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Protein imprinting in polyacrylamide-based gels

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Abstract

Protein imprinting in hydrogels is a method to produce materials capable of selective recognition and capture of a target protein. Here we report on the imprinting of fluorescently-labeled maltose binding protein (MBP) in acrylamide (AAM)/N-isopropylacrylamide (NIPAm) hydrogels. The targeting efficiency and selectivity of protein recognition is usually characterized by the imprinting factor, which in the simplest case is the ratio of protein uptake in an imprinted film divided by the uptake by the corresponding non-imprinted film. Our objective in this work is to study the dynamics of protein binding and elution in imprinted and non-imprinted films to elucidate the processes that control protein recognition. Protein elution from imprinted and non-imprinted films suggests that imprinting results in sites with a distribution of binding energies, and that only a relatively small fraction of these sites exhibit strong binding.

Keywords

Protein imprinting; Polyacrylamide; Hydrogel; Maltose binding protein

1. Introduction

Molecular imprinting seeks to exploit the underlying principles of molecular recognition found in nature to produce artificial recognition elements. Over the last 30 years there have been significant advances in molecular imprinting, particularly related to small molecules [1–6]. Developments in recognition of more complex macromolecules, such as proteins, have been more recent [7–27]. Imprinting involves the formation of a binding cavity in a polymer gel matrix (e.g. thin film or particles) by incorporating functional monomers with side groups that can interact with the target molecule. Imprinted films are synthesized by combining appropriate monomers, initiator, cross-linker, and the target protein. On polymerization, interactions between side groups on the monomers and surface residues on the protein are “frozen” into the polymer gel structure. After polymerization, the protein is extracted from the polymer matrix leaving cavities that are complementary to the target

protein in terms of shape, size, and the location of side groups. On exposing the platform to a protein solution, only the target protein will bind at the binding cavity.

Protein imprinting exploits the interaction between accessible surface residues on the target protein and side groups on the polymer backbone. A key issue in designing polymers for protein imprinting is to find the optimum monomer composition that will interact with the target protein with high affinity. There are four general categories of recognition sites for proteins: hydrophobic interactions, hydrogen bonding, electrostatic interactions, and pi-pi interactions.

In previous work we showed how analysis of surface accessible residues on maltose binding protein (MBP) can be used to guide the selection of functional monomers in the polymer [27]. AAm can form hydrogen bond interactions and NIPAm can form hydrophobic interactions with a target protein. The maximum imprinting factor for MBP in AAm/NIPAm gels was obtained at a mole ratio of AAm/NIPAm of 0.50, very close to the ratio of surface residues that can form hydrogen bonds and hydrophobic interactions (0.53).

The efficiency and selectivity of protein recognition in imprinted films is usually characterized by the imprinting factor, which in the simplest case is the ratio of protein uptake in an imprinted film divided by the uptake by the corresponding non-imprinted film. While the imprinting factor is a convenient figure of merit, it does not contain any information on the processes that contribute to efficiency and selectivity. Therefore, the objective of this study is to elucidate the dynamics of protein uptake and elution in imprinted and non-imprinted films as a function of time. Based on these results we suggest a preliminary model for imprinting that takes into account weakly bound and strongly bound protein.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAm, MW 113.16 g mol⁻¹), acrylamide (AAm, MW 71.08 g mol⁻¹), *N,N*-methylenebisacrylamide (MBA, MW 154.17 g mol⁻¹), ammonium persulfate, *N,N,N',N'*-tetramethylenebisacrylamide (TEMED), 3-(trimethoxysilyl) propyl methacrylate, and Tris buffer were obtained from Sigma-Aldrich. Maltose binding protein (MBP) was expressed and purified as described previously [27]. Bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma. Proteinase K was purchased from New England BioLabs. All chemicals were used as received. All experiments were performed using ultrapure water (Millipore).

2.2. Preparation of molecularly imprinted polymer (MIP) films

Microscope glass slides (1.2 cm × 1.2 cm, Fisher Scientific) were cleaned with piranha solution for 30 min, washed with deionized water and dried under nitrogen. To improve polymer adhesion, the slides were modified by silanization. After incubation in 3-(trimethoxysilyl)propyl methacrylate (1%) in toluene overnight at room temperature, the slides were sequentially washed with toluene and water and then dried at 115°C for 1 h. The silane-modified glass slides were stored under nitrogen at room temperature.

Freshly cleaved mica sheets (1.5 cm × 1.5 cm, grade V-4 from SPI Supplies) were used to ensure that the top surface of the polymer gels was flat. To reduce adhesion to the gel and facilitate separation after gelation the mica wafers were immersed in a solution of PlusOne Repel-Silane ES (GE Healthcare) for 10 min, sequentially washed with ethanol and water, and then air-dried prior to use.

The proteins MBP, BSA and OVA, were labeled with sulfoindocyanine *N*-hydroxysuccinimidyl (Cy3-NHS) ester dye (GE Healthcare, Amersham Cy3 Mono-Reactive Dye Pack, PA23001) following procedures provided by the manufacturer. The average number of Cy3 molecules per protein molecule was 1, as determined by UV-vis spectroscopy. Although the Cy3 dye contains two negative charges and one positive charge, we assume that they do not influence rebinding since MBP has a large number of surface residues with positive and negatively charged side groups (22% of the surface residues are negatively charged and 28% positively charged).

