Networks for Recognition of Biomolecules: Molecular Imprinting and Micropatterning Poly(ethylene glycol)-Containing Films†

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ABSTRACT

Engineering the molecular design of biomaterials by controlling recognition and specificity is the first step in coordinating and duplicating complex biological and physiological processes. Studies of protein binding domains reveal molecular architectures with specific chemical moieties that provide a framework for selective recognition of target biomolecules in aqueous environment. By matching functionality and positioning of chemical residues, we have been successful in designing biomimetic polymer networks that specifically bind biomolecules in aqueous environments. Our work addresses the preparation, behavior, and dynamics of the three-dimensional structure of biomimetic polymers for selective recognition via non-covalent complexation. In particular, the synthesis and characterization of recognition gels for the macromolecular recognition of α-glucose is highlighted. Novel copolymer networks containing poly(ethylene glycol) (PEG) and functional monomers such as acrylic acid, 2-hydroxyethyl methacrylate, and acrylamide were synthesized in dimethyl sulfoxide (polar, aprotic solvent) and water (polar, protic solvent) via UV-free radical polymerization. Polymers were characterized by single and competitive equilibrium and kinetic binding studies, single and competitive fluorescent and confocal microscopy studies, dynamic network swelling studies, and ATR-FTIR. Results qualitatively and quantitatively demonstrate effective glucose-binding polymers in aqueous solvent. Owing to the presence of template, the imprinting process resulted in a more macroporous structure as exhibited by dynamic swelling experiments and confocal microscopy. Polymerization kinetic studies suggest that the template molecule has more than a dilution effect on the polymerization, and the effect of the template is related strongly to the rate of propagation. In addition, PEG containing networks were micropatterned to fabricate microstructures, which would be the basis for micro-diagnostic and tissue engineering devices. Utilizing photolithography techniques, polymer micropatterns of a variety of shapes and dimensions have been created on polymer and silicon substrates using UV free-radical polymerizations with strict spatial control. Micropatterns were characterized using optical microscopy, SEM, and profilometry. The processes and analytical techniques presented are applicable to other stimuli-sensitive and recognition networks.

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for biomolecules, in which hydrogen bonding, hydrophobic, or ionic contributions will direct recognition. Further developments are expected to have direct impact on applications such as analyte controlled and modulated drug and protein delivery, drug and biological elimination, drug targeting, tissue engineering, and micro- or nano-devices. This work is supported by NSF Grant DGE-99-72770. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: free-radical photopolymerization; biomimetic; molecular recognition; molecular imprinting; micropatterning

INTRODUCTION

Contributions to the molecular design and mathematical modeling of carriers for drug delivery, biomaterials for various medical applications, and micro- and nano-devices for the medical and pharmaceutical industries are leading to major new solutions of significant medical problems [1]. No longer is the treatment of diabetes, osteoporosis, asthma, cardiac problems, cancer and other diseases based only on conventional formulations. Many of the methodological advances in biomedicale sciences are the result of molecular understanding.

A significant opportunity has appeared in the medical sciences over the past 25 years with the preparation of advanced medical devices, drug delivery systems, and recognition structures. These formulations do not simply recognize or release a given drug, peptide, or protein at some characteristic rate, but do so in a way that is intended by the molecular designer. For example, insulin may be delivered only when needed [2-4], calcitonin may be directed to bypass the stomach and be delivered only in the upper small intestine [5], and large-molecular-weight molecules are delivered across tissues at acceptable rates [6, 7].

These recent developments involve water-swollen, crosslinked biomedical materials as carriers for novel pharmaceutical formulations in delivery of drugs, peptides and proteins; as targeting agents for site-specific delivery; or as components for preparation of protein or enzyme conjugates. The network structure and the thermodynamic nature of the components of these networks play a key role in their diffusional behavior [8, 9], in their molecular mesh size changes (especially in environmentally responsive hydrogels) [9, 10], and in their associated molecular stability of the incorporated biomolecules or bioactive agents [11, 12].

Gels are crosslinked polymer networks swollen in liquids, which can retain their shape and fluid content. The unique properties of gels make them excellent candidates for numerous applications. Two characteristics of gels make up the basis for most of their applications: (i) their ability to control the diffusion behavior of molecules in or through them; and (ii) the ability to amplify the microscopic events occurring at the mesh chain level into macroscopic phenomena [8, 9]. For example, polymer gels may transit between the macroscopically swollen or collapsed states according to slight changes in the environment and which may break the subtle balance among the interactions exerted on the mesh chains, and induce gel phase transition. These so-called “intelligent” gels can respond to light, temperature, solution pH, magnetic field, radiation, solvent composition, electric field, stress, and the existence of specific molecules in solution, the last enabling them to recognize specific molecules [9, 10].

