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Surface imprinted thin polymer film systems with selective recognition for bovine serum albumin

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ABSTRACT

Molecularly imprinted polymers are synthetic antibody mimics formed by the crosslinking of organic or inorganic polymers in the presence of an analyte which yields recognitive polymer networks with specific binding pockets for that biomolecule. Surface imprinted polymers were synthesized via a novel technique for the specific recognition of bovine serum albumin (BSA). Thin films of recognitive networks based on 2-(dimethylamino)ethyl methacrylate (DMAEMA) as the functional monomer and varying amounts of either N,N′-methylenebisacrylamide (MBA) or poly(ethylene glycol) (400) dimethacrylate (PEG400DMA) as the crosslinking agent were synthesized via UV free-radical polymerization and characterized. A clear and reproducible increase in recognition of the template BSA was demonstrated for these systems at 1.6–2.5 times more BSA recognized by the MIP sample relative to the control polymers. Additionally, these polymers exhibited selective recognition of the template relative to competing proteins with up to 2.9 times more BSA adsorbed than either glucose oxidase or bovine hemoglobin. These synthetic antibody mimics hold significant promise as the next generation of robust recognition elements in a wide range of bioassay and biosensor applications.

1. Introduction

Molecularly imprinted polymers (MIPs) are highly crosslinked polymer networks engineered to specifically recognize target molecules. MIPs are formed by polymerization of organic or inorganic monomers in the presence of an analyte of interest (template molecule). These synthetic systems can be designed to specifically recognize physiologically relevant macromolecule biomarkers and are advantageous over their biological counterparts because they mimic biological recognition pathways while at the same time exhibiting abiotic properties. These favorable properties include stability in a variety of environments, long-term storage without performance loss, reusability, and tunability to almost any macromolecular biomarker. Accordingly, these systems are particularly well suited to replace natural antibodies as diagnostic tools in settings where the health care infrastructure is lacking, including developing countries or after natural disasters.

In spite of the success of MIPs used for recognition of small molecular weight molecules, macromolecular recognitive MIPs have yet to reach their vast potential. This is commonly attributed to the inherent properties of proteins which include conformational instability, large size, and complexity [1,2].

Two recent reports from our lab [3,4] clearly demonstrate the detrimental effect monomers often employed in MIPs have on the secondary structure of common protein templates. Even though only a subset of components used in the literature were investigated with the CD studies, we believe that the same trend holds true for others as these are a representative sample of all monomers and proteins employed to date. As a result, our goal here was to develop a robust and facile procedure which does not require the protein template to be in solution with the monomers prior to polymerization while exhibiting reproducible and selective recognition for the protein template.

A thorough review of the protein MIP literature indicated a limited number of procedures that satisfy the requirement of no protein–monomer(s) contact before polymerization. In general, these procedures adsorb the protein template to a substrate and either coat with a disaccharide layer [5,6], dry with N_2 [7–12], spin-off the supernatant [13], or rinse [14] before addition of monomer solution. Another approach having received significant interest in the past few years is the covalent attachment of a protein template to a spherical surface (silica, chitosan, or Fe_3O_4 particles) via an aldehyde linker before addition to the monomer solution [15–20]. However, this general methodology may not avoid conformational change because the attached protein is not typically dried or rinsed before addition of the monomer solution. Also, permanent protein

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entrapment is a concern as the polymer layer is polymerized on the surface of the protein modified spheres.

Shi et al. [6] employed a procedure in which various templates were adsorbed onto a mica substrate, the protein was coated with a disaccharide layer, then a fluoropolymer layer was deposited via plasma deposition. The selectivity values obtained (ratio of template absorption by MIP to competitor protein absorption by MIP) are some of the best demonstrated to date, especially given that the competing proteins were quite similar to the templates in both size and isoelectric point. However, despite the excellent specificity, minimal affinity was achieved (recognition of template by MIP over control polymer), the synthesis procedure was quite tedious, and no further studies have been published using this approach from this group.

Hu et al. [5] synthesized methacrylic acid (MAA) homopolymers crosslinked with ethyleneglycol dimethacrylate (EGDMA) on the surface of silica beads. A highly concentrated bovine serum albumin (BSA) solution (50 mg mL^{-1}) and a 5% saccharose solution were successively adsorbed onto the silica surfaces by spin coating, the monomer solution was added, covered with a poly(methyl methacrylate) slide, and then polymerized via UV free radical polymerization. BSA affinity (ratio of template adsorption by MIP to template adsorption by non-imprinted polymer) and selectivity against a variety of competitor proteins were both excellent relative to the literature (affinities and selectivities up to 5.5). One drawback to this approach, though, is that it required harsh conditions, namely hydrofluoric acid, to fully etch the silica particles prior to recognition studies. Additionally, the highly concentrated protein solution required for spin coating onto the silica beads would be cost prohibitive if this approach were ever to be extended to a protein biomarker.

In another study, Menaker et al. [14] detailed a novel approach to produce surface imprinted microrods for avidin recognition. The template was adsorbed onto a porous polycarbonate membrane (PCM) after incubation and subsequently rinsed with buffer. The PCM was attached to a gold electrode and immersed into a monomer solution of 3,4-ethylenedioxythiophene (EDOT) and poly(sodium-4-styrenesulfonate) (NaPSS) for electrochemical deposition to yield conductive polymer rods in the pores. After removal of the PCM, rebinding studies demonstrated high affinity (~5) with selectivity not clearly shown. This facile approach shows promise, however further studies are needed to display specific recognition.

