minutes, repeated three times. The clean glass slides were then stored in absolute ethanol prior to use. Before surface modification, the clean microscope slides were thoroughly dried by nitrogen gas. The glass substrates were then incubated in a well mixed 3-GPTS solution (1 ml of 3-GPTS in 99 ml of toluene) for 20 minutes at room temperature. After the reaction, the slides were rinsed with toluene several times in order to wash away any unbound 3-GPTS molecules. Later, the glass slides were dried using nitrogen gas and immediately stored in an oven at 110-120 °C for 30 minutes. Before use, the glass slides were again rinsed with toluene and dried with nitrogen gas.

2.3. Formation of the templating masks

Epoxy-activated microscope slide was fixed in an ArrayIt® Hybridization Cassette and treated with a 0.5 mg/ml protein A in 1X phosphate buffered saline (1X PBS) solution at 4 °C for 24 hrs. Then the protein A coated substrate was rinsed with 1X PBS for 3 times, 5 minutes each time. The substrate was then incubated with a 0.5 wt. %

BSA in phosphate buffered saline-Tween 20 (PBST) solution (PBST, pH 7.4, 0.05 wt. % Tween 20) for 15 minutes at room temperature to block adsorption of other protiens. Finally, the templating mask slides were rinsed with PBST three times.

In order to obtain templating masks with oriented IgG molecules, the protein A coated templating mask were incubated with an IgG solution (2 μg/ml ACM IgG in tris buffered saline, TBS, pH 7.5) for 2 hrs at room temperature. Finally, the substrate was rinsed with PBST for three times (5 minutes each time) to remove any weakly bound IgG molecules. The protein templating mask was dried by nitrogen gas and stored at 4 °C prior to use.

A templating mask with randomly-bound IgG was prepared by incubating epoxy-activated slides in an IgG solution (2 μg/ml ACM IgG in TBS) for 2 hrs at room temperature. The substrate was then rinsed with PBST for three times (5 minutes each time) and incubated with a 0.5 wt. % BSA in PBST solution for 15 minutes at room temperature. Finally, the templating mask slides were rinsed with PBST three times and dried by nitrogen gas. The templating masks were stored at 4 °C prior to use.

Control materials were made by blocking the epoxy coated substrates with a BSA solution (0.5 wt. % BSA in PBST) for 15 minutes at room temperature. The slide was then rinsed with PBST three times (5 minutes each time) and dried with nitrogen gas prior to use.

2.4. Formation of surface imprinted polymers

Photopatterned spots of surface imprinted materials were synthesized using the templating mask by bringing monomer, cross-linker, photoinitiator, and solvent in

contact with the protein surface. In this work, HCPK was used as photoinitiator, HEMA as the functional monomer, EGDMA as the cross-linking monomer, and DMSO as the solvent. The mole ratio between the cross-linking agent and functional monomer was varied from 20 to 100 %. The monomers were mixed with HCPK (2% w/w of the final composition) and DMSO (30% v/v) and pipetted between two clamped glass slides with a Teflon spacer of 0.76 mm. As presented in Scheme 1, the templating mask and photomask were clamped together. The photomask was a piece of black poly(ethylene terephthalate) film with 14 transparent circles of 5.08 mm diameter and center to center distances of 8.64 mm. The polymerization reaction was initiated through irradiation with UV light (365 nm, 150 mw/cm^2, INTELLI-RAY 600, UVi-tron International, West Springfield, MA) at room temperature for 45 seconds in nitrogen environment.

The control polymer (CP) was synthesized in a similar way except that the control material was formed on the BSA coated slide.

2.5. ELISA-like analysis of rebinding ability

After the polymerization reaction, the polymeric discs were carefully peeled from the glass surface and dialyzed in TBS for an hour at 4 °C in order to remove residual solvent and any unreacted monomer. The resulting polymer discs were 0.76 mm in thickness and 5.08 mm in diameter. Next, an enzyme-linked immuno-sorbent assay (ELISA) was applied in order to analyze the recognition ability of the molecularly imprinted and control polymers. A polymeric disc was incubated in 200 µl of an IgG-alkaline phosphatase conjugate solution (2 µg/ml IgG-AP in TBS) contained in a Corning non-binding microplate well for 40 minutes at 4 °C. The polymer sample was then

washed with PBST three times (5 min each time) at room temperature to remove any weakly bound IgG molecules. The quantity of specifically absorbed IgG alkaline phosphatase conjugate was later measured using a colorimetric assay based on the activity of the conjugated alkaline phosphatase enzyme. To accomplish this, the polymeric disc was treated with 100 µl of PNPP solution for 40 minutes at room temperature in darkness. Next, the substrate solution was diluted with 100 µl DI water and an aliquot (100 µl) of the diluted solution was withdrawn from the microwell. Absorbance of this solution at 405 nm was measured using a Multimode Detector (Beckman Coulter, DTX 880, Fullerton, CA) to determine the optical density of the solution. The recognition ability was defined as Excess Selective Binding% (ESB%):

$$ESB\% = [[MIP\ absorbance] - [CP\ absorbance]]/[CP\ absorbance] \times 100\%. \quad (1)$$

All of the experiments in this study were done in triplicate under the same conditions.

