Tailored Binding and Transport Parameters of Molecularly Imprinted Films via Macromolecular Structure: The Rational Design of Recognitive Polymers

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ABSTRACT: Testosterone recognitive networks were synthesized varying feed crosslinking percentage and length of bifunctional crosslinking agent to analyze the effect of manipulating structural parameters on template binding parameters such as affinity, capacity, selectivity, and transport. Crosslinking agent was varied from 50 to 90% and associated networks experienced a twofold increase in capacity and a fourfold increase in affinity, with equilibrium association constants ranging from $0.30 \pm 0.02 \times 10^4\ M^{-1}$ to $1.3 \pm 0.1 \times 10^4\ M^{-1}$, respectively. The higher concentration of crosslinking monomer increased crosslinking points available for interchain stabilization creating an increased number of stable cavities for template association. However, by increasing the length of crosslinking agent $\approx$ 4.5 times, the mesh size of the network increased resulting in 40% faster template diffusional transport. A 77% crosslinked poly(MAA-*co*-PEG200DMA) recognitive network had an association constant of $(0.20 \pm 0.05) \times 10^4\ M^{-1}$ and bound $(0.72 \pm 0.04) \times 10^{-2}$ mmol testosterone/g dry polymer which was less by six and threefold, respectively, compared to a similarly crosslinked poly(MAA-*co*-EGDMA) recognitive network. Structural manipulation of the macromolecular architecture illustrates the programmability of recognitive networks for specific binding parameters and diffusional transport, which may lead to enhanced imprinted sensor materials and successful integration onto sensor platforms. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 3435–3441, 2008

Key words: molecular imprinting; recognitive polymer; microsensor; micropatterning; microdevice

INTRODUCTION

Recognitive networks are synthetic polymeric materials that are rationally designed to recognize or bind target molecules. Noncovalent molecular imprinting can create highly selective recognitive networks by promoting and stabilizing interactions between the chemical functionality of chain building monomers and the functionality of the template or ''guest'' molecules. Noncovalent interactions, such as hydrogen bonding, Van der Waals forces, hydrophobic forces, and ionic interactions, ensure the formation of site-specific cavities between monomer(s) and template molecules. The concept of macromolecular recognition manifests itself from two major synergistic effects: (i) shape specific cavities that match the template molecule, which provide stabilization of the chemistry in a crosslinked matrix; and (ii) chemical groups oriented to form multiple complexation points with the template. A wide variety of molecules have been noncovalently imprinted such as peptides,[1–3] hormones,[4,5] antibodies,[6–9] nucleotides,[10–12] and polysaccharides.[13] The authors point the reader to excellent reviews of the field,[14–16] which include noncovalent and covalent molecular imprinting strategies. Of particular importance, imprinted polymeric networks demonstrate structural integrity and stability in a wide range of pH and temperatures[17] making them excellent tools in the fields of separation,[18–21] drug delivery,[14,22–25] catalysis,[26,27] and sensor materials.[28–41]

The fabrication of selective, inexpensive, robust biosensors has become a topic of major interest in the past decade. Our laboratory is interested in the creation of thin film recognitive structures for use in point-of-care (POC) diagnostic sensors that can function away from the hospital bedside or clinical laboratory and have the potential to drastically decrease analysis time and improve medicinal therapy. The design and fabrication of novel POC sensors will benefit significantly from the development of synthetic recognitive networks as well as the optimization and understanding of the macromolecular structure in relation to enhanced binding properties. Recognitive films are expected to increase sensor shelf life, facilitate sensor use in a variety of environ-

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ments, and decrease sensor production cost compared to biological-containing sensing elements.