The fourth approach, coined microcontact surface imprinting, has been widely studied by the Chou group over the past several years [7–12]. The general procedure is to polymerize between two glass surfaces – a protein stamp and a polymer support slide. The protein stamp is formed by physical adsorption of the template to a cover slip via incubation with a protein solution and subsequent drying with N_2 gas. After pipetting the monomer solution onto the support slide, the protein stamp is placed on top, and a thin polymer film is formed via UV polymerization with 2-dimensional surface imprints of the template. This approach has been used with varying success to recognize ribonuclease A [9,11], C-reactive protein [8], myoglobin [11,12], and creatine kinase [7] with highly crosslinked films composed of either PEG(400)DMA or TEGDMA as crosslinking monomers and various functional monomers. These monomers include styrene [9], methyl methacrylate [12], and methacrylic acid [7,10]. Due to the simplicity of this procedure as well as its robustness with various common model protein templates, we decided to modify our methodology from this microcontact imprinting procedure for the current study. The general schematic for microcontact imprinting is shown in Fig. 1.

It is important to note several key differences between the work presented in this report and those previously published by Chou and colleagues [7–12]. The modifications made by our group include the employment of a different protein template and functional monomer, use of control cover slips that were lysozyme adsorbed rather than just incubated with a buffer solution, and selection of sample concentrators with a UV–Vis plate reader to quantify recognition as opposed to quantification with ELISA.

Surprisingly, there are no published microcontact studies to date with bovine serum albumin (BSA) as the template protein, as it is the most commonly employed model protein template for macromolecular MIPs. This is likely due to the fact that these studies all employ an ELISA based protein detection procedure in which the blocking solutions contain BSA as the blocking agent.

Secondly, we employed 2-(dimethylamino)ethyl methacrylate (DMAEMA) as the functional monomer in order to exploit electrostatic interactions between the monomer and template. DMAEMA has a p*K*a value of ~7.5 [21], which means it will be positively charged at physiological pH. BSA has a isoelectric point of ~4.7 [22], meaning that it will carry a net negative charge at neutral pH. Thus, the positively charged DMAEMA homopolymer will non-covalently bind via ionic interactions with the negatively charged BSA during subsequent rebinding studies.

Third, lysozyme was adsorbed onto the surface of control protein stamps due to difficulty with removal of the control stamp after polymerization. Addition of this control protein allowed for facile removal of the cover stamp for both imprinted and control films. This is most likely due to the fact that the protein would accumulate on the edges of the cover slip after drying with N_2 gas (verified with FITC-BSA adsorbed cover slips, data not shown). This accumulation prevented the monomer solution from falling off the support slide after sandwiching with the cover stamp. As a result, cover slips that were not lysozyme adsorbed resulted in very little polymer forming on the support slide. This caused significant difficulty in subsequent stamp removal. Furthermore, this step created a more specific system overall as the control thin film inherently has some protein imprints present.

Moreover, our goal was to find a method which could determine protein concentration as easily as possible and without natural components. As a result, we employed Vivaspin sample concentrators as they satisfied both of these ends. While the ELISA based detection method used in the previous studies by Chou and colleagues can detect lower amounts of protein, it requires several more steps as well as specific experimental and storage conditions for the antibodies. This was important because protein MIPs are most advantageous over the current gold standard, antibody based systems, in resource-poor settings where harsh conditions may be unavoidable.

2. Materials and methods

N,N′-methylenebisacrylamide (MBA, 99%), albumin from bovine serum (BSA, lyophilized powder, >98%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%), glucose oxidase from Aspergillus niger (GOx, Type X-S, lyophilized powder), lysozyme from chicken egg white (Lys, >90%), and hemoglobin from bovine blood (BHb, lyophilized powder), 3-(trimethoxysilyl)propyl methacrylate (γ-MPS, 98%), and bovine albumin–fluorescein isothiocyanate conjugate (FITC-BSA, >97%) were purchased from Sigma–Aldrich (St. Louis, MO). Polyethylene glycol 400 dimethacrylate (PEG(400)DMA) was purchased from Polysciences, Inc. (Warrington, PA). Irgacure® 2959 (4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone) was purchased from Ciba Specialty Chemicals Corporation (Tarrytown, NY). Contrad® 70 was purchased from Decon Labs, Inc. (King of Prussia, PA). Potassium phosphate (Fisher Scientific, Fair Lawn, NJ) was dissolved in Milli-Q grade deionized water (20 mM), titrated to pH = 7.4 with 1 N NaOH, and filtered (0.2 μm) to prepare potassium phosphate buffer