2.6. Analysis of the IgG immobilized templating masks and BSA coated substrate

Protein A immobilized templating mask and epoxy-activated substrate were treated with ACM IgG-alkaline phosphatase conjugate solution (2 µg/ml ACM IgG-AP in TBS) for 2 hours at room temperature in an ArrayIt® Hybridization Cassette. The templating masks were then rinsed with PBST for three times, 5 minutes each time. Later, the BSA blocked slide, templating masks with oriented and randomly-bound ACM IgG-AP were incubated in a PNPP solution for 40 minutes at room temperature in

darkness. The following absorbance analysis was remained the same as introduced in 2.5.

2.7. Atomic force microscopy of surface imprinted materials

The protein A imprinted and the control materials were rinsed using TBS for an hour and then washed with DI water 3 times, 5 minutes each time in Petri dishes. In addition, the clean protein A MIPs were incubated with 0.05 mg/ml protein A solution for 40 minutes at 4 °C and then washed again with PBST three times, following with DI water rinse for three times. The clean and template treated protein A MIPs, and control materials were then freeze dried overnight. The polymeric discs and protein A templating mask were characterized via AFM (model: Digital Instruments Nanoscope IIIa-phase Atomic Force Microscope, Veeco, NY). The cantilever was made of 0.01-0.025 Ohm-cm Antimony (n) doped Si (model: TESP, Veeco, NY).

3. RESULTS AND DISCUSSION

3.1. Analysis of the templating masks

Protein chips have been widely used in the study of protein-ligand and protein-protein interactions, for instance in the elucidation of antibody and antigen interactions. Recently, aldehyde-, epoxy-, and N-hydroxysuccinimide- activated glass and gold surfaces have been widely applied to immobilize proteins and DNA. In this work, epoxy-activated glass substrates were employed in the immobilization of the protein templates. To determine the relative amount of template molecule located on the template masks, an ELISA like test was completed using antibody sensitive to ACM IgG. Three types of

substrates – oriented IgG templating mask, randomly-bound IgG slide, and BSA coated slide, were studied by ELISA-like analysis, with the colorimetric results presented in Figure 1. The average absorbances of BSA blocked slide, the oriented- and randomly-coupled IgG substrates are 0.097, 0.223, and 0.541, respectively. It was noticed that the absorbance of the randomly-bound IgG templating mask is higher than that of the oriented-coupled IgG templating mask, which indicated there were more IgG molecules adsorbed on the epoxy-activated substrate than on protein A immobilized slides. The absorbance results also reflected that BSA could effectively prevent the adsorption of ACM IgG-AP on the surface and protein A was successfully coupled on the epoxy-activated substrate.

3.2. Optimization of the cross-linker ratio

It is also known that functional and cross-linking monomers are building blocks for the formation of MIPs, in which the functional monomer is capable of binding the template and the cross-linker could remain structural integrity of the imprinted materials after the removal of the template molecules. Therefore, it is of obvious importance to select the cross-linking agent and functional monomer(s) that could mimic the natural receptor of their template. In this work, one of the most studied cross-linker, ethylene glycol dimethacrylate (EGDMA), was selected as the cross-linking agent. HEMA was chosen as the functional monomer, since its hydroxyl group gives the hydrophilic property to the polymer matrix which may affect the selective binding ability of the materials. The chemical structures of the functional and cross-linking monomers are

shown in Table 1. DMSO, a polar aprotic solvent, was employed as the porogenic solvent.