Molecularly imprinted networks have been reported with binding affinities that are not much lower than biological systems. For example, theophylline,[41] morphine,[42] cholesterol,[43] and testosterone[5] recognitive networks have been designed with dissociation constants equal to $(8.1 \pm 0.9) \times 10^{-9}$ M, $(1.2 \pm 0.2) \times 10^{-6}$ M, $(5.9 \pm 1.3) \times 10^{-4}$ M, and 0.9×10^{-4} M, respectively. These values are comparable to dissociation constants found in nature. For example, carbohydrate–protein[14,44] dissociation constants range from 10^{-3} to 10^{-6} M and antigen-antibody[45] dissociation constants range from 10^{-8} to 10^{-10} M.

Researchers have recently utilized imprinted networks on sensor platforms such as quartz crystal microbalance (QCM)[31–40] and surface plasmon resonance (SPR).[28,41] When the target molecule binds to the recognitive network, there is an increase in the acoustic frequency or an increase in the refractive index, allowing for a quick, real-time, label-free analysis. However, the use of imprinted films has had a number of limitations.[46] One limitation is a slow equilibrium response time that is due to inadequate polymer grafting techniques, which produce thick films, and lack of imprinted network structure characterization and optimization (Fig. 1). Recently, controlled/living polymerization techniques were used to synthesize a ethyladenine-9-acetate imprinted poly (MAA-*co*-EGDMA) network with enhanced binding capacity and selectivity while maintaining a similar binding affinity for the target molecule.[47]

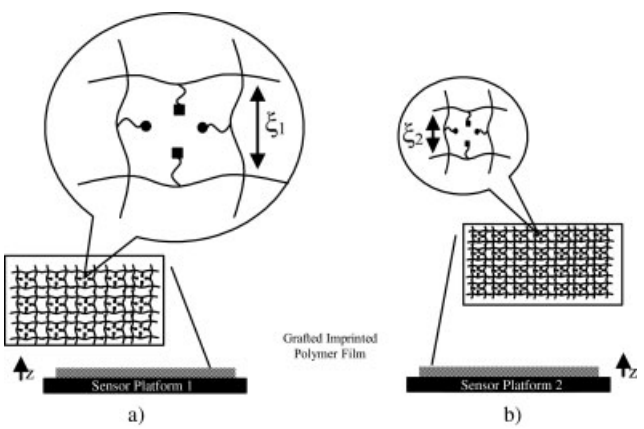

Figure 1 Schematic of recognitive networks grafted onto a sensor surface. (a) Less crosslinked networks or increased molecular size of crosslinking monomers will decrease penetration time of template in the z direction because of the more open network. Depending on the size, shape, and configuration of the molecules in the sample, a less selective, more open network can be used for a quicker response time. (b) Highly crosslinked network has a slower template penetration time in the z direction because of the tight macrmolecular architecture. The mesh size is represented by ξ with $\xi_1 > \xi_2$. The thickness of the film in the z direction is also a major parameter in sensor design.

Promising studies have been conducted grafting exact patterns of recognitive networks onto silicone surfaces using photolithography methods,[48,49] but the thickness of the film yielded large response times. Theoretical calculations[49] predict that recognitive networks less than 1 μm thick will give a response time of seconds. However, even considering a thin film, the imprinted network structure is very important and should be optimized to allow sufficient diffusional transport of template. For example, the film thickness of a bilirubin imprinted polymer-QCM system[31] was approximated, by frequency shift, to be 150 nm thick. Despite this thin film, the sensor had an equilibrium response and regeneration time of 41 min, which is an inadequate response for a diagnostic sensor. This was due to the slow diffusion of the template molecule through the highly crosslinked network. While solvent is typically used in imprinted polymer formulations to create porous networks and increase the transport of template, various grafting techniques limit the use of solvent.