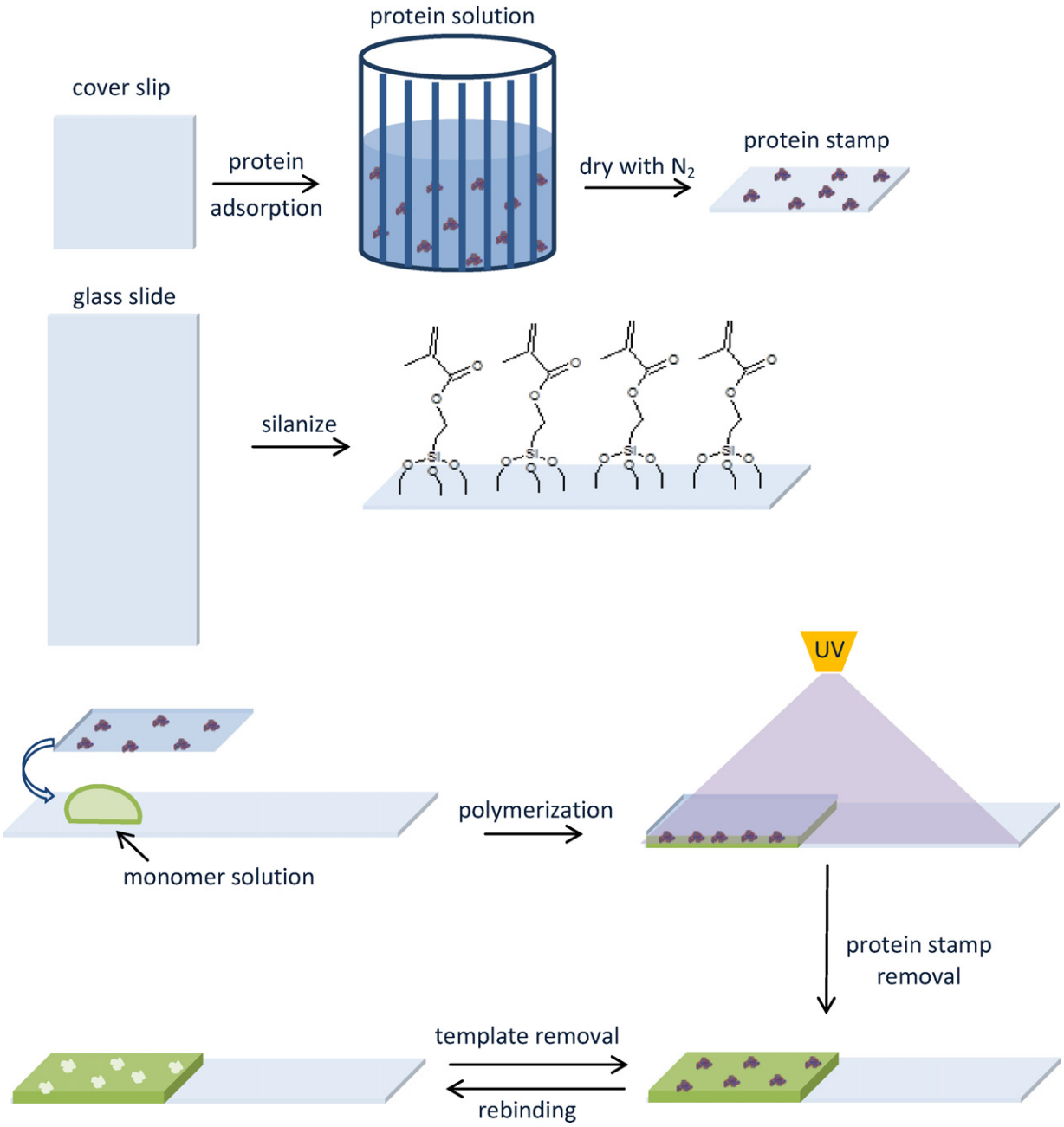

Fig. 1. General schematic for microcontact surface imprinting procedure.

(PPB). Vivaspin 20 (10,000 MW cut-off) sample concentrators were purchased from Sartorius-Stedim Biotech (Aubagne, France). All remaining chemicals were of ACS grade and obtained from standard commercial sources. All chemicals were used as received.

2.1. Preparation of glass surfaces

Standard plain glass microscope slides (75 mm × 25 mm) were employed as supports for the thin films. Prior to use, the support microscope slides were cleaned sequentially via 10 min sonication at room temperature with 1 N NaOH, DI water, 1 N HCl, DI water, and ethanol. They were dried with N_2 gas and stored in a desiccator until use. Immediately prior to use, the substrate slides were silanized by treatment with a solution of 0.5 mL γ-MPS in 100 mL ethanol, 2.7 mL DI water, and 0.3 mL glacial acetic acid for 30 min at room temperature [23]. The treated glass slides were rinsed in ethanol to remove excess γ-MPS and then dried with an N_2 gas stream.

Standard microscope cover slips (22 mm × 22 mm) were used as protein stamps. Prior to use, these cover slips were rigorously cleaned by sequential 30 min bath sonication at 55 °C in solutions of 5% (v/v) Contrad 70®/DI, DI water, 2-propanol, DI water, and ethanol. Cleaned cover slips were dried with an N_2 gas stream and stored in a desiccator until use. Immediately prior to use, protein (either BSA for MIP stamps or lysozyme for the control stamps) was adsorbed onto the previously cleaned cover slips. To do so, 3.7 μM protein solutions were prepared in PPB and cover slips were incubated in one of these solutions for 2 h at room temperature and subsequently dried with an N_2 gas stream. This incubation time was selected because it was previously shown that physical adsorption of various proteins onto quartz slides causes minimal loss of the native structure for 2 h [24]. Longer incubation times resulted in a

loss in protein structure with minimal increase in protein adsorption [24].

2.2. Fabrication of BSA imprinted thin films

Surface imprinted homopolymers of DMAEMA crosslinked with either PEG(400)DMA or MBA were synthesized according to the following procedure. The functional monomer (DMAEMA) and crosslinking monomer (either PEG(400)DMA or MBA) were added into a small glass vial. The ratio of these components was varied to produce thin films with 5% and 20% by molar feed ratio of MBA or 2% and 50% by molar feed ratio of PEG(400)DMA. The UV free radical initiator, Irgacure® 2959, was added to the monomer solution at a concentration of 2% of total monomer weight. To the monomer solutions with MBA as crosslinker, PPB and ethanol were added (85 wt% total solvent out of solvent plus monomers) to the vial at a volume ratio of 3:2 PPB to ethanol for complete dissolution. No additional solvent was required for the monomer solutions containing PEG(400)DMA as the crosslinker.

