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Lysozyme-imprinted polymer synthesized using UV free-radical polymerization

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ABSTRACT

Molecular imprinting is a method to fabricate a polymeric material (molecularly imprinted polymer or MIP) capable of selectively recognizing template molecules. Molecular imprinting of small molecules has been studied widely. Less common, however, is the imprinting of biological macromolecules, including proteins, among which lysozyme is an important molecule in the food, pharmaceutical, and diagnostic sciences. In this study, lysozyme MIP was fabricated in two steps. First, lysozyme, PEG600DMA, and methacrylic acid were used as the template molecule, cross-linking monomer, and the functional monomer, respectively, in a UV free-radical polymerization process to synthesize a polymeric gel. Second, lysozyme was removed by enzymatic digestion. Non-imprinted polymer (NIP) was synthesized without lysozyme addition. To evaluate the preferential binding capability of MIP, lysozyme, RNase A, or a 50:50 mixture of lysozyme and RNase A was added to MIP and NIP and then released by digestion. It was found that when more lysozyme was added to the reaction mixture, the quantity of protein released from the polymer increased, reflecting more potential binding sites. Tests of MIP with a competitive binding mixture of lysozyme and RNase A showed the MIP preferentially bound a greater amount of lysozyme, up to 20 times more than RNase A. NIP bound only small amounts of both proteins and did not show a preference for binding either lysozyme or RNase A. These results demonstrate that lysozyme was successfully imprinted into the MIP by UV free-radical polymerization, and the fabricated MIP was able to preferentially bind its template protein.

1. Introduction

Molecular imprinting is the technique of producing artificial recognition sites by forming a polymer around a molecule used as a template. Both the template molecule's geometry and its specific configuration of functional moieties are mirrored in a complementary manner after removing the template from the polymer following polymerization. The resulting imprinted polymers have recognitive sites with a high affinity for the molecules used to create them. Typical applications of molecularly imprinted polymer (MIP) networks rely on the selective binding provided by their precise molecular chemical architecture and include mimics of immunoassays/antibodies, recognitive elements in biosensors, drug delivery devices, and catalysis and artificial enzymes [1–7].

Molecular imprinting has been used successfully for imprinting small molecules and metal-ions, such as glucose, steroids, pesticides, cobalt(II) acetate, and UO_2–$(NO_3)_2$ [e.g., 5–11]. For example Hilt et al. [10] studied the imprinting of small molecule D-glucose using UV-activated free-radical polymerization. Imprinting of larger molecules, such as proteins, has become an active area of research but has shown limited success [e.g., 2,12]. Major obstacles in protein imprinting include their large molecular size and conformational complexity and flexibility. In spite of these difficulties, potential applications of protein imprinting in biology and the life sciences have led to numerous attempts to prepare protein-imprinted polymers via different strategies, including sol–gel processing [1,5,11] and free-radical polymerization [2,6,12]. Generally protein-imprinted polymers using sol–gel processing are porous in nature, and those fabricated by free-radical polymerization are dense.

In this study, lysozyme was chosen as the protein template. A simplified ribbon diagram of chicken egg white lysozyme is shown in Fig. 1 [13]. Lysozyme is an enzyme found in a wide variety of locations, including chicken egg white, tears, saliva, and other body fluids. It hydrolyzes β-linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides in the bacterial cell wall and thereby acts as a mild antiseptic, providing protection against infection [14–20]. Lysozyme is also a commercially valuable enzyme used for different applications, such as a food additive in milk products and a cell-disrupting agent for extraction of