The precursor solution for producing the hydrogel films was prepared by mixing functional monomers (NIPAm, AAm) at a 1:1 mol ratio along with the cross-linker (MBA) and ammonium persulfate (1 mg mL⁻¹) in 10 mM Tris buffer (pH 7) to obtain desired total monomer concentration. In all cases the total amount of monomer (including cross-linker) was 1.69 × 10⁻³ mol. Subsequently, TEMED was added to initiate polymerization. The total monomer concentration (AAm, NIPAm, and MBA) was varied from 10 to 50 wt.% and the cross-linker concentration was varied from 1 to 5 mol.%.

As an example, a non-imprinted polymer with a 1:1 mol ratio of AAm and NIPAm monomers and 2 mol% cross-linker was prepared as follows. AAm (8.27 × 10⁻⁴ mol), NIPAm (8.27 × 10⁻⁴ mol), MBA (3.38 × 10⁻⁵ mol), ammonium persulfate (1 mg mL⁻¹) were mixed in a volume of 10 mM Tris buffer (pH 7) to obtain a required total monomer concentration. To initiate polymerization, 5 µL of TEMED (6.5% v/v, aqueous solution) was added to 50 µL of precursor solution, purged with nitrogen for 20 s and then immediately deposited on a silane-modified glass slide and covered by a mica wafer.

The imprinted polymers (MIPs) were prepared in the same way by adding MBP labeled with Cy3 (MBP-Cy3) at a concentration of 1 mg mL⁻¹ in 10 mM Tris buffer to the precursor solution. Most imprinted films were prepared with 0.21 mg mL⁻¹ MBP. Polymerization was carried out at 37°C for 1.5 h. After polymerization, the mica was removed from the surface of the polymer film. The thickness of the films was determined using a confocal microscope (Nikon Spinning Disk). After incubating in 0.50 mg mL⁻¹ MBP solution, the films were removed from solution and placed upside down on a cover slip in a dish with 10 mM Tris buffer to ensure that the films remained hydrated during the measurement. Z-stack images were obtained using a 2 µm spacing from the cover slip to the glass slide. The thickness of the imprinted films was 120 µm and the non-imprinted films was 100 µm.

MBP-Cy3 was extracted from the imprinted films by digestion with proteinase K (400 µg mL⁻¹ in a solution containing 100 mM NaCl and 50 mM CaCl₂) for 12 h at 40°C in the dark. The polymer films on the glass slides were then washed for 1 h in 10 mM Tris buffer to remove the protein fragments and proteinase K. Non-imprinted polymers were subjected

to the same treatment to avoid any differences in comparing to the imprinted polymers. Proteinase K was selected for protein extraction from imprinted films for its lack of specificity in cleaving peptide bonds and its ability to breakdown proteins to very short peptides [28].

2.3. Protein uptake in imprinted and non-imprinted films

The affinity of the hydrogel films for the template protein was verified by rebinding experiments in which imprinted films after protein extraction (MIPs) and non-imprinted films (NIPs) were incubated in 1 mL MBP-Cy3 solution in Tris buffer pH 7 on a rocker at room temperature in the dark. Imprinted films were incubated in 0.5 mg mL⁻¹ MBP-Cy3 solution unless otherwise stated. The incubation time for most experiments was 5 h, however, for some experiments rebinding was studied up to 63 h. To study the influence of protein concentration on binding to non-imprinted films, a series of experiments were performed in MBP-Cy3 solution with concentrations from 0.01 to 0.5 mg mL⁻¹.

After incubation in protein solution, the films were rinsed once in Tris buffer for 5 min and then imaged to determine the amount of remaining protein. In some experiments, films were immersed in Tris buffer for several hours to study desorption of non-specifically bound and weakly bound protein. To determine the selectivity of imprinting, MBP-imprinted films and non-imprinted films were incubated in the same concentration of BSA-Cy3 and OVA-Cy3.

After each step (protein imprinting, protein extraction, and protein rebinding), the films were imaged by fluorescence microscopy using a Nikon Eclipse ME 600 epifluorescence microscope. SPOT 5.0 software (Spot Imaging Solutions) was used to acquire fluorescence images using a 10× objective (NA 0.3). Images were collected using a SpotRT 229044 camera with 2×2 binning yielding 1600 × 1200 pixels. For measuring Cy3 fluorescence (Ex 550 nm, Em 570 nm) we used a Nikon G-2A filter cube (Ex 510e560 nm, DM 565 nm, BA 590 nm). The microscope was focused on the top surface of the films and three images recorded at random locations near the center of the film. Each image was 1040 μm × 780 μm (800 × 600 pixels). The background intensity was determined from fluorescence images of as-prepared non-imprinted films using the same imaging procedures as described above. The average background intensity (per pixel) was obtained from three images at random locations near the center of the film. The average fluorescence intensity (per pixel) for each image was obtained using ImageJ software (NIH), and the average background intensity subtracted. For all experiments, the background corrected average intensity was averaged over three independent experiments and converted to a protein concentration as described below. For most experiments, fluorescence images of the imprinted and non-imprinted films, and of protein solutions for calibration, were obtained at an exposure time of 100 ms. For experiments with thicker films (160 μm–520 μm) the exposure time was 25 ms.