To facilitate imprinted polymer integration within sensor platforms and optimize the response times, imprinted macromolecular structural parameters need to be studied to determine relationships between network architecture and template binding affinity, capacity, selectivity, and diffusional transport. Imprinted networks typically demonstrate increased affinity and selectivity in a highly crosslinked structure which significantly limits template diffusion. Thus, this article focuses on the rational design and characterization of recognitive polymeric networks altering parameters such as the degree of network crosslinking and the size of the crosslinking monomer (i.e., feed or initial formulation crosslinking percentage and the length of the crosslinking monomer) to alter network architecture. We hypothesize by tailoring the network structure, favorable and selective template binding can occur without significantly limiting the diffusion of the template molecule. In effect, imprinted networks can have programmable binding and diffusion parameters by rational design of the network that can integrate quite well in a number of sensing schemes. It is also important to note that depending on the diversity of molecules within a given sample environment and how similar in size and chemical functionality other molecules are to the template molecule, a highly-crosslinked, transport-restrictive, highly-selective network may not be needed to achieve adequate recognition.

EXPERIMENTAL

Materials and methods

In this work, we synthesized methacrylate copolymer networks imprinted for testosterone using ethyl-

ene glycol dimethacrylate (EGDMA) and poly(ethylene glycol)200dimethacrylate (PEG200DMA) as crosslinking agents, which have approximately 1 and 4.5 ethylene glycol units, respectively. Methacrylic acid (MAA), testosterone (template molecule), chloroform, azobisisobutyronitrile (AIBN), and EGDMA were purchased from Sigma Aldrich (Milwaukee, WI). PEG200DMA was purchased from Polysciences (Warrington, PA). All chemicals were analytical grade and used as received except for MAA which had inhibitor removed by vacuum distillation prior to use.

An 8 : 1 functional monomer to template molar ratio was used to produce testosterone recognitive networks with MAA as the functional monomer, testosterone as the template molecule dissolved in chloroform, AIBN as the initiator, and EGDMA or PEG200DMA as the crosslinking monomer. Control polymers were produced by the same method as the imprinted polymers but without testosterone. Monomer solutions were prepared with the molar ratio of initiator to double bonds held constant at 0.015 and with the weight percentage of the solvent constant at 50% (w/v). The crosslinking percentage, defined as the moles of crosslinking monomer divided by the total moles of all monomers including crosslinking monomer, was varied from 50, 77, and 90% to study the influence of the macromolecular structure on the affinity, capacity, selectivity, and diffusional transport of the template.

Recognitive network synthesis

In a typical experiment, functional monomer, template molecule, crosslinking monomer, and solvent were added to a glass vial and sonicated for 30 min to ensure the solution was well mixed. For example, a solution to form a 50% crosslinked poly(MAA-*co*-EGDMA) recognitive network consisted of EGDMA (9.40 mmol), MAA (9.40 mmol), testosterone (1.20 mmol), AIBN (0.41 mmol), and chloroform (25.0 mmol). To eliminate dissolved oxygen, which is a free-radical scavenger, nitrogen was bubbled through the solution for 30 min. The monomer solution was transferred to an aluminum mold that maintained a nitrogen atmosphere with a cooling system that held the temperature constant at 0°C. A Novacure 2100 (Exfo, Ontario, Canada) mercury spot cure lamp was used as the light source with intensity of 50 mW/cm^2, as determined via radiometer, for a duration of 17 min. After synthesis, the polymer was crushed using a mortar and pestle and then separated into particles of sizes ranging between 53 and 150 μm using sieves. The polymer particles were washed using a Soxhlet apparatus with ethanol for 1 week or until testosterone and unreacted monomers were not detected in the effluent via spectrophotometric monitoring (Biotek Synergy, Winooski, VT). The particles were then dried in a vacuum oven (T = 30°C at 27 mmHg) for 24 h in preparation for the template rebinding studies.