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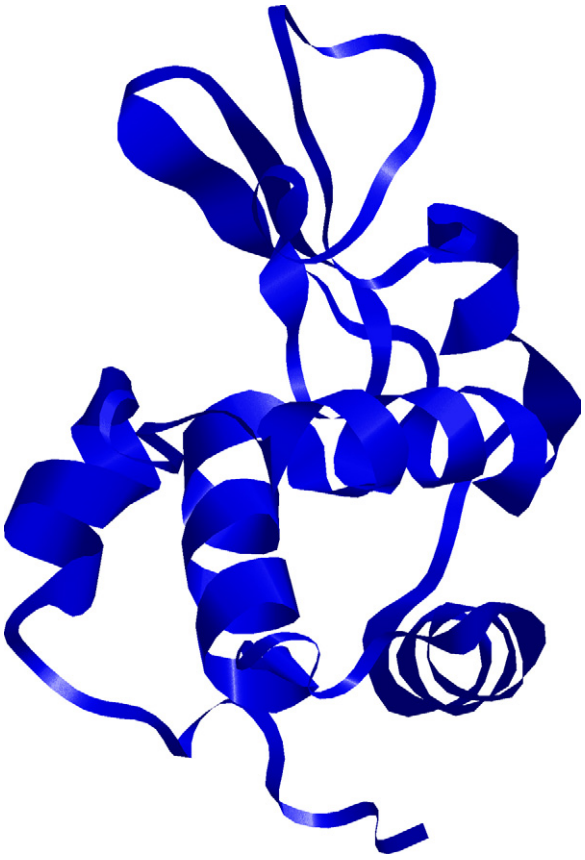

Fig. 1. A ribbon diagram of the three-dimensional structure of chicken egg white lysozyme. It was created from PDB entry 1GXX [13].

intracellular bacterial products. The potential use of lysozyme as an anticancer drug has been demonstrated by *in vitro* and cell culture experiments [14,20]. Lysozyme is also an important index in the diagnosis of various diseases, e.g., tuberculosis meningitis, neurosyphilis, fungal meningitis, leukemia, and various kidney diseases [17,21,22]. All the applications of lysozyme will require more efficient and cost-effective techniques for its separation and identification. Therefore, polymer with specific recognition sites for lysozyme may be useful for measurement of lysozyme concentration and the diagnosis of certain diseases.

Protein-imprinted polymers have the advantage of being prepared in an easy and inexpensive way. Although a few reports about imprinting inorganic materials have been found [1,16,18], most molecular imprinting research has used methacrylic polymers imprinted with low molecular weight compounds. In this study, we investigated the feasibility of imprinting lysozyme into an MIP using a UV free-radical polymerization technique and evaluated ability of the MIP to recognize the template in a binary protein solution. Because variation in the amount of lysozyme incorporated during MIP synthesis could impact the protein-binding behavior of the MIP, this variable was also investigated.

2. Materials and methods

2.1. Materials

Lysozyme (chicken egg white; Sigma, St. Louis, MO); ribonuclease A (RNase A; Sigma); Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 594 (Molecular Probes, Eugene, OR); methacrylic acid (MAA; Aldrich, Milwaukee, WI); poly(ethylene glycol)600 dimethacrylate (PEG600DMA; 600 indicating the average molecular weight of the PEG chain and corresponding to 14 repeating units; Polysciences, Warrington, PA); protease (Pronase E; Sigma); anhydrous ethanol (Sigma); BCA Protein Assay Reagent (Pierce, Rockford, IL), phosphate-buffered saline (PBS); 2,2-dimethoxy-2-phenyl acetophenone (DMPA, Aldrich, Milwaukee, WI).

2.2. Protein labeling

To enable their identification at different stages of MIP synthesis and testing (described in subsequent sections), proteins were labeled with the different fluorophores according to manufacturer instructions. Lysozyme, the template molecule, was labeled by Alexa Fluor 350 before polymerization. For selectivity experiments, separate batches of lysozyme were labeled with Alexa Fluor 488, and the competitor protein RNase A was labeled with Alexa Fluor 594. A Dynatech MR5000 microplate reader was used for determining the concentration of the labeled protein after reaction with the BCA Protein Assay reagent. The fluorescence of serially diluted protein solutions was measured with a Spectra Max Gemini XS. According to the related fluorescence and concentration of the labeled protein, a standard curve was obtained and used for calculating protein concentration from fluorescence measurements.