Quantitative analysis of protein incorporation into the films was achieved by measuring the average fluorescence intensity for known concentrations of MBP-Cy3. The average fluorescence intensity (per pixel) of MBP-Cy3 with concentrations of 0.001–0.2 mg mL⁻¹ was determined by pipetting 10 μL of the protein solution on a microscope slide and covering with a circular cover slip (1.13 cm²) such that the solution was constrained to a

fixed height and a fixed area. An example of a calibration curve is shown in Supplemental Information.

The ability of imprinted polymers to bind a target protein is analyzed quantitatively in terms of the imprinting factor, IF:

$$IF = \frac{m^{IP} - m_0^{IP}}{m^{NIP} - m_0^{NIP}} \quad (1)$$

where m^{IP} is the amount of protein bound to the imprinted polymer, m^{NIP} is the amount of protein bound to the non-imprinted polymer, m_0^{IP} is the amount of residual protein in the imprinted polymer after extraction, and m_0^{NIP} is the intrinsic (autofluorescence) signal of the non-imprinted polymer. If the fluorescently-labeled protein is completely removed from the polymer film and the non-imprinted polymer has no autofluorescence then $m_0^{IP} = m_0^{NIP} = 0$. Note that an imprinting factor of 1.0 corresponds to no selectivity to the target protein.

2.4. Structural characterization

The structure of imprinted and non-imprinted polymers were examined by scanning electron microscopy (SEM). To preserve three-dimensional structure of the gels, the samples were prepared by a freeze-drying method to avoid shrinkage and structural deformation associated with air-drying [29, 30]. The polyacrylamide hydrogels were prepared in 24-well flat-bottom polystyrene plates using the same precursor solution as for producing the hydrogel films on glass slides. Hydrogel disks approximately 5 mm in thickness were prepared by using 500 μ L of precursor solution. To initiate polymerization, 50 μ L TEMED (6.5% v/v, aqueous solution) was added to 500 μ L of precursor solution, purged with nitrogen for 45 s and then immediately transferred to one of the wells. The imprinted polymers (MIPs) were prepared in the same way by adding MBP labeled with Cy3 (MBP-Cy3) to achieve a final concentration of 0.21 mg mL⁻¹. Polymerization was conducted at 37 °C for 1.5 h. After polymerization, the hydrogel disks were removed from the wells and hydrated with water for 2 h. The hydrated gels were sectioned with a razor blade parallel and perpendicular to the top surface. In some cases the films were cut after freezing to ensure that there were no artifacts from the cutting process. The hydrogel samples were then placed in small glass bottles, frozen in liquid nitrogen, and freeze-dried (Labconco) for 17 h. The freeze-dried gels were mounted on SEM stubs using adhesive carbon double-sided tabs, outlined with conductive silver paint, and sputtered with platinum for 2 min. The coated gels were imaged on an FEI Quanta 200 Environmental SEM at a low beam voltage of 2.5 V.

Pore sizes and wall thicknesses were obtained from analysis of SEM images. Pore sizes were obtained using NIS Elements to trace individual pores. The pore diameter is defined as the diameter of the circle with the same area as the pore. Wall thicknesses were obtained from analysis of high magnification images. Each measurement represents the thickness between two adjacent pores obtained using NIS Elements.

3. Results and discussion

3.1. Influence of monomer concentration and cross-linker concentration

Protein recognition was studied in imprinted acrylamide (AAm)/N-isopropylacrylamide (NIPAm) hydrogel films [27]. The target protein, maltose binding protein (MBP), is a 41 kD protein with surface residues capable of both hydrogen bonding interactions and hydrophobic interactions [27]. The steps involved in preparing imprinted polymer films are illustrated in Fig. 1. Briefly, a fixed volume of precursor solution containing the monomers, cross-linker (methylene-bisacrylamide, MBA), initiators (ammonium persulfate and tetramethylethylenediamine, TEMED), and the fluorescently labeled target protein is placed on a modified glass slide and covered by a mica wafer to ensure that the surface of the polymer is flat. After polymerization, the mica is removed and the protein extracted by digestion with proteinase K. The affinity of the imprinted polymer films to the target protein is determined by exposing the imprinted and non-imprinted films to a solution containing the target protein.

The amount of protein taken up by imprinted and non-imprinted films is obtained from analysis of fluorescence images [31]. To relate the fluorescence intensity to protein concentration, fluorescence images were obtained from known concentrations of protein solution under identical imaging conditions (Fig. S1, Supporting Information). Representative fluorescence images before and after incubation of imprinted and non-imprinted (control) AAm/NIPAm gels with MBP-Cy3 show that protein binding is uniform throughout the film (Fig. 1). In control experiments, non-imprinted films are also incubated with fluorescently labeled protein. In this case the level of non-specific binding is very low.