Cross-linking density is an important parameter in the design of recognitive polymeric networks. The cross-linker serves as the structural skeleton of the MIP, restricting chain mobility and ultimately retaining the complementary structure of the binding sites. Therefore, the ratio of the cross-linker in the prepolymerization mixture is of obvious importance to the performance of the MIPs. In this work, different ratios of the functional and cross-linking monomers were evaluated in an effort to optimize the recognition abilities of the MIPs. The absorbance results of protein A and ACM IgG imprinted materials in comparison with that of the control materials are presented in Figures 2 and 3. In addition, the corresponding Excess Selective Binding% of protein A and ACM IgG imprinted materials were calculated and are shown in Tables 2 and 3, respectively. We found that the optimal mole ratio of functional monomer/cross-linker was 20/80 for both protein A imprinted polymers and well-oriented ACM IgG imprinted polymers. For protein A MIPs, when the mole ratio of EGDMA was lower than 60%, the imprinted polymeric discs lost recognition ability towards their template molecules. For ACM IgG imprinted materials, the best ESB% results were obtained when the mole ratio of EGDMA in the prepolymerization mixture was larger than 60%. As a result, 80 mole% of the cross-linking agent and 20 mole% of the functional monomer were employed in the remaining experiments.

3.3. Selectivity of protein imprinted materials

In order to evaluate the effectiveness of this surface imprinting technique, the protein A MIP, the well-oriented ACM IgG MIP, and the CP were treated by three different protein enzyme conjugates – protein A-AP, ACM IgG-AP, and RAP IgG-AP. The control materials, synthesized using the immobilized BSA templating mask, demonstrated poor recognition abilities to all three protein targets (Figure 4). When the protein A MIP was tested with ACM IgG-AP and RAP IgG-AP solutions, the binding of the recognitive materials was similar to that of the control materials, suggesting good selectivity towards the target. More importantly, the ACM IgG MIP showed no significant binding affinity to protein A-AP or to RAP IgG-AP. While many reports on protein imprinting probe selectivity using molecular analogs of various size, charge, and shape, RAP IgG may be considered a close structural analog of ACM IgG (differences are animal source: rabbit vs. mouse, and origin: serum derived IgG vs. monoclonal IgG). The produced materials showed significant recognition towards their target IgG molecules and little affinity towards competing species.

3.4. Investigation of the importance of template molecule orientation

In this work, we attempt to imprint the Fab domain of the IgG molecule so as to produce materials which may differentiate between various immunoglobulins. To improve the chances of this happening, orientation of the IgG molecule with the Fc domain down and away was attempted through the use of protein A as a coupling molecule. To test this hypothesis and the importance of controlling the molecular orientation, surface imprinted materials formed using templating masks with well-

oriented ACM IgG were compared against materials produced with randomly-coupled (adsorbed) IgG. In the first set of experiments, it was investigated whether materials formed using these oriented template masks showed recognition for the IgG target molecule, or for both IgG and protein A. As presented in Figure 4, although there were two proteins bound on the template mask surface, the MIPs showed specific recognition towards ACM IgG only. This result demonstrates that protein A is a useful linker molecule which does not introduce additional cross-reactivity.

Next, the recognitive abilities of the materials formed with the well-oriented template mask were compared to those formed using the randomly oriented template. As illustrated in Figure 5, the ESB% of the MIPs synthesized on randomly-bound ACM IgG substrate was lower than that of the materials polymerized on the directed IgG templating mask, which indicated homogenous imprinting sites are better than heterogeneous binding sites for the improvement of the MIP performance. Even though significantly more IgG was bound on the randomly-oriented templating mask than on the mask with the controlled orientation, effectiveness of the mask was markedly lower. Future work to increase the amount of oriented IgG bound on the protein A surface is warranted.

3.5. AFM analysis of the imprinted materials and templating masks

The surface morphologies of the protein A imprinted polymer before and after treating with its template molecules, the templating mask immobilized with protein A, and the control material were characterized via AFM. Figures 6 (a), (b) and (c) are surface images of protein A MIP before and after incubation by its template molecules.

When comparing Figures (b) and (c), the scattered holes on the clean protein A MIP surface disappeared after the MIP was treated with protein A, which suggested the cavities on the protein A MIP surface were formed by protein A imprinting. Figures 6 (d) and (e) show images of the control material and protein A templating mask used to polymerize the MIPs. It is noticeable that protein A molecules were adsorbed like islands on the templating mask, which is consistent with Demirel et al.'s study of protein A immobilization [42]. The result suggested physically adsorbed protein A molecules were removed because of washing with 1X PBS and the cavities on the MIP surface are complementary to the peaks on the templating mask. In Figures 7 (a) and (b), the imprinted cavity is shown to be approximately 50 nm in depth and 90 nm in diameter. These dimensions are much larger than an individual protein and are likely the result of protein aggregation.