The sample vial, silanized substrate slides, and protein adsorbed cover slips were placed into a glove box and the monomer solution was purged with N_2 for 10 min. 50 μL of monomer solution was pipette onto a substrate slide and the appropriate protein stamp was placed onto the droplet, either BSA adsorbed for a MIP or lysozyme adsorbed for a control. The glass plate assemblies were placed into a UV reactor (Dymax Ultraviolet Flood Cure System, Torrington, CT) and photopolymerization was initiated by the UV light flood source with intensity ~15–17 mW cm^{-2} for 10 min. BSA imprinted and control thin films were synthesized in triplicate by either using BSA or lysozyme adsorbed protein stamps, respectively. After polymerization, the cover slip was removed with forceps. The remaining surface imprinted thin film on the glass substrate was placed into a 50 mL centrifuge tube and was washed repeatedly with PPB to remove any unreacted monomers or protein.

2.3. Bovine serum albumin affinity recognition studies

To determine the efficacy of the BSA imprinted thin films, washed thin films were incubated in a solution containing the model protein template, BSA. A 3.7 μM (0.25 mg mL^{-1}) stock solution of BSA in PPB was prepared, and 15 mL of the solution was added to each 50 mL centrifuge tube containing a crosslinked film with support in addition to a conical vial with no thin film as a blank sample. After incubation for 2 h on a plate shaker, the supernatant was poured into a Vivaspin 20 sample concentrator and concentrated via centrifugation for 15 min at 3500 rpm. This was sufficient to concentrate the supernatant ~30 times with minimal loss of protein. To calculate the mass of BSA loaded into the imprinted films after the 2 h incubation, the following mass balance was used:

$$M_{L,i} = C_B * V_B - C_i * V_i \quad (1)$$

where $M_{L,i}$ is the mass of BSA absorbed for sample i; C_B and C_i are the concentrations of the blank and sample i, respectively, after centrifugation with Vivaspin; V_B and V_i are the volumes of the filtrand remaining after Vivaspin concentration for the blank and sample i, respectively. Protein concentrations were determined by measuring the filtrand absorbance at $\lambda = 277$ nm using a UV–Vis spectrometer 96-well plate reader (Bio-Tek, Synergy HT, Winooski, VT). Affinity or imprinted efficiency, IE, can be defined as shown below in Eq. (2),

$$\mathrm{IE} = \frac{M_{L,\mathrm{MIP}}}{M_{L,\mathrm{control}}} \quad (2)$$

where $M_{L,\mathrm{MIP}}$ and $M_{L,\mathrm{control}}$ are averages of three independent samples.

Table 1
General properties of proteins used in the selectivity studies – bovine serum albumin (BSA), bovine hemoglobin (BHb), and glucose oxidase (GOx).

Protein	MW (kDa)	Hydrodynamic diameter (nm)	pI
BSA	66.4	14	4.7
BHb	64.5	5.5	6.9
GOx	160	8.6	4.2

Protein properties references: MW for BSA [6]; diameter for BSA [32]; pI for BSA & BHb [22]; MW and diameter for BHb [33], MW of glucose oxidase [34]; and pI & diameter of glucose oxidase [35].

2.4. Specific recognition studies

For specificity studies, proteins with varying molecular weight, hydrodynamic diameters, and isoelectric points (pI), were selected to assess the specificity of the BSA imprinted thin films (Table 1). Hemoglobin (BHb) is the iron containing protein used for oxygen transport in the red blood cells of all vertebrates. BHb was chosen as a competitor protein because it has a similar molecular weight as BSA as well as being a common protein template in protein MIP literature. Glucose oxidase (GOx) is a dimeric enzyme which oxidizes glucose into gluconic acid and has been used for decades to determining blood glucose levels in diabetic patients. Although the molecular weight for GOx is much larger than that of BSA, its isoelectric point is very close (Table 1); therefore, these two templates will have similar overall charges in aqueous buffers.

DMAEMA homopolymer thin films used in the selectivity studies were prepared as described earlier in Section 2.2. The higher crosslinked thin film formulations for each crosslinker were chosen to assess the specificity as they demonstrated superior performance in the BSA affinity studies. Surface imprinted films were then incubated in 3.7 μM solutions of the competitor proteins in PPB for 2 h at room temperature on a plate shaker. The supernatant solutions were then poured into Vivaspin 20 sample concentrators and centrifuged for 15 min at 3500 rpm for GOx based samples or 30 min at 3500 rpm for BHb based samples. The absorbance values of the resultant filtrand were read on a UV–Vis spectrometer 96-well plate reader (Bio-Tek, Synergy HT, Winooski, VT) at $\lambda = 274$ nm for BHb samples and $\lambda = 276$ nm for GOx samples. These absorbance values were converted to protein concentrations via previously constructed calibration curves for each competitor and mass of competitor protein loaded was determined via Eq. (1).

To analyze the selectivity of the surface imprinted thin films for BSA over the competitor proteins, selectivity, α, was calculated by the following equation (Eq. (3)):

$$\alpha_{t/c} = \frac{M_{L,\mathrm{template}}}{M_{L,\mathrm{competitor}}} \quad (3)$$

where $M_{L,\mathrm{template}}$ is the average mass BSA loaded into three independent MIP films and $M_{L,\mathrm{competitor}}$ is the average mass of competitor protein loaded into three independent MIP films.

2.5. Thin film characterization

Fluorescence microscopy was used to visually show the increase in BSA loading for the imprinted versus control films. To do so, thin films were incubated in a 3.7 μM solution of FITC-BSA in PPB for 2 h at room temperature on a plate shaker. Subsequently, the films with support slides were rinsed in PPB, dried with N_2, and stored in a desiccator until ready for use. Fluorescence microscopy images were acquired with a CoolSnap digital camera attached to the Nikon Eclipse ME600 fluorescent microscope operating with a FITC filter.