2.3. MIP synthesis

The molecularly imprinted polymer was synthesized by UV free-radical polymerization. In a typical experiment, 2.18 g PEG600DMA (cross-linking monomer), 795 μL deionized (DI) water and 994 μL anhydrous ethanol were mixed in a 6 mL vial, followed by the addition of 1.0 g MAA (functional monomer). This composition was based on a pilot study that showed formation of good gels. Different amounts of Alexa Fluor 350-labeled lysozyme, i.e., 0.2, 0.4, 0.6, or 0.8 mg, were added to the monomer solution and thoroughly mixed. The initiator DMPA was added last in the amount of 1-2 wt% and mixed with the solution. Non-imprinted polymers (NIP) with the same composition mentioned above were made but without lysozyme. The monomer mixtures were pipetted into a chamber created by an 820 μm thick Teflon spacer between two 26 mm by 76 mm glass slides clamped together. In a typical experiment, 4–6 assemblies were prepared from one batch of solution. The assembly was flood-exposed to a UV source (Spectroline, SB-100P, 365 nm) with an intensity of 10.0–15.0 mW cm^{-2} for 10 min, during which free-radical polymerization was initiated and completed. The ~15 mm × 50 mm × 0.8 mm (width by length by thickness) polymer gel was carefully removed from the glass assembly and rinsed with DI water five times to remove unreacted monomer and template molecules, as well as any debris created during fabrication.

2.4. Loading test

The gels were cut into 17 mm diameter discs using a cork borer. Each sample was soaked in 1 mL 0.4 mg mL^{-1} protease solution in PBS (pH 7.0) in the wells of 12-well plate and shaken at room temperature for 24 h. The fluorescence of lysozyme digested from each disc was measured, and the amount of lysozyme released was calculated. The disc samples were rinsed with DI water, air-dried, and stored in a dark and dry environment until needed for the selectivity test. The results from all the discs cut from the gels synthesized from one batch of solution were averaged, and the total amount of lysozyme released was extrapolated.

2.5. Selectivity test

To evaluate the preferential binding capability of the lysozyme-imprinted MIP samples, RNase A (subsequently referred to as