4. CONCLUSIONS

In this work, we have successfully developed a surface imprinting technique, which employs protein templating masks to synthesize protein recognitive polymeric discs. The MIPs demonstrated very good rebinding ability towards their template molecules and also showed excellent selectivity when challenged with competing molecules. In addition, the influence of template orientation was analyzed and materials formed with templating mask featuring oriented target molecules showed better recognition ability than materials formed with randomly-oriented template molecules. Moreover, we used two different proteins as templates and both of them presented very

good recognition abilities, which suggested this surface imprinting technique is a promising method to synthesize imprints of medium-sized and larger proteins, and may therefore be applied as elements in sensors for the detection of these species.

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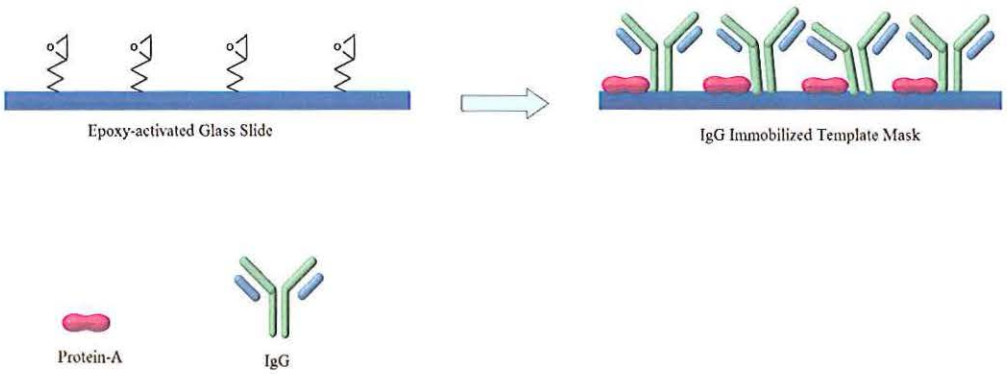
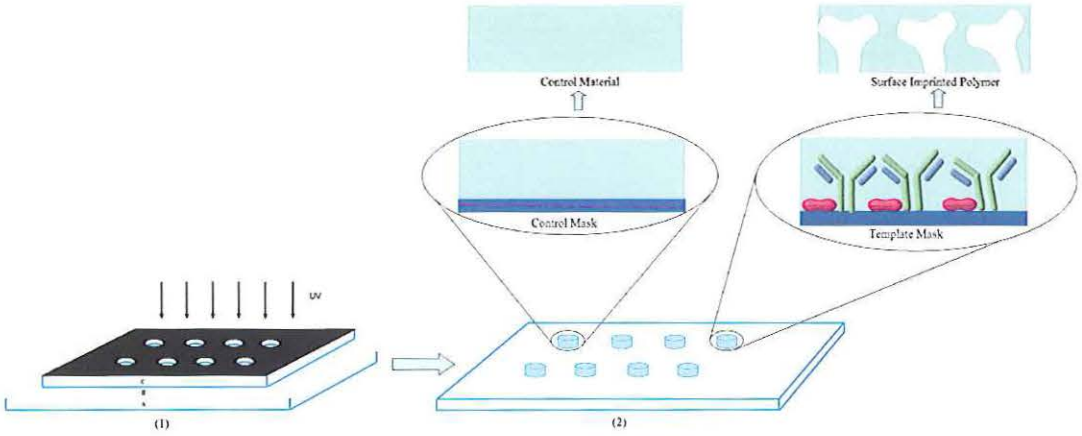


(a)



(b)

Scheme 1: Formation of the templating mask and synthetic materials.
(a): Formation of templating mask with oriented IgG molecules,
(b): Preparation of control material and surface imprinted polymer. A: the BSA coated substrate or templating mask immobilized with oriented IgG, B: Teflon spacer, C: glass microscope slide attached with photomask.

Table 1: Molecular weight and chemical structure of the cross-linker and functional monomer.

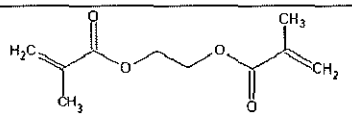
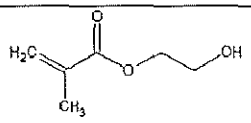
	Molecular Weight	Chemical Structure
EGDMA	198	
HEMA	130	

Table 2: Excess Selective Binding% of protein A MIPs composed of different mole percentages of EGDMA.

	100% EGDMA	80% EGDMA	60% EGDMA	40% EGDMA	20% EGDMA
ESB (%)	66	364	135	-19	-2

Table 3: Excess Selective Binding% of ACM IgG MIPs composed of different mole percentages of EGDMA.