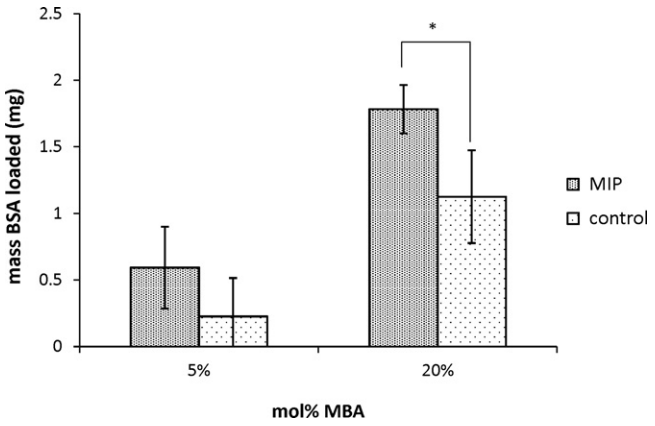

Fig. 2. Recognition studies results for DMAEMA homopolymers crosslinked with different amounts of MBA ($n = 3$, ±S.D.) (*$p < 0.1$).

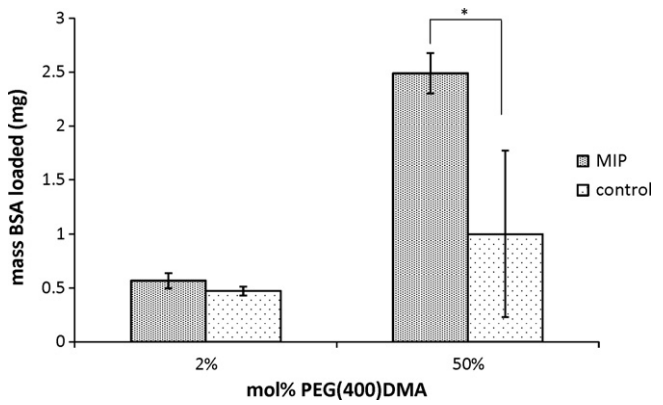

Fig. 3. Recognition studies results for DMAEMA homopolymers crosslinked with different amounts of PEG(400)DMA ($n = 3$, ±S.D.) (*$p < 0.1$).

3. Results and discussion

3.1. Rebinding of BSA to surface imprinted thin films

Two sets of molecularly imprinted thin films were synthesized, those with MBA as crosslinker and those with PEG(400)DMA as crosslinker and DMAEMA as the functional monomer for both. As mentioned above, the MBA based thin films were synthesized with either 5 or 20 mol% crosslinker (relative to DMAEMA), 2 wt% initiator (relative to mass of both MBA and DMAEMA), and 75 or 85 wt% solvent for low crosslinker (PPB only) or high crosslinking (PPB/EtOH mixture) concentration, respectively. Addition of ethanol was required for dissolution of the MBA at the higher concentration.

Fig. 2 shows the BSA recognition results for the MBA crosslinked imprinted and control thin films. These resultant films were relatively rigid, especially for the more crosslinked formulation, due to the addition of the ethanol in the pre-polymerization solution. The 5 mol% MBA thin films adsorb 0.6 ± 0.3 and 0.2 ± 0.3 mg of BSA for the imprinted and control samples, respectively. This yields an affinity or imprinting efficiency of ~2.6. However, as can be seen in Fig. 2, when the experimental errors are accounted for, there is no statistical significance between the two values. The 20 mol% MBA films absorb 1.8 ± 0.2 and 1.1 ± 0.3 mg BSA for the imprinted and control samples, respectively. This gives an IE of 1.6 from Eq. (2) and a *t*-test analysis on these two values shows a statistical difference for $p < 0.1$. It is clear that the higher crosslinked network adsorbs more BSA for both imprinted and control films overall and that the imprinted samples recognize more of the protein template relative to the control as 60% more BSA is adsorbed. Attempting to incorporate even higher amounts of MBA (50 and 85 mol%) required larger volumes of solvent in general (and ethanol more specifically) for dissolution and caused the resultant thin films to be highly rigid and thus adsorb very low amounts of BSA overall. As a result, 20 mol% appears to be the optimal amount of crosslinker for this formulation.

The second set of thin films synthesized and characterized was done so with PEG(400)DMA as the crosslinker. As mentioned above these films were polymerized with either 2 or 50 mol% crosslinker (relative to DMAEMA), 2 wt% Irgacure® 2959 (relative to mass of both crosslinker and functional monomer), and without additional solvent. Since no ethanol was required for dissolution and the fact that PEG(400)DMA is a much longer crosslinker, the resultant films obtained after synthesis were more rubbery than the MBA based films, even with the highly crosslinked films. Consequently, these were able to adsorb a higher mass of BSA overall. Fig. 3 shows the BSA recognition results for these systems which included 2 mol% and 50 mol% PEG(400)DMA in the monomer solution.

There is no increase in protein rebinding for the 2 mol% imprinted films relative to the controls as 0.6 ± 0.1 mg and 0.5 ± 0.1 mg BSA are adsorbed to the imprinted and control, respectively. On the contrary, there is a statistically significant increase ($p < 0.1$) in BSA rebinding with the imprinted relative to the control samples with the 50 mol% PEG(400)DMA system as 2.5 ± 0.2 and 1.0 ± 0.8 mg BSA is adsorbed into the imprinted and control samples, respectively. This produces an IE value of 2.5, from Eq. (2). Once again, it is clear that the more highly crosslinked formulation rebinds more BSA overall and that the imprinted samples are able to recognize a statistically significant higher amount than the control as evidenced by the 250% increase in adsorption. The 50 mol% formulation appears to be the optimal amount of crosslinker as films polymerized with higher amounts of PEG(400)DMA required addition of PPB for dissolution of the monomer solution, which resulted in an increased porosity that negated the impact of an increase in crosslinker.