Polymer recognition studies: Template affinity, capacity, and selectivity

A standard curve was constructed with testosterone in chloroform and indicated a linear absorbance versus concentration region until 0.18 mM and a concentration-independent region after 0.18 mM where the absorbance did not change with increasing concentration of testosterone. Therefore, a 0.025 mM to 0.0167 mM concentration range was used in all experiments. Rebinding studies were performed in triplicate by introducing 5 mg of recognitive or control polymer particles into 2.5 mL of varying concentrations of testosterone in chloroform for a predetermined equilibrium time of 24 h. Dynamic binding studies were conducted on separate samples measuring the supernatant at various time points. Final equilibrium values of testosterone concentration were obtained by spectrophotometric analysis at a wavelength of 238 nm (i.e., the wavelength of maximum absorbance of testosterone). A mass balance was used to determine the bound testosterone within the polymeric network.

The Freundlich isotherm was used to assess binding affinity since the Freundlich isotherm gave the best fit to the data compared to either a Langmuir isotherm or Scatchard plot. The Freundlich isotherm is represented by eq. (1) below,

$$q = AC^n \tag{1}$$

where A and n are constants relating to the number of binding sites and association constant. The constant n provides a measure of binding heterogeneity, which decreases as n increases, with one representing a linear isotherm ($0 < n < 1$).

The equilibrium, weighted-average affinity or association constant (K_{avg}) was calculated using eq. (2),

$$K_{avg} = \left(\frac{n}{n-1}\right) \cdot \left[\frac{K_1^{1-n} - K_2^{1-n}}{K_1^{-n} - K_2^{-n}}\right] \tag{2}$$

where K_1 and K_2 depict the affinity range which is determined to be between K_{min} and K_{max} which are calculated by the reciprocal of C_{max} and C_{min}, respectively.[50,51] To properly compare different polymers, K_1 and K_2 were the same for all polymers analyzed. When weighted-average affinity values are high, the molecules, on average, bind tightly to the receptor. Conversely, low weighted-average affinity values are

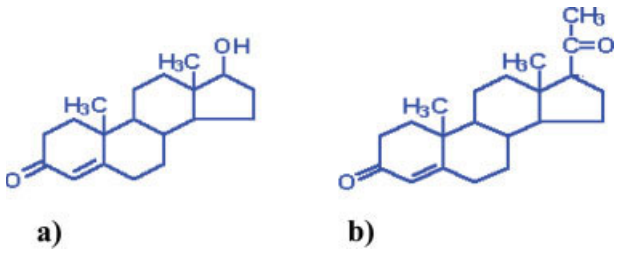

Figure 2 Structures of template and template analogue (a) testosterone and (b) progesterone. Progesterone differs in the chemistry of the carbonyl group located at the C17 location. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

indicative of weak binding systems. The reciprocal of the equilibrium association constant is the dissociation constant.

Selectivity studies followed a similar procedure as the template binding studies except that progesterone, dissolved in chloroform (wavelength of max. absorbance equal to 240 nm) was used as the rebinding solution. Progesterone was chosen because of the similar structure of progesterone compared to testosterone, where progesterone differs only by the carbonyl group located at the C17 chemistry (Fig. 2).

The selectivity, α, for the testosterone recognitive network was determined by eq. (3),

$$\alpha = \frac{K_{\text{avg, template}}}{K_{\text{avg, other molecule}}} \tag{3}$$

where, K_{avg} is the equilibrium, weighted-average affinity or association constant determined by Freundlich analysis.

Template transport studies and calculation of diffusion coefficients

Disks of each polymer network were made under nitrogen as previously described within an aluminum mold that held the temperature constant at 0°C. The discs had a diameter of 28 mm and thicknesses ranged from 0.8 to 1.0 mm. The disks were washed in a modified Soxhlet extraction device to ensure the disks were immersed in solvent at all times. After the washing procedure, the disks were placed in 0.18 m*M* of testosterone in chloroform and allowed to reach equilibrium. Release studies were performed using 50 mL polypropylene vials with 10 mL of chloroform. To ensure an infinite sink for the release studies, the fluid was changed every 8 h for the first 36 h and thereafter every 24 h until equilibrium. At every fluid change, a 200 μL aliquot of the solution was taken and the testosterone concentration was measured at 238 nm.