To determine the influence of total monomer concentration and cross-linker concentration on protein imprinting, we performed a series of experiments in AAm/NIPAm (1:1 mol ratio) hydrogel films. For all monomer concentrations, the maximum imprinting factor was obtained at a cross-linker concentration of 2 mol.% (Fig. 2). Cross-linker concentrations of 1 mol.% and 5 mol.% resulted in low imprinting factors in the range from 1.0–1.5. Films with 2 mol.% cross-linker had imprinting factors from 2–4, with a maximum of 3.8 ± 0.3 (SD) at 20 wt.% monomers.

The maximum imprinting factor at 2 mol.% cross-linker and 20 wt.% monomer can be explained by the balance between transport and the number of points of interaction around each protein. Increasing cross-linking decreases the pore size and hence attenuates protein transport in the film. However, decreasing cross-linking increases spatial fluctuations in the recognition sites and hence reduces binding efficiency. The monomer concentration determines the number of points of interactions between the target protein and the polymer film. Increasing monomer concentration provides more points of interaction, and improves the fidelity of the binding cavity to the target protein, however, increasing the monomer concentration also results in decreasing pore size and hence limits protein transport in the film. However, decreasing the monomer concentration reduces binding efficiency by increasing the pore size and spatial fluctuations in the polymer backbone.

The selectivity of films imprinted with MBP (MW 41 kDa, $3 \times 4 \times 6.5$ nm, pI 5.22) was investigated by incubating MBP imprinted and non-imprinted films with reference proteins of similar molecular weight, dimensions, and isoelectric point: bovine serum albumin (BSA, MW 66 kDa, $4 \times 4 \times 14$ nm, pI 4.7) and ovalbumin (OVA, MW 45 kDa, $5 \times 4.5 \times 7$ nm, pI 4.6). The imprinting factor was determined for films with 20 wt.% monomer and 2 mol.% cross-linker and imprinted with MBP. As described above, the imprinting factor for MBP was 3.8, whereas the imprinting factors for BSA and OVA were 1.02 and 1.09, respectively, very close to the value of 1.0 that indicates no selectivity. These results confirm very high selectivity of imprinted films to the target protein.

3.2. Structure of AAm/NIPAm films

Acrylamide films exhibit a characteristic cellular structure [30, 32, 33]. In contrast, collagen type 1 films usually exhibit a fibrillar structure, and hyaluronic acid and matrigel films typically exhibit a sheet-like structure [34–36]. The AAm/NIPAm films studied here are characterized by an interpenetrating porous network with a cellular structure (Fig. 3). The structure is isotropic and appears the same both in parallel and perpendicular sections to the top surface.

The pore size and wall thickness were obtained from analysis of the images. The imprinted films had an average pore size of 13.4 ± 6.8 μm (SD) and an average wall thickness of 200 ± 60 nm (SD) (Fig. 4). The pore size represents the diameter of the circle with the same area as the pore. The structure of the non-imprinted films is very similar with a pore size of 6.2 ± 1.7 μm (SD) and a wall thickness of 240 ± 80 nm (SD) (Fig. 4). The shape of the pores was characterized by the circularity $C = 4\pi A/P^2$ where A is the area and P is the perimeter. The circularity was 0.81 ± 0.11 (SD) for the imprinted films and 0.86 ± 0.09 (SD) for the non-imprinted films, indicating that the pores are relatively circular.

The porosity of the films was estimated from the same images by measuring the total length of pore wall in a given area and multiplying by the average wall thickness. Using this approach we calculate a porosity of 91% for the imprinted films and 80% for the non-imprinted films.

The structure of the AAm/NIPAm films are very similar to images of AAm films reported in the literature. AAm films show an identical cellular structure with similar pore size [30, 32, 33]. For example, 11.6 wt.% AAm films with 2.4 mol.% MBA cross-linker have 1–2 μm pores [30]. Increasing or decreasing the cross-linker concentration results in increasing pore size. For example 10 wt.% AAm films with 4.6 mol.% MBA cross-linker have pores that are 5–10 μm in size [32]. NIPAm/AA (acrylic acid) particles with 23 wt.% monomer and 1.9 mol.% MBA cross-linker have a similar cellular structure to the AAm/NIPAm films reported here with a pore size of about 10 μm [37].

The thickness of the imprinted AAm/NIPAm films (20 wt.% monomer, 2 mol.% cross-linker, 0.21 mg mL⁻¹ MBP) was about 120 μm . In contrast the thickness of the non-imprinted films was about 100 μm . For films imprinted with 0.21 mg mL⁻¹ MBP, the average spacing between proteins is about 31 nm (using an average porosity of 90%). The additional volume of the protein accounts for about 20% of the increase (0.2 μm). From

analysis of the SEM images, it is evident that the presence of the protein increases the average pore size and decreases the wall thickness resulting in an increase in porosity of 11%, and accounting for most of the increase in film thickness.