	100% EGDMA	80% EGDMA	60% EGDMA	40% EGDMA	20% EGDMA
ESB (%)	183	194	74	54	75

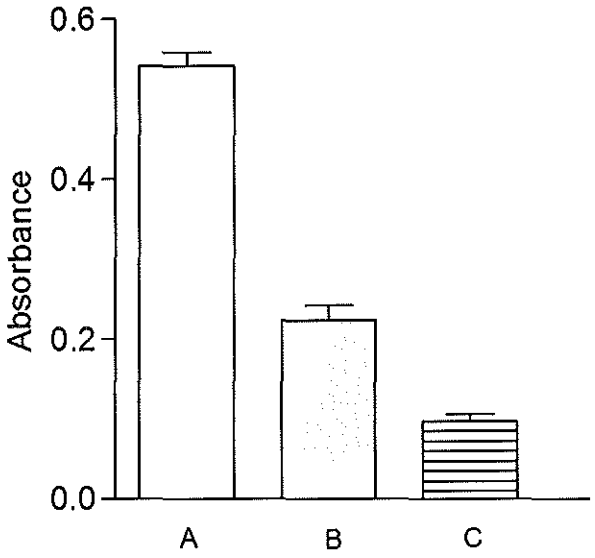


Figure 1: ELISA-like analysis of the templating masks.
A: Templating mask with randomly-bound IgG, B: Templating mask with oriented IgG, and C: BSA coated slide.

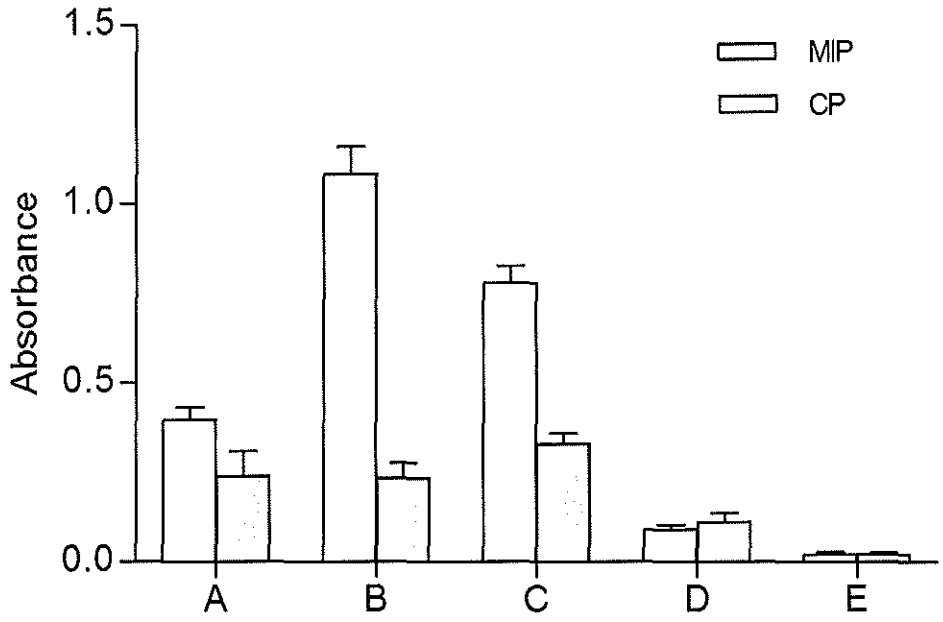


Figure 2: Optimization of protein A MIP recognition ability by varying mole ratio of functional and cross-linking monomers.
A: materials synthesized using 100 mole% EGDMA + 0% HEMA, B: 80 mole% EGDMA + 20 mole% HEMA, C: 60 mole% EGDMA + 40 mole% HEMA, D: 40 mole% EGDMA + 60 mole% HEMA, E: 20 mole% EGDMA + 80 mole% HEMA.