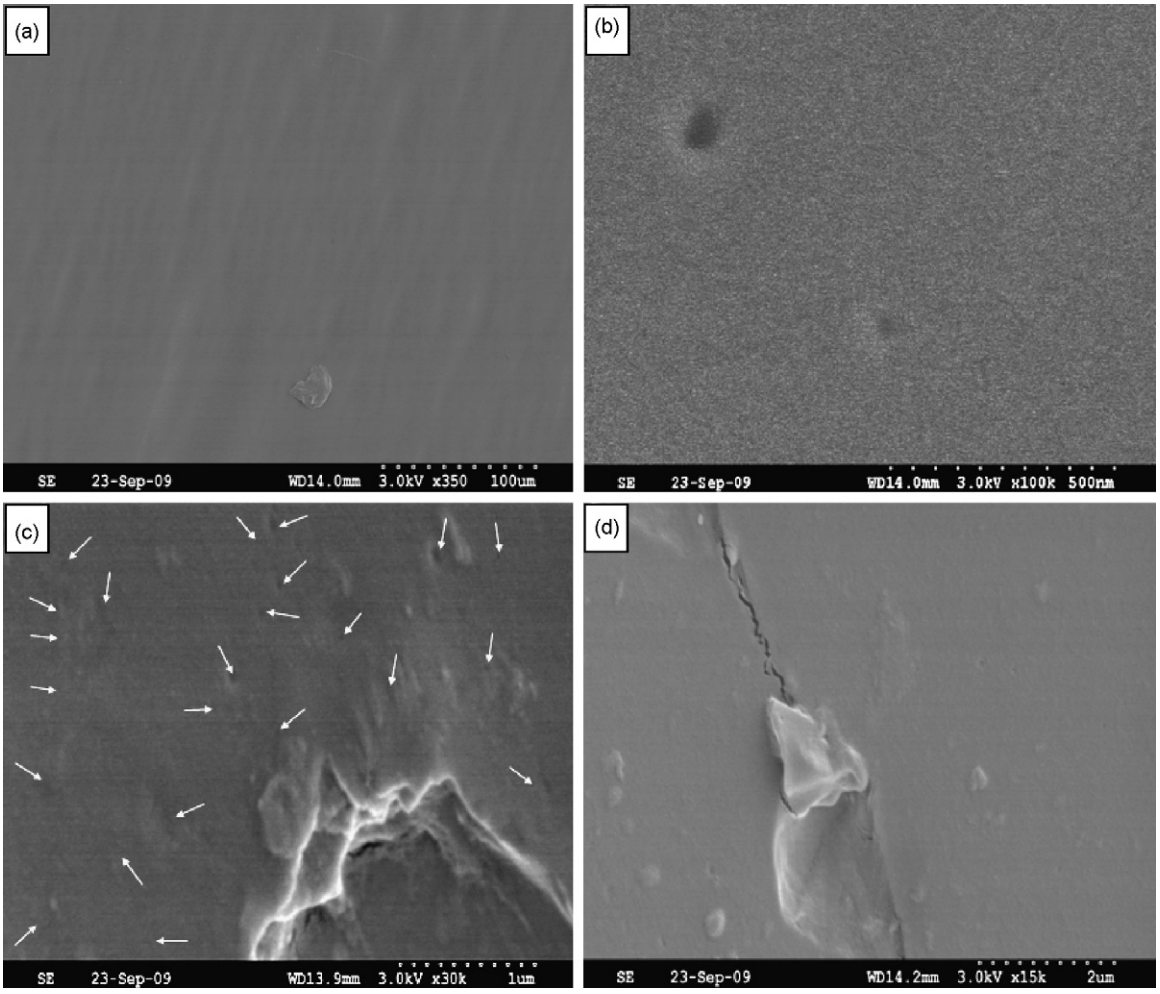

Fig. 2. Surface morphology of: (a) MIP synthesized with 0.6 mg lysozyme added, before removal of lysozyme by protease digestion; (b) high magnification of (a); (c) 0.6 mg lysozyme MIP after digestion; and (d) NIP after digestion. Note that arrows in (c) are used to mark the locations of small pits, which may be recognitive sites created when lysozyme molecules were released into the protease solution.

RNase) was chosen to act as a competitor protein because its molecular weight is similar to that of lysozyme. As previously mentioned, lysozyme was labeled with Alexa Fluor 488 so it could be differentiated from the lysozyme used during imprinting. RNase was labeled with Alexa Fluor 594. Three protein solutions were prepared with lysozyme to RNase ratios of 1:0 (lysozyme only), 1:1 and 0:1 (RNase only). The total protein concentration of each solution was 0.1 mg mL^{-1} so there was enough protein to occupy all binding sites. MIP samples loaded with 0.2 mg lysozyme were placed in a 12-well plate with each well containing 1 mL lysozyme/RNase solution and shaken at room temperature for 24 h. MIP discs with bound lysozyme and RNase were digested in 0.4 mg mL^{-1} protease solution at room temperature for 24 h with shaking. The amounts of protein released into the protease solution were measured in a similar way as described for the Loading Test. The same method was used as in the loading test to obtain the total amount of protein that was bound to the polymer gels. Selectivity tests were repeated for the 0 mg lysozyme polymer (NIP), 0.4, 0.6 and 0.8 mg lysozyme-imprinted MIP samples.