3.3. Influence of time on imprinting factor

To study the role of time on imprinting factor, we exposed imprinted and non-imprinted films to MBP (0.50 mg mL^{-1}) for 44 h (Fig. 5a). Films were prepared with 20 wt.% monomer and 2 mol.% cross-linker. At each time point the film was removed from MBP solution, rinsed with 10 mM TRIS buffer for 5 min, imaged using fluorescence microscopy, and then re-immersed in MBP solution. For imprinted films, protein uptake increases quickly in the first 40 min, and then increases more slowly with time. After about 5 h, protein uptake reaches a maximum of about 0.07 mg mL^{-1} , or about 33% of the imprinted concentration (0.21 mg mL^{-1}), and remains constant until the end of the experiment after 44 h. In contrast, protein uptake by the non-imprinted films increases quickly to about 0.018 mg mL^{-1} in the first 20 min, but then remains constant.

The increase in protein uptake in the imprinted films during the first 5 h follows a $t^{1/2}$ dependence indicating that uptake is limited by protein diffusion into the film (Fig. 5b). Assuming a constant concentration of protein in bulk solution, the total amount of protein taken up by the imprinted polymer film is given by:[38]

$$\frac{M}{c_0} = 2 \left(\frac{Dt}{\pi} \right)^{1/2} \quad (2)$$

where D is the diffusion coefficient ($\text{cm}^2 \text{ s}^{-1}$), c_0 is the protein concentration in solution during rebinding (mg cm^{-3}) and M is the amount of protein taken up by the polymer film (mg cm^{-2}). Quantitative analysis is complicated by the fact that the internal surface area of the polymer film is unknown. Based on the average pore size of $13.4 \text{ }\mu\text{m}$ and the film thickness of $120 \text{ }\mu\text{m}$, we estimate a roughness factor (surface area divided by geometric area) of about 80. Assuming a roughness factor of 100, a least squares fit to the data gives a diffusion coefficient of $1.0 \times 10^{-14} \text{ cm}^2 \text{ s}^{-1}$. From the time at which protein uptake saturates (5 h) and the diffusion coefficient of $1.0 \times 10^{-14} \text{ cm}^2 \text{ s}^{-1}$ we estimate a diffusion length ($x = (Dt)^{1/2}$) of 130 nm. Since protein can diffuse into the polymer from both sides of the walls that make up the cellular structure of the films, we would expect the diffusion length at the time at which the protein concentration saturates to be about half of the wall thickness. From the SEM images, the wall thickness of the imprinted films is about 200 nm, and hence the diffusion length is expected to be about 100 nm in good agreement with the value estimated from the diffusion length. While there are a number of assumptions in this analysis, the experimental results are consistent with a model where protein rapidly diffuses into the pores and then slowly diffuses into the polymer walls that define the cellular structure of the films.

From the protein uptake curves for imprinted and non-imprinted films, we can determine the time dependence of the imprinting factor (Fig. 5c). The imprinting factor increases rapidly over 5 h and then remains constant at about 3.5. These experiments show that protein uptake in imprinted films increases over about 5 h and then saturates. For non-imprinted polymers,

uptake increases very rapidly, within 20 min, and then remains constant. Therefore, the imprinting factor increases up to the time when protein uptake in the imprinted films saturates. As we show below, however, the amount of protein bound to the imprinted and non-imprinted films is strongly dependent the amount of weakly bound protein removed during washing.

3.4. Protein uptake in non-imprinted films

Protein uptake in non-imprinted films increases rapidly and remains constant with time (Fig. 5a). Non-imprinted films incubated with different concentrations of MBP showed similar behavior, although the maximum protein uptake was dependent on protein concentration (Fig. 6a). Assuming that uptake in non-imprinted films is non-specific or weak binding to the internal surface of the films, these results indicate a fast surface process leading to equilibrium between the bulk and surface concentrations. The steady state protein uptake in non-imprinted films increases with increasing protein concentration in solution (Fig. 6b) and can be fitted to a Langmuir isotherm with protein uptake, $c_p = 0.024 (c_0/(c_0 + 1/b))$ where c_0 is the protein concentration in solution and $1/b = 0.13 \text{ mg mL}^{-1}$.

After incubating the non-imprinted polymers with different concentrations of MBP solution, the films were incubated in buffer for 5 h to study protein elution (Fig. 6c). The non-imprinted films show fast elution over the first 60 min, independent of the initial MBP concentration, and almost all of protein is eluted after 5 h. After 11 h, the protein concentration in the films is $0.0015\text{--}0.0018 \text{ mg mL}^{-1}$. These results suggest weak non-specific binding of MBP to the internal surface of the hydrogels.

The structure of the imprinted and non-imprinted films shows an isotropic cellular structure with an interpenetrating porous network (Fig. 3). To test the hypothesis that protein uptake in non-imprinted films is dominated by non-specific or weak binding to the internal surface of the hydrogels, we incubated non-imprinted films of different thickness with 0.5 mg mL^{-1} MBP for 5 h. Protein uptake increases linearly with film thickness (Fig. 6d), providing further evidence for non-specific binding to the internal surface of the non-imprinted films.