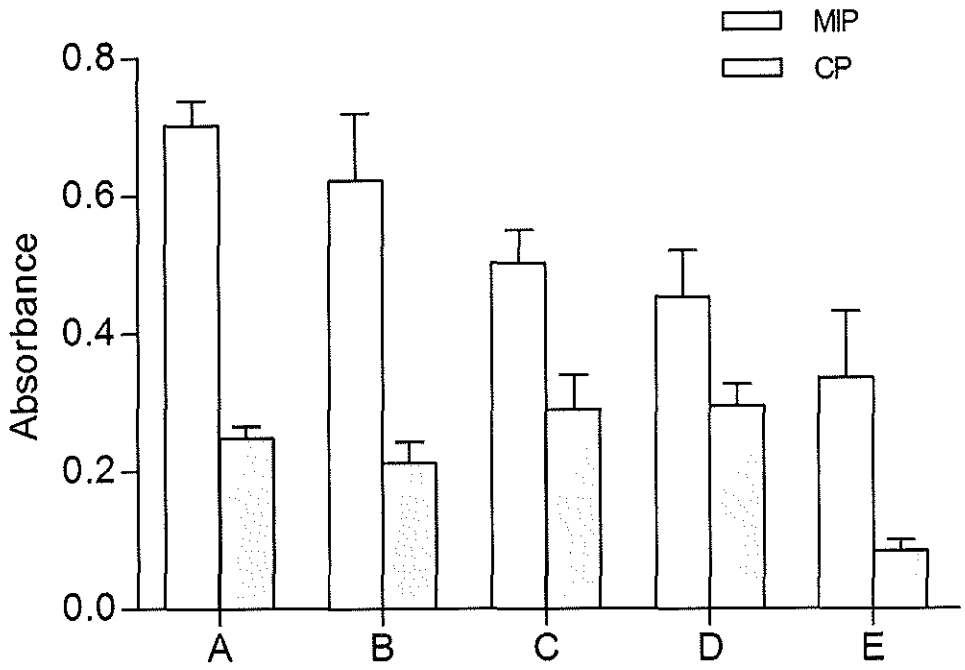


Figure 3: Optimization of ACM IgG MIP recognition ability by varying mole ratio of functional and cross-linking monomers.
A: materials synthesized using 100 mole% EGDMA + 0% HEMA, B: 80 mole% EGDMA + 20 mole% HEMA, C: 60 mole% EGDMA + 40 mole% HEMA, D: 40 mole% EGDMA + 60 mole% HEMA, E: 20 mole% EGDMA + 80 mole% HEMA.

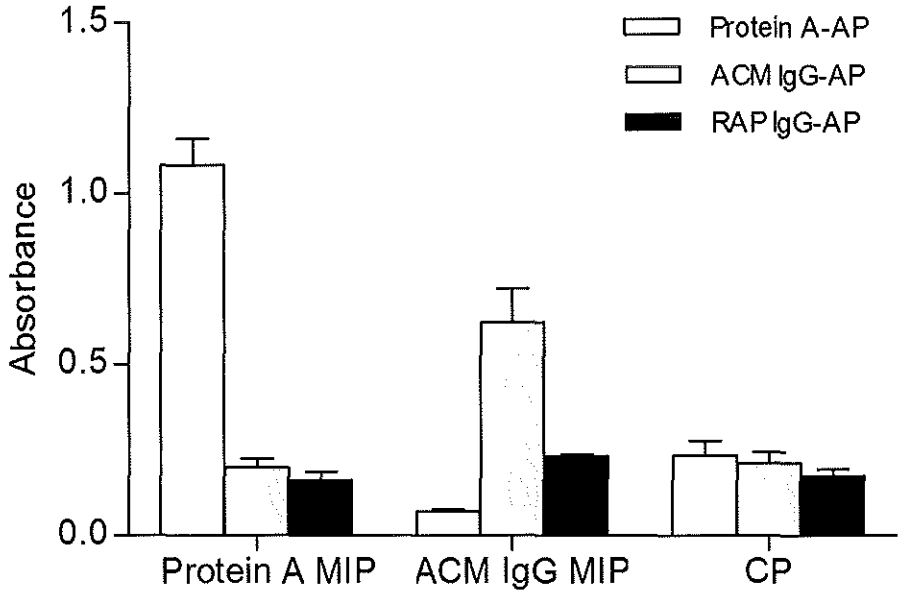


Figure 4: Investigation of protein binding for the surface imprinted materials.