In the past few decades, biomaterials have evolved from non-medical, off-the-shelf materials to materials specifically designed for medical applications [13]. In the design of novel functional biomaterials, this development focuses on orchestrating complex physiological interactions with a fundamental understanding of natural principles and a thorough examination of biological processes [14-16].

Since molecular recognition is ubiquitous and essential in life processes, the design and synthesis of polymeric materials with molecular recognition ability has tremendous opportunity. Natural processes such as enzymatic reactions, ligand-receptor interactions, and the molecular mechanisms during the repilcation of genetic information through DNA/RNA enzyme, DNA/protein, and DNA/DNA interactions are all based on molecular recognition. Through these recognition processes, molecules of interest are recognized from many structurally and/or functionally similar compounds and bound to exact sites on a receptor molecule or molecules. Molecular recognition, in this sense, can be defined as the ability of a polymer to interact with a designated target molecule usually amidst a vast range of other molecules, some of which may be almost identical to the target [17].

Directed Macromolecular Recognition and Specific Ligand Complexation Mechanisms

At this stage in the evolving field of biomaterials science, major emphasis is being focused toward engineering the architectural design of biomaterials on a molecular level. By controlling recognition and specificity, the preparation of synthetic macromolecular gels with designed artificial recognition domains is soon to be the next hurdle crossed in polymer and biomaterials development. Therefore, the next generation of biomaterials will include recognition-oriented design and intelligent complexation mechanisms that originate from specific macro-molecular chemistry, itself present in the polymer matrix or surface in a controlled manner, which manipulate the surface and bulk properties of the material in reproducible and tunable ways. The interest in this technology stems from a larger interest within our laboratory in artificially duplicating complex biological and physiological processes such as biomolecule modulated drug and protein release [18], targeted drug and protein
FIGURE 1. Biomimetic approach to producing recognition networks. (A) Mimic recognition proteins and enzymes by analyzing the amino acids involved in binding a particular molecule and duplicating complexation interactions. (B) Solution mixture of biomolecule (template), functional monomer(s) (triangles and circles), crosslinking monomer, solvent, and initiator (I). (C) The prepolymerization complex is formed via covalent or non-covalent chemistry. (D) The formation of the network (imprinting process). (E) Wash step where original template is removed.

delivery (site or ligand-specific interaction with cells and tissues), biomolecule recognition and removal of undesirable biologicals, ligand-directed cooperative allosteric recognition processes, directed on-off ligand binding processes, tissue engineering devices, biosensors, and novel therapeutic and micro-sensing devices, etc. For example, surface patterning of recognition gels on silicon substrates can create micro- or nano-binding regions with areas of differing chemistry [19, 20], which would be the basis for micro- or nano-scale diagnostic, drug delivery, or tissue engineering devices [21].

The study of recognition proteins and protein binding domains reveal molecular architectures with specific chemical moieties that provide a framework for selective recognition of biomolecules in aqueous environment. Since proteins are heteropolymers of amino acids, proper matching and positioning of chemical residues can lead to artificial macromolecular structures capable of specific recognition. By analyzing binding proteins,
we have been successful in designing biomimetic (mimicking biological recognition) polymer networks that specifically bind biomolecules.

Our goal, in general, is to produce stereospecific, three-dimensional binding cavities for biologically significant molecules that function in aqueous environments. By tailoring the polymer network architecture and composition, effective recognition sites can be created in polymer gels. Our approach has been to mimic recognition proteins and enzymes by analyzing the amino acids involved in binding a particular molecule and to duplicate the complexation interactions involved (Fig. 1). The techniques that were developed to create synthetic polymers that can recognize, bind, and react with specific molecules involve polymerization in the presence of those specific molecules. The polymerization solvent is chosen to not interfere with the pre-polymerization complex formation, but to provide mutual solubility of all components. If proper complexation occurs in the pre-polymerization stage, the network formation will proceed with effective recognition domains. For example, many binding proteins contain non-covalent binding mechanisms (e.g., hydrogen bonding, hydrophobic interactions) that bind specific molecules quite well in water (polar, protic solvent) [22]. Our scientific rationale is based on the hypothesis that effectively designed recognition networks will have superior binding properties and directed recognition in aqueous environments by properly tuning the non-covalent pre-polymerization complexation interactions between the gel functionality and template biomolecule (increasing or decreasing macromolecular chain hydrophobicity [23], including strong hydrogen bond donors and acceptors [24], or including strong ionic directed recognition sites [25]).