The diffusion coefficient was calculated from Fick's law, which describes one-dimensional planar solute release from gels.[52] For geometries with aspect ratios (exposed surface length/thickness) greater than 10, edge effects can be ignored and the problem approached as a one-dimensional process.[52]

Solution of Fick's law for short times of diffusion is given by eq. (4),

$$\frac{M_t}{M_\infty} = 4\left[\frac{Dt}{\pi L^2}\right]^{\frac{1}{2}} \tag{4}$$

where M_t is the mass of testosterone released at time t, M_∞ is the mass of testosterone released at time equal to infinity, D is the diffusion coefficient independent of position and concentration, and L is the thickness of the disk.[52] For each polymer network, the fractional release of testosterone (M_t/M_∞) versus ($t^{0.5}/L$) was plotted and the diffusion coefficient was calculated from the slope.

RESULTS AND DISCUSSION

Polymer recognition studies: Template affinity and capacity

Rebinding studies were conducted on testosterone recognitive networks to determine the effect of varying structural parameters, such as the crosslinking agent type and the feed crosslinking percentage, on the binding affinity and capacity. Dynamic binding studies indicated equilibrium was reached for all particles by 24 h. Figure 3 highlights the differences

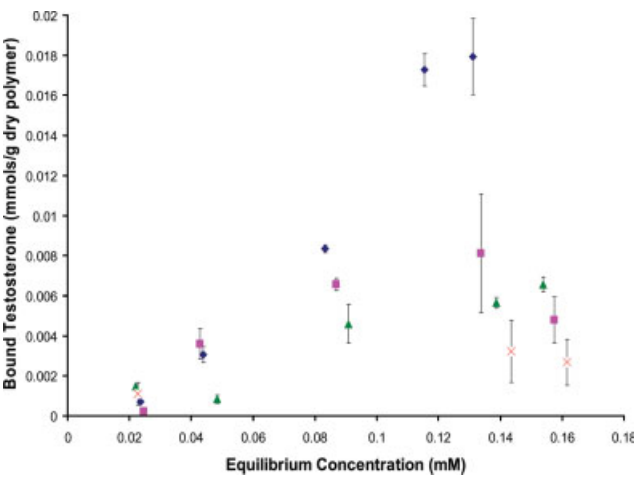

Figure 3 Testosterone equilibrium binding isotherms of poly(MAA-*co*-EGDMA) and poly(MAA-*co*-PEG200DMA) networks at 77% feed crosslinking. Poly(MAA-*co*-EGDMA) recognitive network (◆) and control network (■). Poly-(MAA-*co*-PEG200DMA) recognitive network (▲) and control network (×). Error bars represent standard deviation with (n = 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