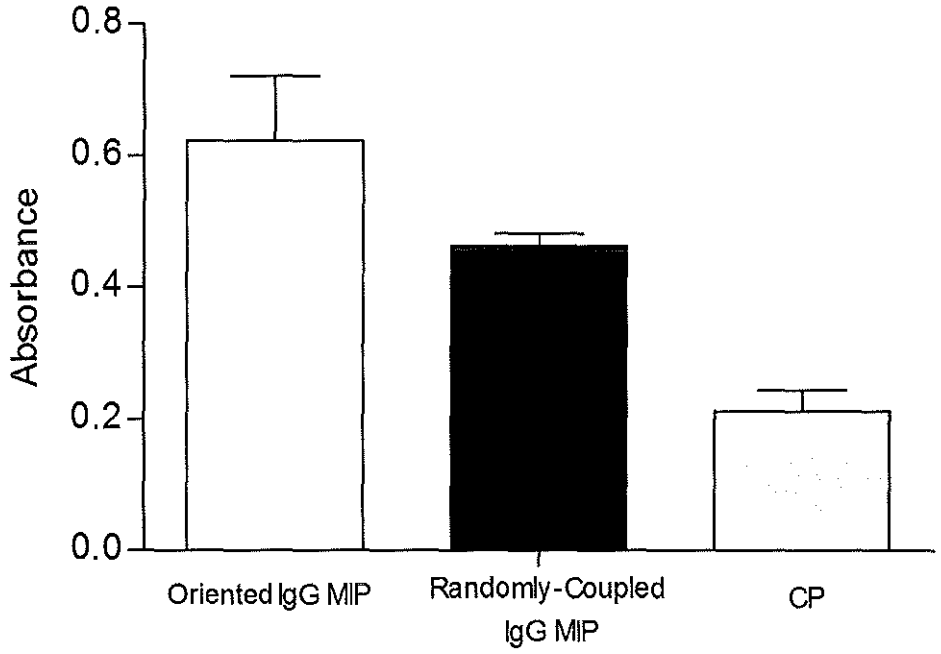
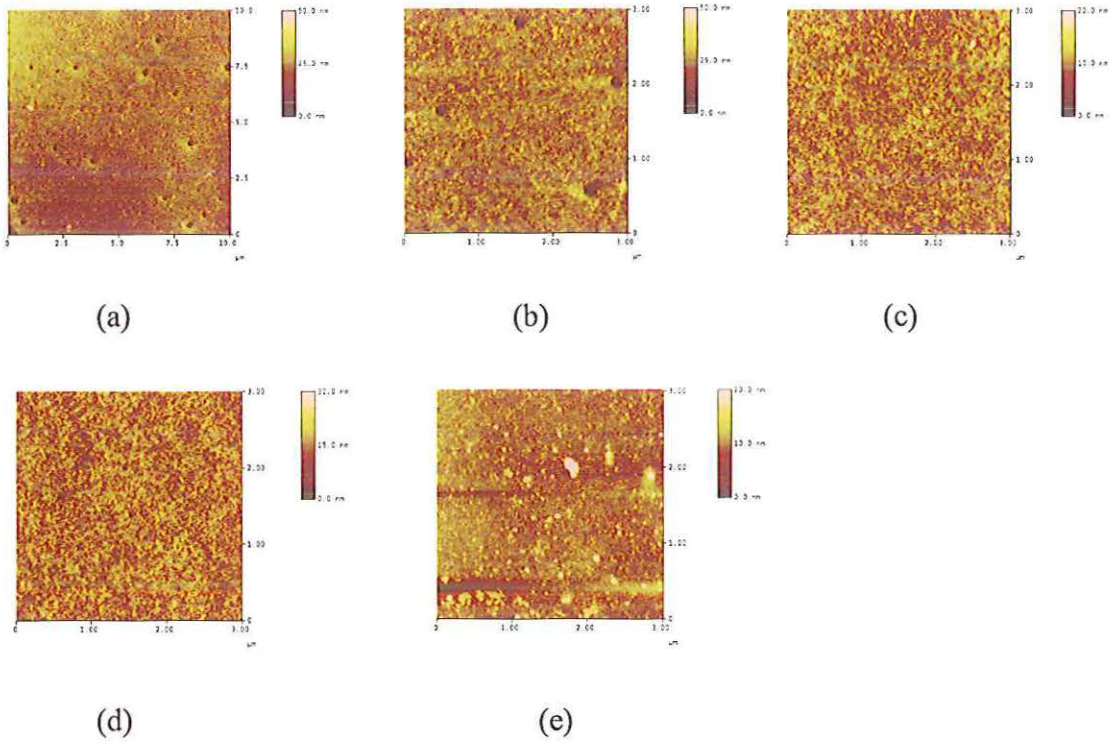


Figure 5: The influence of IgG orientation on the recognition abilities of ACM IgG MIPs.