2.6. Morphology

A Hitachi S-4300 scanning electron microscope was used for observation of polymer morphology at an accelerating voltage of 3.0 kV. Samples were sputter-coated with gold before SEM examination.

2.7. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using InStat (Graphpad Software, San Diego, CA). Post hoc comparisons were made using the Tukey–Kramer Multiple Comparisons Test when the p-value was significant ($p < 0.05$). A minimum of three replicates was used for each experiment, and experiments were repeated at least once.

3. Results and discussion

Macroscopically, the surfaces of the NIP, MIP before digestion, and MIP after digestion were smooth and nonporous (Fig. 2), which is similar to the observations of Liu et al. for poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels [23]. At higher magnification, however, the surface morphology of undigested and digested MIP gels was different. Although small depressions or other features were not totally absent from the surface of gels before digestion, numerous nanometer-sized pits were observed after treatment with protease to remove imprinted protein. The limited number of surface features on undigested samples could be a result of bubbles introduced when loading the monomer solution between the glass slides as well as from regions of aggregated lysozyme or unreacted monomer removed during washing [10]. Considering the molecular size of lysozyme to be about 4.5 nm × 3.0 nm × 3.0 nm [24], extraction of lysozyme molecules from the gel surface during the

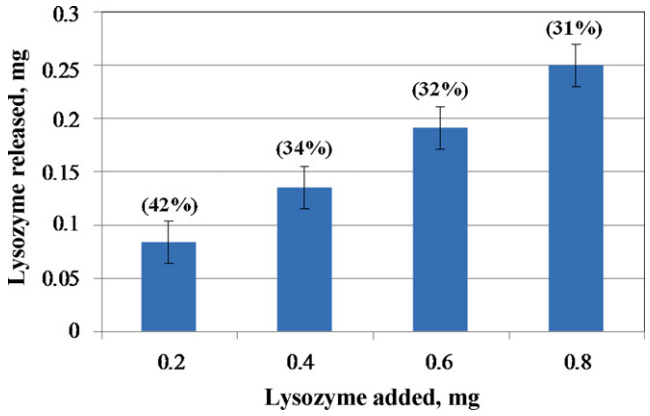

Fig. 3. Amount (and percentage) of lysozyme released from MIP as a function of that added to the monomer solution. Data are mean ± standard deviation for $n = 22$ samples.

digestion process will leave behind surface cavities in the size range of nanometers. Thus, the nanopits on digested samples are believed to be the recognitive or binding sites for lysozyme. Microstructure of the present materials is quite different from that of MIP synthesized using sol–gel methods [1]. For the latter, surfactant was used to create macropores for cell ingrowth. It could be possible to incorporate a porogen in the UV-polymerized MIPs.

Fig. 3 summarizes and compares graphically the amount of template molecule lysozyme added before polymerization synthesis and that released by digestion from the synthesized gel. The percentage of lysozyme released during digestion, i.e., 42% for 0.2 mg, 34% for 0.4 mg, 32% for 0.6 mg and 31% for 0.8 mg lysozyme loading, was also calculated and shown in the figure. ANOVA showed a significant ($p < 0.0001$) loading-dependent increase in the amount of surface-accessible lysozyme. It can be seen that when the amount of lysozyme increased in the range of 0.2–0.8 mg, the quantity of protein removed from the MIP, and therefore the number of potential binding or recognitive sites, also increased, as expected. Interestingly, only 1/3–1/2 of the total lysozyme incorporated into the gel was released, less than the ideal case where 100% of the protein molecules would be removed. Furthermore, the percentage of lysozyme released from the MIP decreased from 42% down to 31% with increasing lysozyme addition before polymerization, indicating that the varying quantity of template molecules can affect the relative amount of binding sites on the fabricated MIP. Ou et al. reported that approximately 27% of the lysozyme template was not extracted from their molecularly imprinted acrylamide polymers in the form of ~150 μm diameter particles [18]. The present relatively low removal efficiency was most likely due to the dimension of the nonporous MIP polymer gel. Our experiments used monolithic 17 mm diameter × 0.8 mm thick discs. During digestion, the template molecules on and close to the gel surface were easily extracted, but those in the bulk were inaccessible. While further reducing the gel thickness (via the Teflon spacer dimension) could enhance removal, the advantages that our large size (width and length) lysozyme-imprinted MIP has to offer are easy handling and assembly in producing potential devices for food, pharmaceutical, and diagnostic sciences.