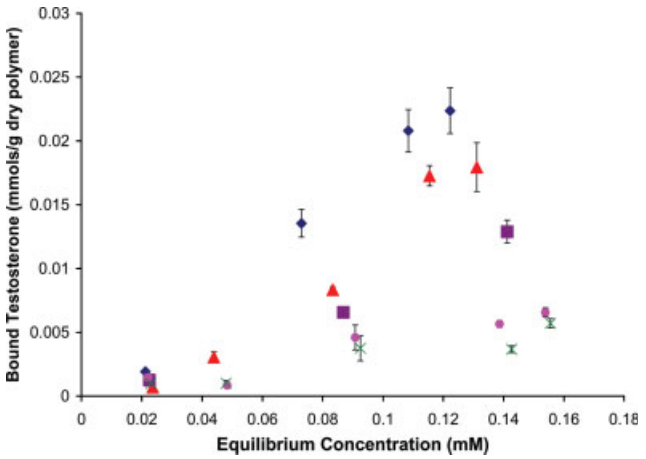

Figure 4 Testosterone equilibrium binding isotherms of poly(MAA-*co*-EGDMA) and poly(MAA-*co*-PEG200DMA) recognitive networks at various feed crosslinker percentages. Poly(MAA-*co*-EGDMA) networks: 90% (◆), 77% (▲), and 50% (■). Poly(MAA-*co*-PEG200DMA) networks: 77% (●) and 50% (×). A higher crosslinking percentage increases the binding capacity and affinity of the network. An increase in length of the crosslinking monomer lowers the binding capacity and affinity of the network. Error bars represent standard deviation with (n = 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in binding capacity for 77% crosslinked poly-(MAA-*co*-EGDMA) networks and poly(MAA-*co*-PEG200DMA) networks. The poly(MAA-*co*-EGDMA) recognitive network bound significantly more testosterone, $(1.8 \pm 0.2) \times 10^{-2}$ mmol/g dry polymer, compared to the corresponding control network, which bound $(0.5 \pm 0.2) \times 10^{-2}$ mmol/g dry polymer. As mentioned previously, the control network was synthesized from the same formulation without the template molecule. This demonstrates the imprinting effect with enhanced capacity since there was a fourfold increase in recognitive network testosterone binding compared to control networks. At the same crosslinking percentage but longer crosslinking chains, the poly(MAA-*co*-PEG200DMA) recognitive networks bound $(0.72 \pm 0.04) \times 10^{-2}$ mmol/g dry polymer of testosterone, which was threefold more than its corresponding control and threefold less than the 77% crosslinked recognitive poly(MAA-*co*-EGDMA) network. By increasing the crosslinking monomer linear size, which affected the distance between crosslinked chains, the mobility of the network increased as well as the spacing between the functional chemistry, creating less stable cavities for the binding of the target molecule.

Figure 4 highlights the variation of the feed crosslinking percentage of the poly(MAA-*co*-EGDMA) network, from 50–90%, to evaluate the effect on the binding parameters. The highest crosslinked network, 90% poly(MAA-*co*-EGDMA), bound two times more testosterone $((0.22 \pm 0.03) \times 10^{-1}$ mmol/g dry polymer) compared to the least crosslinkined network, 50% poly(MAA-*co*-EGDMA) $((0.13 \pm 0.03) \times 10^{-1}$ mmol/g dry polymer). Increasing the crosslinking percentage of the network increases the amount of crosslinking points available for interchain stabilization, creating not only more cavities for binding but more stable sites for the template-macromolecular receptor interactions. The trend of decreasing binding capacity with a lower concentration of crosslinking monomer is also evident with a longer bifunctional crosslinking monomer. The 77% crosslinked poly(MAA-*co*-PEG200DMA) recognitive network bound $(0.72 \pm 0.04) \times 10^{-2}$ mmol testosterone/g dry polymer while the 50% crosslinked poly-(MAA-*co*-PEG200DMA) recognitive network bound $(0.51 \pm 0.03) \times 10^{-2}$ mmol testosterone/g dry polymer, demonstrating that polymeric networks can be rationally tailored to specific binding capacities.

Figure 5 demonstrates that recognitive networks can have programmable binding affinity. As shown in Figure 4, testosterone binding affinity increases as the feed crosslinking percentage increases. A higher feed concentration of the bifunctional monomer supports the formation of more stable cavities for template association. For example, the association constant for the 90% crosslinked poly(MAA-*co*-EGDMA) recognitive network was equal to $(1.3 \pm 0.1) \times 10^4\ M^{-1}$ and decreased approximately fourfold to $(0.30 \pm 0.02) \times 10^4\ M^{-1}$ for the 50% crosslinked network. Also, the association constant for the 77% crosslinking poly-(MAA-*co*-EGDMA) recognitive network (K_a = $(1.20 \pm 0.07) \times 10^4\ M^{-1}$) is in experimental agreement with a