3.2. Reproducibility of BSA recognition to thin films

Based on previous work in our lab and a recent critical review of the literature [25], it seems that a lack of reproducibility is common occurrence in protein imprinting. As a result, it was important for the results obtained in these studies to be reproducible. To illustrate this, the higher performing systems (20 mol% MBA and 50 mol% PEG(400)DMA) were synthesized two additional independent times in triplicate, and BSA rebinding studies were conducted following the same procedure. The results from these reproducibility studies are shown in Figs. 4 and 5. Fig. 4 shows consistent mass loaded and IE values for all three sets of 20% MBA crosslinked systems. Once again, the BSA quantities loaded for the MIP samples are significantly larger ($p < 0.1$) than those of the control. An overall IE value of 1.5 was obtained. Therefore, we can say that our novel surface imprinting procedure yielded reproducible BSA recognition results.

Fig. 5 shows the results of the three independent studies with the PEG(400)DMA crosslinked thin films. Even though the mass loaded for the imprinted samples fluctuates from 1.5 ± 0.2 to 2.5 ± 0.2, these values are statistically significantly larger ($p < 0.1$) than those of the control samples. An overall IE for all three sets was determined to be 2.3.

3.3. Specific recognition studies results

Similar to the reproducibility studies, the selectivity studies were performed on the two systems with the best IE results,

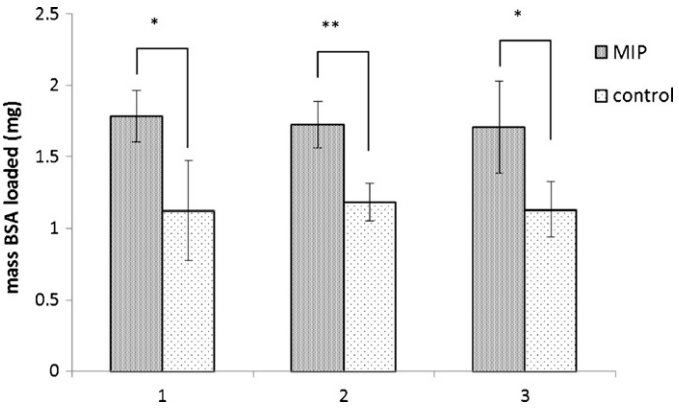

Fig. 4. Reproducibility of BSA recognition for 20 mol% MBA crosslinked thin films ($n = 3$, ±S.D.) ($^{*}p < 0.1$, $^{**}p < 0.05$).

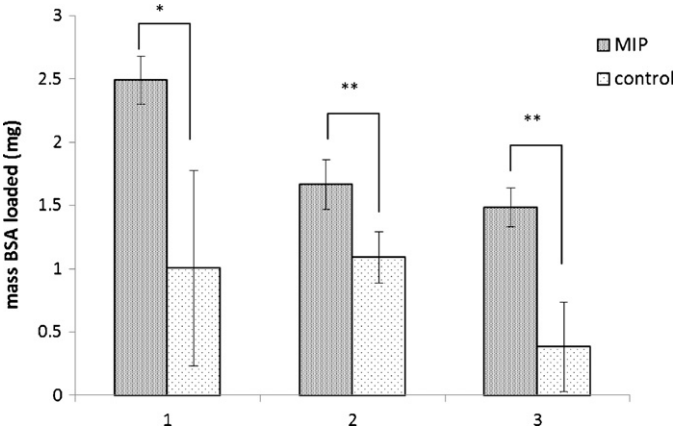

Fig. 5. Reproducibility of BSA recognition for 50 mol% PEG(400)DMA crosslinked thin films ($n = 3$, ±S.D.) ($^{*}p < 0.1$, $^{**}p < 0.05$).

20 mol% MBA and 50 mol% PEG(400)DMA. To do so, imprinted and control thin films were incubated in 3.7 μM single protein environments of either bovine hemoglobin (BHb) or glucose oxidase (GOx) for 2 h at room temperature. After concentrating the supernatants with Vivaspin, the resultant filtrand solutions were analyzed with UV spectroscopy to determine the change in competitor protein concentration. Fig. 6 shows that there is no difference in mass loaded of competitor protein, either BHb or GOx, between the

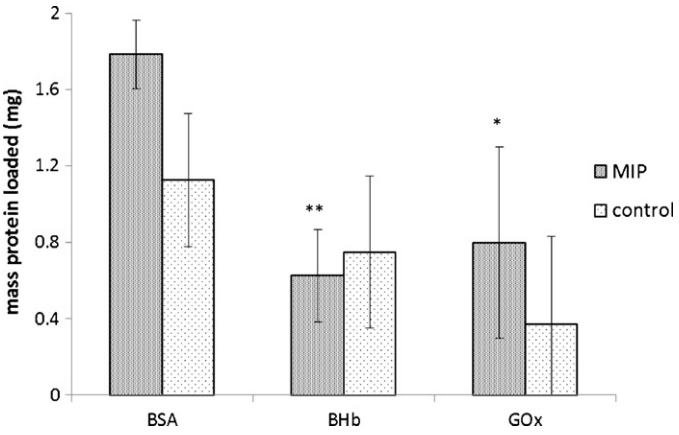

Fig. 6. Selectivity studies results for MBA crosslinked thin films for the template, BSA, and competitor proteins, BHb and GOx ($n = 3$, ±S.D.). Bovine serum albumin (BSA), bovine hemoglobin (BHb), and glucose oxidase (GOx) ($^{**}p < 0.01$, $^{*}p < 0.05$ relative to MIP with BSA).