Results of MIP selectivity tests are shown in Table 1 and also graphically in Fig. 4. For protein solutions containing only lysozyme (lysozyme:RNase = 1:0), the amount of lysozyme bound to MIP increased with increasing amount of template protein used for imprinting, consistent with the trend in the loading test shown in Fig. 3. For solutions with only RNase (lysozyme:RNase = 0:1), the amount of protein bound to MIP did not show an observable trend with loading, and significantly ($p < 0.001$) less RNase bound compared to lysozyme. In addition, the amount of RNase bound to lysozyme-imprinted MIP was statistically similar to the lysozyme and RNase amounts bound to the NIP. This result demonstrates that binding of RNase to MIP was non-specific, because an increased number of nanopits or binding sites on the surface did not affect RNase binding. More importantly, competitive binding tests using equal amounts of the two proteins (lysozyme:RNase = 1:1) showed 2–4 times more ($p < 0.05$) lysozyme than RNase bound to MIP when the amount of lysozyme imprinted during polymerization was low (0.2–0.6 mg added); with imprinted lysozyme increased to 0.8 mg, the MIP bound a much greater amount of lysozyme than RNase, approximately 20 times more ($p < 0.01$). Overall, when the MIP was exposed to both the template protein lysozyme and the competitor protein RNase, it preferentially bound lysozyme instead of the similarly sized RNase, indicating a chemical imprinting effect in addition to a geometrical effect.

These findings provide evidence that the template protein lysozyme was successfully imprinted into UV-polymerized MIP, and the resulting polymer demonstrated preferential binding of its template protein. Different amounts of lysozyme used in MIP synthesis altered the density of the binding sites on MIP surface, hence influencing its binding or recognition capability. It can be seen that control polymer bound a limited amount of both the template (lysozyme) and competitor (RNase), which is reasonable because there were no specific geometrical or chemical binding sites created by the template molecules. In this case, protein binding to the NIP was of non-specific nature (random surface adsorption). RNase bound to MIP under the same non-specific mechanism as both lysozyme and RNase bound to the NIP.

Protein-imprinted polymers have the advantage of being prepared to have a variety of physical forms in an easy and inexpensive way. Particles, slabs, and macroporous materials have been fabricated. Ou et al. [18] studied the system of polyacrylamide incorporated with methacrylic acid and 2-(dimethylamino)ethyl methacrylate for the imprinting of lysozyme. Particles ~150 μm in diameter were synthesized. As mentioned previously, approximately 27% (of the lysozyme template was not extracted from the molecularly imprinted acrylamide polymers. Addition of methacrylic acid in the polymerization increased the extracted amount of lysozyme. Good selective binding capability was also shown, with MIP adsorbing 83% more lysozyme than did the NIP. Odabaşi et al. [16] sought to prepare lysozyme-imprinted polymer

Table 1
Selectivity testing results.