3.5. Protein uptake and elution in imprinted films

On polymerization of hydrogels containing protein, some of the monomer units in the polymer backbone form non-covalent points of contact (e.g. hydrogen bond or hydrophobic interactions) with surface residues on the protein. Removing the protein from the gels leaves binding cavities or pockets in the solid polymer phase of the hydrogel with a spatial distribution of interaction points with the target protein [39]. Since a monomer unit is more likely to add to a growing polymer chain than initiate a new chain, the number of interaction points around a protein is expected to have a broad distribution.

To investigate protein binding in imprinted films, we studied protein elution and rebinding in steady state. Incubating an imprinted film in buffer results in elution of the imprinted protein (Fig. 7a). The protein concentration decreases from the imprinted concentration (0.21 mg mL^{-1}) to about 0.041 mg mL^{-1} MBP after about 5 h, corresponding to 20% of the initial imprinted protein concentration. The constant concentration in the film up to 58 h

suggests that the remaining protein is bound in sites where the interaction energy (E_b) is greater than the thermal energy. We define the interaction energy as the sum of all hydrogen bonding and hydrophobic interactions around a protein. These results suggest that 20% of the protein is strongly bound ($E_b \gg kT$) in the imprinted films with a large number of interaction points, and that 80% of the protein is weakly bound ($E_b \sim kT$) either at the internal surface or within the solid phase.

After digestion in proteinase K for 1000 min, the strongly bound protein is removed from the imprinted film (Fig. 7b). The film was then incubated with 0.5 mg mL^{-1} MBP for 5 h and rinsed, resulting in uptake of 0.063 mg mL^{-1} in the film (Fig. 7c). The amount of protein taken up represents 30% of the original imprinted concentration of 0.21 mg mL^{-1} .

After rebinding, the film was incubated in buffer for 5 h (Fig. 7d). The protein in the film decreases from 0.063 mg mL^{-1} to about 0.01 mg mL^{-1} after 1 h and then decreases slightly over the subsequent 4 h. The strongly bound protein remaining in the film after 5 h washing in buffer ($0.0074 \text{ mg mL}^{-1}$) is 18% of the strongly bound protein concentration after rebinding (or 3.6% of initial protein concentration after imprinting).

These experiments suggest that imprinted films have binding sites with a distribution of binding energies. For the system studied here, 20% of the binding sites remain after elution and are characterized as strong binding ($E_b \gg kT$). After digestion of the remaining protein, incubation in protein solution, and subsequent elution, only 18% of the strongly bound protein or 3.6% of the imprinted protein remains in the film. This suggests that not all of the binding cavities formed during imprinting are accessible from protein solution.

As described above, the protein concentration in an imprinted film after digestion, incubation in 0.50 mg mL^{-1} MBP, and elution in buffer for 5 h is $0.0074 \text{ mg mL}^{-1}$ (Fig. 7d). As shown in Fig. 6c, the protein concentration in non-imprinted films after incubation in $0.01\text{--}0.50 \text{ mg mL}^{-1}$ MBP and subsequent elution in buffer for 11 h is about $0.0015\text{--}0.0018 \text{ mg mL}^{-1}$. From these concentrations, the imprinting factor is 4–5, slightly larger than the imprinting factors obtained after incubation in protein solution followed by washing in buffer for 5 min (Fig. 2). From Figs. 6c and 7d it is evident that the protein concentration in the imprinted and non-imprinted films is still decreasing slightly even at very long elution times. Therefore, we do not have definitive evidence that the protein concentration in the films reaches a steady state even after long incubation times in buffer.

The rebinding experiments described above were performed with films imprinted with 0.21 mg mL^{-1} MBP. To confirm that protein binding in imprinted films occurs at binding cavities in the solid phase in the hydrogels, we prepared films imprinted with different MBP concentrations from 0.01 mg mL^{-1} to 0.5 mg mL^{-1} . After removing the imprinted protein by digestion in proteinase K, we incubated imprinted films with 0.5 mg mL^{-1} MBP for 5 h. Protein uptake increases linearly with imprinted MBP concentration (Fig. 8), indicating that binding occurs at imprinted sites in the polymer.

3.6. Model for protein binding in imprinted films

Based on the results presented here we can begin to build a model of protein imprinting (Fig. 9). In imprinted AAm/NIPAm films, protein is distributed throughout the polymer sheets that define the cellular structure. On immersion in buffer, 80% of the protein is eluted, indicating that it is weakly bound in the polymer. The 20% of the protein remaining in the film after elution is strongly bound in sites where $E_b > kT$. The weakly bound protein ($E_b < kT$) may be located either at the surface or inside the polymer but with no interaction points, i.e. no hydrogen bond or hydrophobic interactions.

After removal of all strongly bound protein by digestion, incubation in protein solution, and washing in buffer for 5 min, the protein concentration in the film is 30% of the original imprinted protein concentration. Subsequent, incubation in buffer results in elution of protein over about 5 h to a concentration that is 3.6% of the initial imprinted protein concentration. This suggests that some of the imprinted strong binding sites are inaccessible to protein diffusing into the film, and that only 3.6% of the imprinted binding sites are accessible strong binding sites.