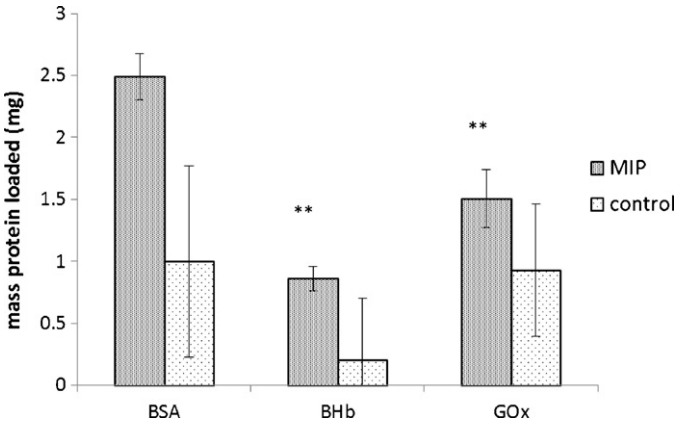

Fig. 7. Selectivity studies results for PEG(400)DMA crosslinked thin films for the template, BSA, and competitor proteins, BHb and GOx ($n = 3$, ±S.D.). Bovine serum albumin (BSA), bovine hemoglobin (BHb), and glucose oxidase (GOx) ($^{**}p < 0.01$, relative to MIP with BSA).

imprinted and control samples for the MBA crosslinked samples. More importantly, this plot also shows that the amount of competitor protein adsorbed by the imprinted thin films (0.6 ± 0.2 mg for BHb and 0.8 ± 0.5 mg for GOx) is significantly lower ($p < 0.05$) than the amount of BSA loaded (1.8 ± 0.2 mg). These rebinding amounts result in selectivity values (α) of 2.8 for BSA over BHb and 2.2 for BSA over GOx.

Along the same lines, Fig. 7 shows the selectivity results for the PEG(400)DMA crosslinked thin films for BSA against BHb and GOx. Once again, it is clear that there is no difference in mass of competitor protein adsorbed between the imprinted and control samples. Also, Fig. 7 shows that the mass of competitor protein adsorbed by the BSA MIP (0.9 ± 0.1 mg for BHb and 1.5 ± 0.2 mg for GOx) is statistically significantly lower ($p < 0.05$) than that of BSA (2.5 ± 0.2 mg). The resultant selectivity values are $\alpha_{BSA/BHb} = 2.9$ and $\alpha_{BSA/GOx} = 1.7$. Not surprisingly, the same trend applies to both the MBA and PEG(400)DMA crosslinked systems in that the selectivity is lower for GOx. This is because of the similarity in isoelectric point, as ionic interactions will dominate between the positively charged DMAEMA and overall negatively charged BSA and GOx proteins. These selectivity values are on the lower end of what has been previously reported in the literature for BSA, in general [6,26–28], however compare quite favorably for studies using competitor proteins with similar MW and isoelectric points [29–31], as was done in this work.

3.4. Thin film characterization

Fig. 8 shows fluorescence microscopy images of MBA crosslinked imprinted and control thin films after incubation with FITC-BSA, respectively. These images were taken with identical instrument settings and qualitatively show an increased amount of FITC-BSA adsorbed onto the imprinted polymer (Fig. 8A) relative to the control (Fig. 8B). This provides further evidence for increased recognition of BSA to the imprinted thin films relative to the control samples.

Armed with the knowledge of our previous work [3,4] which clearly demonstrates a large conformational change in protein templates when in solution with common ligands from protein MIP literature; we have highlighted a novel, fully synthetic, approach to BSA recognition. In order to continue employing traditional monomers and crosslinkers, there is a significant need to further study strategies which obviate the need for protein contact with monomers before polymerization, such as the one detailed in this

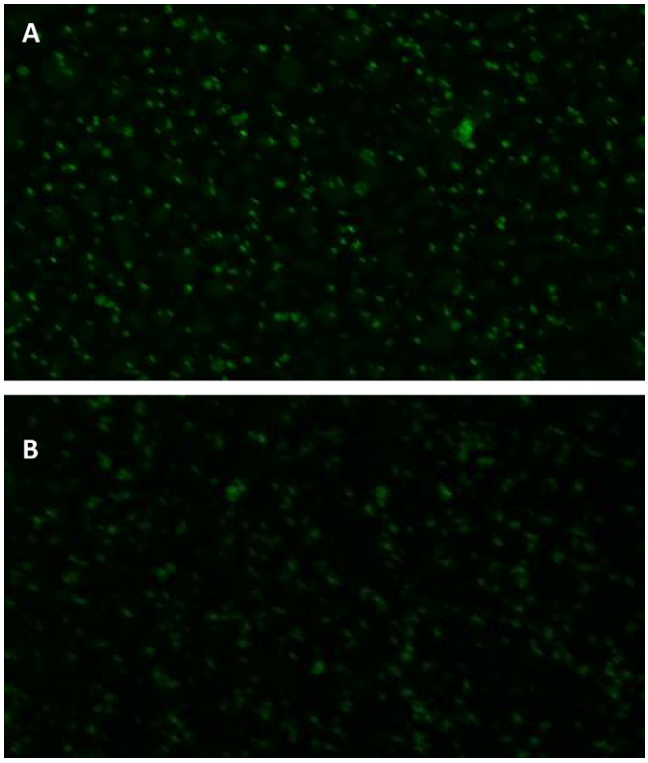

Fig. 8. Fluorescence microscopy of (A) BSA imprinted thin film and (B) control thin films (i.e. lysozyme imprinted) after incubation with FITC-BSA.

report as well as those previously reported which were mentioned in Section 1.