Polymer type	Lysozyme imprinted, mg	Lysozyme:RNase re-bound to MIP tested in lysozyme:RNase = 1:0, 1:1 and 0:1 solutions, μg		
		1:0	1:1	0:1
Control	0	10 ± 2 : 0	8 ± 1 : 13 ± 3	0 : 12 ± 1
MIP	0.2	83 ± 17 : 0	74 ± 2 : 15 ± 3	0 : 18 ± 3
MIP	0.4	76 ± 13 : 0	49 ± 16 : 28 ± 0	0 : 16 ± 5
MIP	0.6	107 ± 23 : 0	29 ± 3 : 18 ± 2	0 : 7 ± 3
MIP	0.8	112 ± 26 : 0	124 ± 17 : 6 ± 1	0 : 7 ± 2

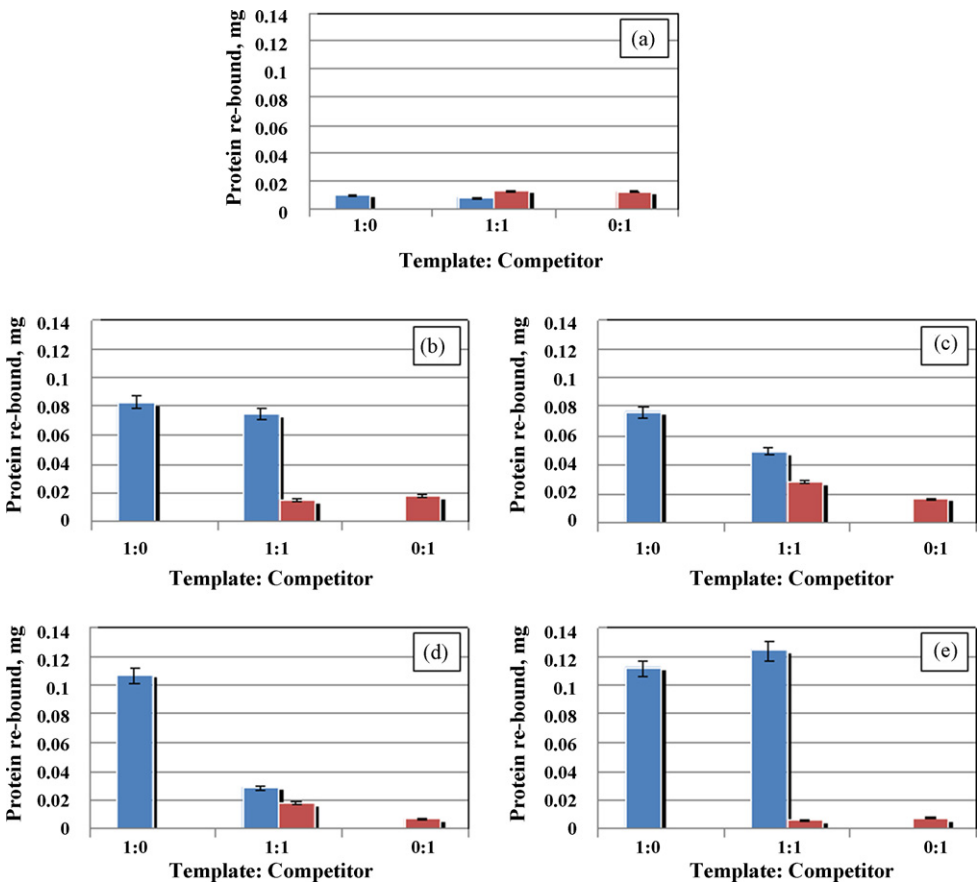

Fig. 4. Binding results of NIP and MIPs in solutions of lysozyme:RNase = 1:0, 1:1 and 0:1. The amount of lysozyme imprinted in these polymer samples was (a) 0 mg, (b) 0.2 mg, (c) 0.4 mg, (d) 0.6 mg, and (e) 0.8 mg. Lysozyme is shown in blue, and RNase in red. Data are mean ± standard deviation for $n = 3$ samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Lys-MIP) for use in purifying the enzyme from aqueous solutions and egg white. N-Methacryloyl-(L)-histidinemethylester (MAH) was complexed with Cu^{2+} to form functional monomer, to which lysozyme was added and imprinted in a poly(HEMA–MAH) particles through UV-initiated bulk polymerization. The resulting 20–63 μm diameter Lys-MIP particles could be used many times without decreasing their adsorption capacities significantly. Using the same polymer composition as Ou et al. [18], Kimhi and Bianco-Peled [25] formed fragmented particles that had ~1 μm surface pores. About 1.7 times more lysozyme bound to the MIP than to the NIP. Even though competition from cytochrome c decreased the amount of adsorbed lysozyme, approximately 50% remained even when six times more competitor was present. He et al. [26] used surface grafting of the Ou et al. polymer composition [18] to imprint lysozyme on silica nanoparticles. The Lys-MIP bound 1.5 times more lysozyme than did the NIP, and eight times more lysozyme than cytochrome *c* competitor was adsorbed.