Protein uptake in the non-imprinted films is dominated by rapid, non-specific, weak binding to the surface, but may also include protein that can diffuse into the polymer sheets in the film. Subsequent washing for 5 min results in removal of most of the protein. Subsequent extended incubation in buffer for 11 h results in removal of almost all of the remaining protein, confirming that it is only weakly bound. These results suggest a distribution of binding energies in imprinted protein films where only a small fraction of binding sites have energy $E_b > kT$. For non-imprinted films incubated in protein solution, the weakly bound protein is predominantly in low energy binding sites with $E_b < kT$.

4. Conclusions

In summary, the binding of fluorescently-labeled maltose binding protein (MBP) to imprinted and non-imprinted acrylamide (AAm)/N-isopropylacrylamide (NIPAm) hydrogels was used to elucidate details of protein imprinting. The highest imprinting factor (3.8 ± 0.3 (SD)) was obtained for films with 2 mol.% cross-linker and 20 wt.% monomers. The dynamics of protein binding and elution were studied in films with this composition. Protein uptake in imprinted films increased over the first 5 h reaching a maximum corresponding to about 33% of the imprinted protein concentration. The initial uptake followed a $t^{1/2}$ dependence suggesting that binding is dominated by diffusion in the imprinted hydrogel. In contrast, uptake in non-imprinted films reached a maximum in the first 20 min. Consequently the imprinting factor is initially time dependent, reaching a maximum when uptake in the imprinted film saturates.

Protein uptake in non-imprinted films increased with increasing concentration in bulk solution, consistent with weak surface or near surface binding following a Langmuir isotherm. Protein uptake increased linearly with film thickness further supporting the conclusion that uptake in non-imprinted films is predominantly a surface process. After incubation of non-imprinted films in protein solution, incubation in buffer showed fast

elution over the first 1 h, independent of the initial MBP concentration, and almost complete elution after 5 h.

The dynamics of protein elution from imprinted films was studied both after imprinting and after incubation in protein solution. Elution from imprinted films resulted in 20% of the imprinted protein remaining after 5 h. After digestion of the remaining protein, rebinding, and elution, about 3.6% of the imprinted protein remained in the film. These experiments suggest that imprinting results in sites with a distribution of binding energies, and that only a relatively small fraction represent strong binding. While protein imprinting can be used to selectively bind a target protein, many details of the imprinting process remain to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.05.079>.

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Fig. 1.

Schematic illustration of synthesis of imprinted polymer films. A glass slide (a) is modified with a 3-(trimethoxysilyl)propyl methacrylate silane (b) to improve adhesion of the polymer film. A fixed volume of precursor solution with fluorescently-labeled protein is placed on the modified glass slide (c) and a mica wafer placed on top of the droplet (d) prior to polymerization. Finally, the mica wafer is removed (e) and the protein extracted (f). The imprinted film is then incubated in protein solution (g). In control experiments precursor solution with no protein is placed on a modified glass slide (h) and a mica wafer placed on top of the droplet (i) prior to polymerization and removal of the mica wafer (j). In control experiments the non-imprinted film is incubated in protein solution (k). (l) Representative fluorescence images of an MBP-Cy3 imprinted polymer before and after incubating with MBP-Cy3. (m) Illustration of cross-section of an imprinted gel. (n) Representative fluorescence images of a non-imprinted polymer incubated with fluorescently labeled protein. (o) Illustration of cross-section of a non-imprinted gel.

Fig. 2.

Influence of cross-linker (CL) concentration and monomer concentration on the imprinting factor of MBP in AAm/NIPAm films. Imprinted and non-imprinted films were incubated in 0.50 mg mL^{-1} MBP-Cy3 solution for 5 h and rinsed in 10 mM TRIS buffer for 5 min prior to imaging. The influence of monomer concentration was obtained at cross-linker concentrations of 1, 2, and 5 mol.%: (a) 10 wt.% monomer, (b) 20 wt.% monomer, (c) 30 wt.% monomer, (d) 50 wt.% monomer. The influence of cross-linker concentration was obtained at monomer concentrations of 10, 20, 30, and 50 wt.%: (e) 1 mol.% cross-linker, (f) 2 mol.% cross-linker, (g) 5 mol.% cross-linker. (h) Selectivity of AAm/NIPAm films with 20 wt% monomer and 2 mol.% cross-linker imprinted with 0.21 mg mL^{-1} MBP and incubated in 0.50 mg mL^{-1} MBP, OVA, or BSA. The total monomer concentration includes AAm, NIPAm, and MBA cross-linker. All measurements were performed in triplicate. Error bars represent standard deviation.

Fig. 3.

Electron microscope images of imprinted (MIP) and non-imprinted (NIP) AAm/NIPAm films (20 wt.% monomer and 2 mol.% cross-linker). The MIPs were imprinted with 0.21 mg mL^{-1} MBP. (a,b) Top surface of an imprinted film. (c) Section of an imprinted film cut parallel to the surface. (d) Top surface of a non-imprinted film. (e) Section of a non-imprinted film cut parallel to the surface. (f) Section of a non-imprinted film cut perpendicular to the surface.

Fig. 4.