4. Conclusions

We have developed a facile synthesis procedure for reproducible and selective recognition of BSA. This procedure was selected due to the fact that it does not require the protein to be in solution with the monomers prior to polymerization in addition to its previous success with other protein templates. In this work, we have shown that the imprinted polymers synthesized with higher amounts of crosslinker, either MBA or PEG(400)DMA, rebind statistically significantly larger amounts of BSA than the control. These recognition studies were also shown to be reproducible, which is an important result not often demonstrated in the literature. In addition, our optimized thin films are highly selective to the template protein over similar competitor proteins. Consequently, we feel that this approach shows great promise for integration with a transducer (i.e. microcantilever or quartz crystal microbalance) as a platform technology for a fully synthetic biosensor which could one day be used in various applications, including the diagnosis of diseases in settings where medical infrastructure is deficient.

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References

[1] N. Bergmann, N.A. Peppas, Prog. Polym. Sci. 33 (2008) 271–288.
[2] N.W. Turner, C.W. Jeans, K.R. Brain, C.J. Allender, V. Hlady, D.W. Britt, Biotechnol. Prog. 22 (2006) 1474–1489.
[3] D.R. Kryscio, M.Q. Fleming, N.A. Peppas, Macromol. Biosci., submitted for publication.
[4] D.R. Kryscio, M.Q. Fleming, N.A. Peppas, Biomed. Microdevices, submitted for publication.
[5] X.B. Hu, G.T. Li, J. Huang, D. Zhang, Y. Qiu, Adv. Mater. 19 (2007) 4327–4332.
[6] H.Q. Shi, W.B. Tsai, M.D. Garrison, S. Ferrari, B.D. Ratner, Nature 398 (1999) 593–597.
[7] Y.W. Chen, J. Rick, T.C. Chou, Org. Biomol. Chem. 7 (2009) 488–494.
[8] P.C. Chou, Anal. Chim. Acta 542 (2005) 20–25.
[9] Hsu, Nanotechnology 17 (2006) s77–s83.
[10] P.C. Liao, Y.C. Tyan, C.Y. Wang, J.F. Hsu, T.C. Chou, H.Y. Lin, J. Biomed. Mater. Res. A 91A (2009) 597–604.
[11] H.Y. Lin, Biosens. Bioelectron. 22 (2006) 534–543.
[12] H.Y. Lin, J. Rick, T.C. Chou, Biosens. Bioelectron. 22 (2007) 3293–3301.
[13] O. Hayden, P.A. Lieberzeit, D. Blaas, F.L. Dickert, Adv. Funct. Mater. 16 (2006) 1269–1278.
[14] A. Menaker, V. Syritski, J. Reut, A. Opik, V. Horvath, R.E. Gyurcsanyi, Adv. Mater. 21 (2009) 2271–2275.
[15] F. Bonini, Biosens. Bioelectron. 22 (2007) 2322–2328.
[16] F. Li, J. Li, S.S. Zhang, Talanta 74 (2008) 1247–1255.
[17] T. Shiomi, M. Matsui, F. Mizukami, K. Sakaguchi, Biomaterials 26 (2005) 5564–5571.
[18] C.J. Tan, M.G. Chua, K.H. Ker, Y.W. Tong, Anal. Chem. 80 (2008) 683–692.
[19] W. Zhang, L. Qin, R.R. Chen, X.W. He, W.Y. Li, Y.K. Zhang, Appl. Surf. Sci. 256 (2010) 3000–3005.
[20] W. Zhang, L. Qin, X.W. He, W.Y. Li, Y.K. Zhang, J. Chromatogr. A 1216 (2009) 4560–4567.
[21] D. Fournier, R. Hoogenboom, H.M. Thijs, R.M. Paulus, U.S. Schubert, Macromolecules 40 (2007) 915–920.
[22] D. Malamud, J.W. Drysdale, Anal. Biochem. 86 (1978) 620–647.
[23] H. Garoff, W. Ansorge, Anal. Biochem. 115 (1981) 450–457.
[24] E.P. Vieira, S. Rocha, M.C. Pereira, H. Mohwald, M.A.N. Coelho, Langmuir 25 (2009) 9879–9886.
[25] E. Verheyen, J.P. Schillemans, M. van Wijk, M.A. Demeniex, W.E. Hennink, C.F. van Nostrum, Biomaterials 32 (2011) 3008–3020.
[26] C.J. Tan, S. Wangrangsimakul, R. Bai, Y.W. Tong, Chem. Mater. 20 (2008) 118–127.
[27] Y.T. Wang, Y.X. Zhou, J. Sokolov, B. Rigas, K. Levon, M. Rafailovich, Biosens. Bioelectron. 24 (2008) 162–166.
[28] S.T. Yuan Lin, X. Mao, L. Bao, J. Biomed. Mater. Res. A 85a (2008) 573–581.
[29] G. Demirel, G. Ozcetin, E. Turan, T. Caykara, Macromol. Biosci. 5 (2005) 1032–1037.
[30] X. Pang, React. Funct. Polym. 66 (2006) 1182–1188.
[31] K.Y. Zhao, G.X. Cheng, J.J. Huang, X.G. Ying, React. Funct. Polym. 68 (2008) 732–741.
[32] F.W. Putnam (Ed.), The Plasma Proteins: Structure, Function and Genetic Control, Academic Press, New York, 1975.
[33] S. Papadopoulos, K.D. Jurgens, G. Gros, Biophys. J. 79 (2000) 2084–2094.
[34] H. Tsuge, O. Natsuaki, K. Ohashi, J. Biochem. (Tokyo) 78 (1975) 835–843.
[35] M. Burow, N. Minoura, Biochem. Biophys. Res. Commun. 227 (1996) 419–422.