Bereli et al. [17] used free-radical polymerization initiated by N,N,N,N-tetramethylene diamine (TEMED) to fabricate lysozyme-imprinted poly(HEMA–MAH) cryogels. The relative selectivity coefficient of lysozyme-imprinted cryogels was about three times greater than that for non-imprinted NIP cryogels. Purification of lysozyme from egg white was also studied using *Micrococcus lysodeikticus* as substrate. The purity of the desorbed lysozyme was about 94%, with recovery about 86%. The Lys-MIP cryogel could be used many times without significantly decreasing the adsorption capacity.

A novel approach to reducing non-specific binding in MIPs was recently reported by Tov et al. [27]. The two stage process based on formation of interpenetrating networks (IPN) involves first imprinting lysozyme into acrylamide-methacrylic acid hydrogels followed by polymerization of an identical composition in and around the "conventional" MIP. The second network was hypothesized to block charged clusters that can mediate non-specific adsorption. Compared to traditional MIP, the IPN materials had higher imprinting efficiency and greater selectivity for lysozyme.

Lee et al. reported synthesis and testing of macroporous polysiloxane (silica) scaffolds imprinted with either lysozyme or RNase A in a sol–gel process [1]. The quantity of surface-accessible protein (number of potential binding sites) was varied by changing the amount of protein loaded into the sol–gel solution. Up to 62% of loaded lysozyme was extracted, which is comparable with the results reported by Ou et al. [18]. The protein-imprinted polysiloxane scaffolds bound up to 3.6 times the amount as NIP, and they preferentially recognized the template biomolecules when incubated in mixtures containing both the imprinted protein and a competitor protein, adsorbing up to three times more template.

From these reports, it can be seen that lysozyme was imprinted into either particle polymer materials or a porous type material. Particulate [16–18,24] and macroporous [1] materials have relatively higher rebinding efficiency in recognitive capability tests because of their greater specific surface area. In the present studies, monolithic and dense polymers imprinted with lysozyme were fabricated by using UV free-radical polymerization. The advantage of monolithic lysozyme MIP is that different physical shapes or geometries can be produced from a fabricated MIP bulk material as needed for lysozyme recognition or separation devices. Besides, easy handling and assembling of bulk MIP are additional merits for producing potential devices.

4. Conclusions

The present study demonstrates use of a simple polymerization strategy to create molecularly imprinted substrates having the ability to preferentially bind protein molecules. Lysozyme was molecularly imprinted into a polymer with MAA as functional monomer and PEG600DMA as cross-linking monomer by UV free-radical polymerization. Incorporation of more lysozyme in the reaction mixture led to an increased quantity of binding sites in the MIP. More importantly, the lysozyme-imprinted MIP had good selectivity for the template lysozyme over the competitor RNase; the MIP bound up to 20 times more lysozyme than RNase when exposed to a lysozyme/RNase mixture. In contrast, low binding of RNase was measured, and it did not show an observable trend compared with lysozyme binding. RNase binding to MIP was of a non-specific nature, similar to the control (non-imprinted) polymer, which showed low binding of both lysozyme and RNase. This facile method may be extended to fabricate materials having recognitive sites that selectively bind other proteins and, therefore, may have a range of applications, including artificial antibodies, biosensors, and drug delivery devices.

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