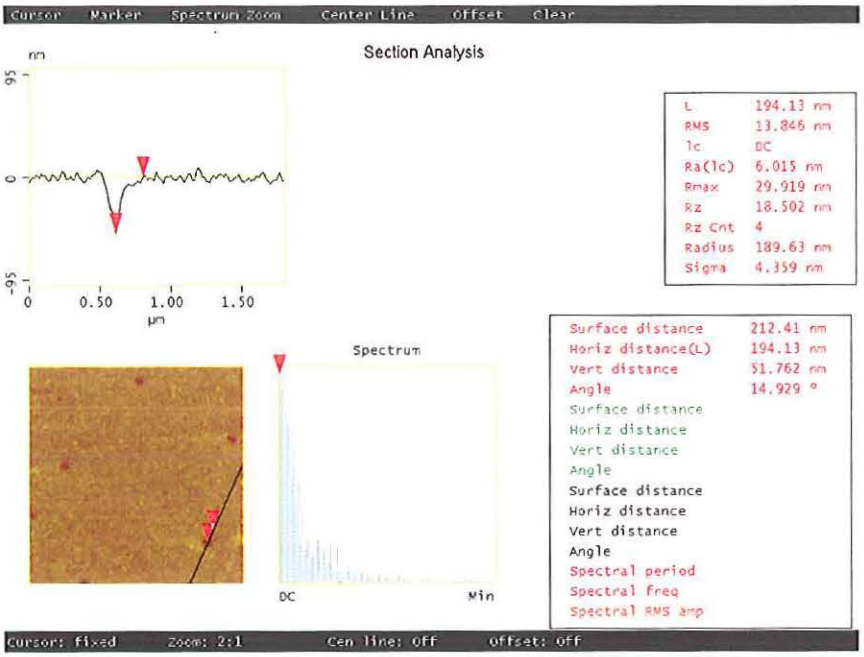


(a) (b) (c)

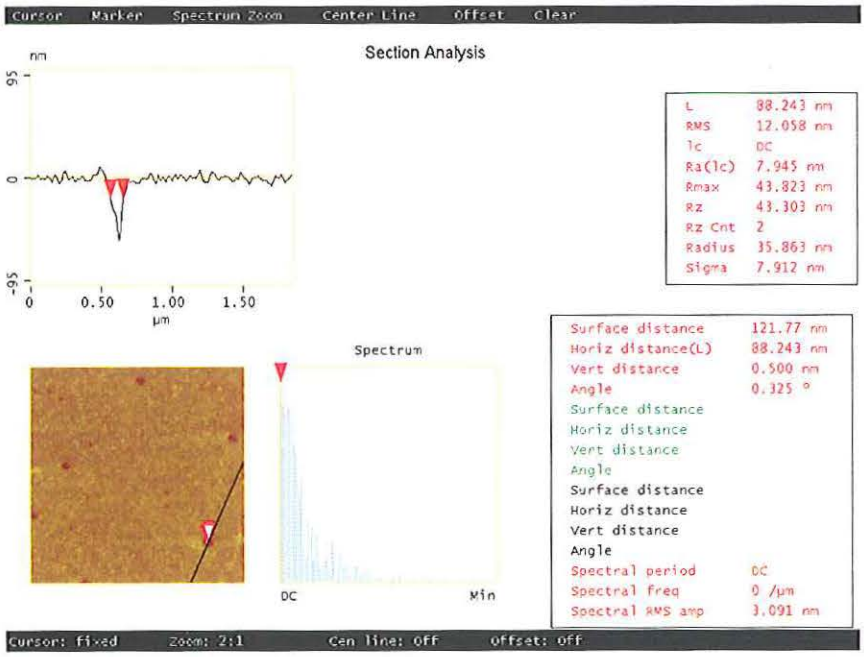
(d) (e)

Figure 6: AFM images of the control material, imprinted material and protein A templating mask.

(a) surface morphology of protein A MIP after wash (10 μm × 10 μm),
(b) surface morphology of protein A MIP after wash (3 μm × 3 μm),
(c) surface morphology of protein A MIP after incubation in protein A (3 μm × 3 μm),
(d) surface morphology of the control material (3 μm × 3 μm),
(e) surface morphology of the protein A templating mask (3 μm × 3 μm).



(a)



(b)

Figure 7: AFM images of protein A MIP.
(a) vertical distance of an imprinting site,
(b) horizontal distance of the imprinting site.

3. CONCLUSIONS

In this study, a surface imprinting technique was successfully developed to synthesize molecularly imprinted materials with recognition abilities. This technique was first applied to form molecularly imprinted materials specific for monoclonal anti-c-myc IgG in a manner compatible with standard 96-well microplates. The ELISA-like analysis that resulted was optimized to enhance rebinding efficiency of the recognitive materials and to promote target specificity. In the second part of this work, this technique was applied to two dimensional surfaces to study whether the surface imprinting method may one day find use in protein microarrays or in the fabrication of microelectronics-based biosensors. In addition, the reusability of the templating mask was studied. Finally, the importance of molecular orientation as a way to control which epitopes are presented for imprinting was studied using the two dimensional surface imprinting method. The recognition and rebinding ability of these materials was found to be enhanced through controlled template molecule orientation, further demonstrating the usefulness of the surface imprinting technique over conventional MIP production schemes.

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