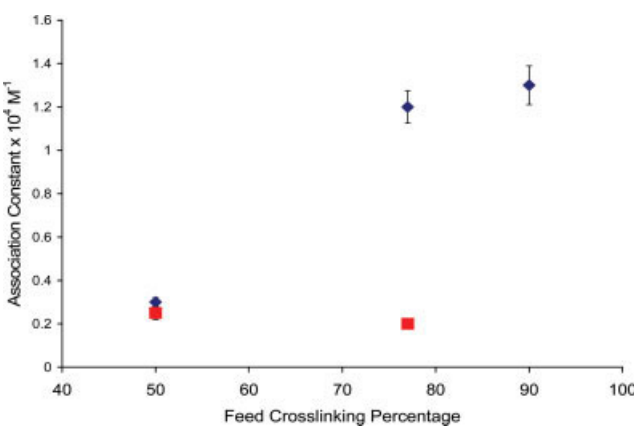

Figure 5 Testosterone binding affinity constants for poly(MAA-*co*-EGDMA) and poly(MAA-*co*-PEG200DMA) networks at various feed crosslinking percentages. Poly-(MAA-*co*-EGDMA) networks, (◆), and poly(MAA-*co*-PEG200DMA) networks, (■). The higher the feed crosslinking percentage, the higher the association constant due to the increase stability of binding sites. Error bars represent standard deviation with (n = 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

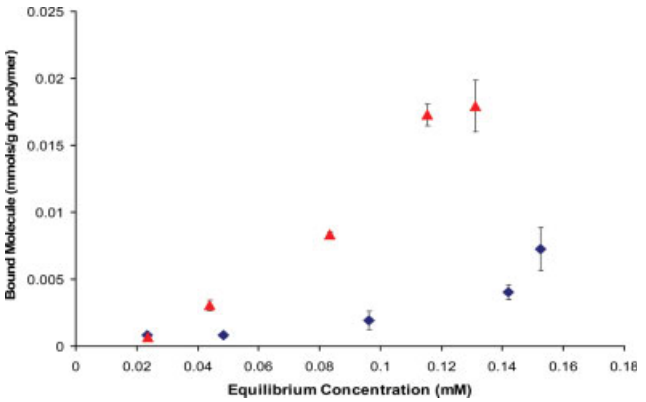

Figure 6 Selectivity study of poly(MAA-*co*-EGDMA) recognitive networks at 77% crosslinking. For the recognitive network in testosterone solution, (▲) and the recognitive network in progesterone solution, (■). The testosterone recognitive network binds more testosterone than progesterone illustrating that the imprinted network is more selective and has a higher affinity for testosterone. Error bars represent standard deviation with (n = 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

literature value[5] (K_a = 1.10 × 10^4 M^{-1}). Comparing the 77% crosslinked polymers, the poly(MAA-*co*-PEG200DMA) recognitive network had an association constant of (0.20 ± 0.05) × 10^4 M^{-1} which was a sixfold decrease compared to the poly(MAA-*co*-EGDMA) recognitive network (K_a = (1.20 ± 0.07) × 10^4 M^{-1}). When the length of the crosslinking agent increased within the polymer network, the template bound with less affinity and had less stable cavities for association.

Polymer recognition studies: Selectivity studies

Equilibrium rebinding studies were conducted using a progesterone solution to determine how selective the testosterone recognitive poly(MAA-*co*-EGDMA) network was to a molecule that was similar in size, functionality, and configuration. The 77% crosslinked poly(MAA-*co*-EGDMA) testosterone recognitive polymer demonstrated two times lower binding capacity for progesterone (0.80 ± 0.20) × 10^{-2} mmol/g dry polymer) compared to testosterone (1.8 ± 0.2) × 10^{-2} mmol/g dry polymer) (Fig. 6). Progesterone differs from testosterone only at the C17 chemistry (Fig. 2). The macromolecular memory formed during polymerization has the same configuration and complementary functionality as testosterone allowing for the poly(MAA-*co*-EGDMA) network to decipher between the molecules with higher selectivity towards testosterone. The selectivity ratio, α, for the testosterone recognitive network was equal to 1.2. The 77% crosslinked poly(MAA-*co*-PEG200DMA) testosterone recognitive polymer did not demonstrate a statistically different selectivity ratio.