Pore size and wall thickness of imprinted (MIP) and non-imprinted (NIP) films. (a) Pore size for imprinted film: $13.4 \pm 6.8 \mu\text{m}$ (SD, $N = 237$). (b) Wall thickness for imprinted film: $200 \pm 60 \text{ nm}$ (SD, $N = 80$). (c) Pore size for non-imprinted film: of $6.2 \pm 1.7 \mu\text{m}$ (SD, $N = 247$). (d) Wall thickness for non-imprinted film: $240 \pm 80 \text{ nm}$ (SD, $N = 92$). The pore size and wall thickness were obtained from analysis of the top surface of SEM images. Similar results were obtained from analysis of sections cut parallel to the surface. The pore size is the diameter of the circle with the same area as the pore. Wall thicknesses were obtained from analysis of high magnification images. Each measurements represents the thickness obtained between two adjacent pores.

Fig. 5.

Influence of incubation time on protein uptake and imprinting factor. Imprinted and non-imprinted films prepared from 20 wt.% monomers and 2 mol.% cross-linker were incubated in 0.50 mg mL⁻¹ MBP-Cy3 solution. At each time point films were rinsed in 10 mM TRIS buffer for 5 min, imaged, and then incubated in MBP-Cy3 solution. (a) Protein uptake in imprinted and non-imprinted films over 44 h. Each experiment was performed in triplicate. Error bars represent standard deviation. (b) Protein uptake plotted versus $t^{1/2}$. From the slope we obtain a diffusion coefficient of 1.0×10^{-14} cm² s⁻¹ assuming a roughness factor of 100. (c) Imprinting factor, obtained from protein uptake in the imprinted and non-imprinted films, versus time.

Fig. 6. Protein uptake in non-imprinted films. Films prepared from 20 wt.% monomers and 2 mol.% cross-linker were incubated in MBP-Cy3 solution. Each experiment was performed in triplicate. Error bars represent standard deviation. (a) Protein uptake versus time for different concentrations of MBP-Cy3. At each time point films were rinsed in 10 mM TRIS buffer for 5 min, imaged, and then replaced in MBP-Cy3 solution. (b) Steady-state protein uptake after 6 h versus concentration in solution. The solid line shows a fit to a Langmuir isotherm with protein uptake, $c_p = 0.024 (c_0 / (c_0 + 1/b))$ where c_0 is the protein concentration in solution and $1/b = 0.13 \text{ mg mL}^{-1}$. (c) Protein elution in non-imprinted films. After

incubation in MBP-Cy3 solution, films were immersed in 10 mM TRIS buffer. At each time point films were rinsed in 10 mM TRIS buffer for 5 min, imaged, and then returned to the MBP-Cy3 solution. (d) Protein uptake in non-imprinted films of different thickness incubated in 0.50 mg mL⁻¹ MBP-Cy3 solution for 5 h.

Fig. 7.

Protein uptake and elution in imprinted films. (a) AAm/NIPAm films (20 wt.% monomer and 2 mol.% cross-linker) imprinted with 0.21 mg mL⁻¹ MBP were incubated in 10 mM TRIS buffer for 58 h. At each time point films were rinsed for 5 min, imaged, and then incubated in clean buffer. Each experiment was performed in triplicate. Error bars represent standard deviation. (b) After protein elution, films were incubated in proteinase K for 1000 min resulting in almost complete removal of the remaining protein. (c) After protein digestion, films were incubated in 0.50 mg mL⁻¹ MBP solution for 5 h. Error bars represent standard deviation. (d) Following protein rebinding, the films were incubated in 10 mM TRIS buffer to measure protein elution. Error bars represent standard deviation.

Fig. 8.

Influence of imprinted protein concentration on rebinding. AAm/NIPAm films (20 wt.% monomer and 2 mol.% cross-linker) were imprinted with 0.0083, 0.045, and 0.21 mg mL⁻¹ MBP. After protein removal by digestion in proteinase K, films were incubated in 0.50 mg mL⁻¹ MBP for 5 h. Protein uptake was determined from fluorescence images after rinsing in 10 mM TRIS buffer for 5 min. Each experiment was performed in triplicate. Error bars represent standard deviation. The dotted line has a slope of 1.0.

Fig. 9.

Proposed model for protein binding. (a) On incubating imprinted films in buffer for extended times, non-specifically bound and weakly bound protein is eluted. Digestion removes remaining strongly bound protein. On incubation with protein solution, some strongly bound sites and weakly bound sites are reoccupied, along with other non-specifically and weakly bound protein. Some of the strongly bound sites may be inaccessible to protein on rebinding. Washing removes a large amount of the non-specifically and weakly bound protein. Extended washing removes most of the remaining weakly bound protein. (b) On exposing non-imprinted films to protein solution non-specifically bound and weakly bound protein taken up. After washing, most of the non-specifically bound protein is removed leaving some weakly bound protein in the film. Most weakly bound protein is removed on extended washing. (c) The distribution of binding energies for non-imprinted films is dominated by non-specifically bound and weakly bound protein. The distribution of binding energies for imprinted films includes non-specifically bound, weakly bound, and strongly bound protein.