Biomaterials 35 (2014) 8659–8668

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Protein imprinting in polyacrylamide-based gels

Maya Zayats [a], Andrew J. Brenner [a], Peter C. Searson [a, b, *]

[a] Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
[b] Institute for Nanobiotechnology, Johns Hopkins University, Baltimore, MD 21218, USA

ARTICLE INFO

Article history:
Received 19 March 2014
Accepted 28 May 2014
Available online 14 July 2014

Keywords:
Protein imprinting
Polyacrylamide
Hydrogel
Maltose binding protein

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.

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*-methylenebisacrlamide (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

* Corresponding author. Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA.
E-mail address: searson@jhu.edu (P.C. Searson).

http://dx.doi.org/10.1016/j.biomaterials.2014.05.079
0142-9612/

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 $CaCl_2$) 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 510–560 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 cm^2) 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 μL of precursor solution. To initiate polymerization, 50 μL TEMED (6.5% v/v, aqueous solution) was added to 500 μL 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