Template diffusion studies

Figure 7 demonstrates that higher crosslinked networks had lower diffusion coefficients, and the diffusion coefficients increased with an increase in the crosslinking monomer size. Also, the imprinting process may have had an effect upon the diffusion of testosterone. All control gels had higher diffusion coefficients than corresponding recognitive networks, suggesting that imprinting decreased the diffusion coefficient. However, additional structural analysis along with equilibrium weight and volume swelling studies are needed to prove this conclusively. For each feed crosslinking percentage, poly(MAA-*co*-PEG200DMA) recognitive networks had approximately a 1.4-fold or 40% higher testosterone diffusion coefficient compared to poly(MAA-*co*-EGDMA) recognitive networks. It is important to note that lower crosslinking percentages for recognitive EGDMA and PEG200DMA based copolymers resulted in 17 and 12% higher diffusion coefficients, respectively.

CONCLUSIONS

This work demonstrates the rational design, synthesis, and characterization of synthetic intelligent polymeric materials that display tremendous potential for application as recognitive films in micro/nanoscale sensor applications such as POC diagnostics. By manipulating key structural parameters such as the feed concentration and length of the crosslinking agent, the macromolecular architecture can be rationally tailored to have tuned capacity, affinity, selectivity, and diffusional transport. In this work, we have shown that a highly crosslinked recognitive network, 90% crosslinked poly(MAA-*co*-EGDMA), had a twofold increase in the binding capacity and a fourfold increase in binding affinity compared to a similar

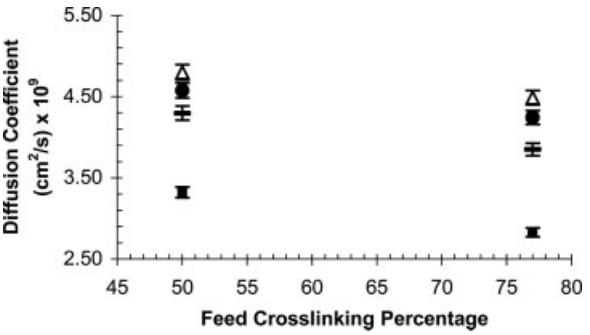

Figure 7 Diffusion coefficients for poly(MAA-*co*-EGDMA) and poly(MAA-*co*-PEG200DMA) networks at varying crosslinking percentages. Poly(MAA-*co*-PEG200DMA) control network (△), and recognitive network, (▬). Poly(MAA-*co*-EGDMA) control network (●), and recognitive network (■). Error bars represent standard deviation with (n = 3).

lower crosslinked recognitive network, 50% crosslinked poly(MAA-*co*-EGDMA). Also, by increasing the length of the crosslinking monomer within imprinted networks (i.e., comparing 77% crosslinked poly(MAA-*co*-EGDMA) to poly(MAA-*co*-PEG200DMA)), there was a threefold decrease in the amount of testosterone bound. However, the longer crosslinking agent increased the mesh size and mobility of the macromolecular network allowing for a 40% increase in the diffusional transport.

Ultimately, in any sensor design incorporating recognitive thin films, there will be trade-off involving binding parameters (e.g., affinity, selectivity, capacity) and transport considerations. A more structurally open network will have faster template permeation, allowing for a decrease in response time. Also, a more structurally open network may provide bulk template binding rather than only on the surface, which would increase binding capacity. The affinity and selectivity may be lower; however, depending on the size, shape, and configuration of molecules in the sample fluid (i.e., how difficult a sensing environment), the lower values may be a workable tradeoff for a faster response time.

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