**Producing Recognitive Networks for Biomolecules**

Producing macromolecular networks with recognition ability has been a field of research for a number of years [15, 16, 26]. Research on polymers with recognition capabilities by complexation has mainly included polymer–biomolecule immobilization (protein, enzyme, lectin bioconjugation techniques) [26–34] and molecular imprinting methods (surface or bulk template-directed polymerization) [16, 35–38]. Both have included intelligent environment-sensitive mechanisms, which have the potential to turn the active recognition site on and off, similar to induced recognition or cofactor-induced protein recognition. Hoffman and collaborators have demonstrated site-specific conjugation, in which a tethered chain or chains can modulate entrance to a given protein active site [27–29]. Also, synthetic intelligent-imprinted gels have been prepared that memorize their binding conformation and can be switched on and off by external stimuli which modify their swelling behavior [39–43]. Theoretical and experimental evidence exists that points toward the next generation of recognitive polymers which may form from synthetic oligomers and polymers that have the potential to fold in a controlled manner to create an active binding site and therefore direct recognition [44, 45].

The method of template polymerization, which had been used to increase polymerization rates by the complexion of large polymer chains, took on a broader application area when it was discovered that the use of a template during polymerization, not only changes the kinetics of polymerization, but also affects the structure and binding properties of the polymer network immensely [46]. “Template-mediated” polymerizations, which involved small molecules instead of oligomers or polymers, were performed by taking advantage of complexation between templates and monomers before and during polymerization [47, 48].

The process of molecular imprinting (template-mediated polymerization) calls for the mixing of various functional monomers and the desired template molecule. The template molecule may be the molecule that is to be extracted out of solution. In the case of a desired catalytic reaction, it might be the substrate or a closely related molecule. The mixing of the monomers and the template may or may not involve a solvent to dissolve the monomers and the template depending on the required polymer morphology, the desired porosity, and ease of template diffusion. Mixing allows the formation of interactions between the monomers and the template by either covalent bonding [49], non-covalent bonding [38, 50], or metal coordination [48, 51]. With the addition of an initiator, polymerization takes place by photo or UV polymerization. The last step involves the extraction of the template by a suitable extraction solution or solvent. As a result, sites are created that are complementary to the template in shape and function (Fig. 1). Several reviews exist describing the field of molecular imprinting [37, 38, 52].

The network structure depends upon the type of monomer chemistry (anionic, cationic, neutral, amphiphilic), the association interactions between monomers and pendant groups, the solvent, and the relative amounts of comonomers in the feed from which the structure is formed. Recognitive success, i.e., the ability to correlate high template-binding affinity and specificity, depends on the relative amount of cross-interaction between the solvent and the intended interactions. For non-covalent complexation, this translates to the strength of hydrogen bonding, hydrophobic interactions, π-π orbital interactions, ionic interactions, and van der Waals forces employed during template–monomer complex formation.

The technique has been used in imprinting drugs [53–59], steroids [49, 60–63], nucleic acids and derivatives [64, 65], amino acids [38], metals [41, 66, 67], and other molecules [68]. Imprinting of large molecules and proteins have had greater success utilizing two-dimensional surface imprinting rather than within the bulk polymer matrix [35, 36]. Although, three-dimensional imprinting of proteins within a bulk matrix has
demonstrated success by using an epitope approach [69]. Most commonly, the functional monomer of choice is methacrylic acid (MAA), although itaconic acid [55], acrylamides [24], 4-vinyl pyridine [70, 71], β-cyclodextrin [61, 72], and some specially designed monomers [64] have been used. The most commonly used crosslinking agent is ethylene glycol dimethacrylate, where the polymerization is performed by free-radical reactions. The most common initiator is 2,2′-azobis(2-methylpropionitrile) (AIBN), and the reaction is initiated either by thermal methods at 60–65°C or by photochemical homolysis by exposure to UV light at 366 nm at 4–20°C.

The major constraints with non-covalent molecular imprinting processes have been the heterogeneity of binding sites, the presence of water as a hydrogen-bonding solvent, and polymer morphology limitations to diffusion [73–76]. Owing to mutual solubility constraints between monomers and template molecules as well as the strength of the non-covalent complexation interactions between the functional chemistry and the solvent, developments have not progressed far with aqueous (polar, protic) recognition systems as compared to organic (polar and non-polar, non-protic) based solvent systems.

Polymerization in the Presence of a Template Molecule

As it first evolved, template polymerization involved the polymerization of one monomer using a previously prepared polymer as a pattern [77]. The two different types of monomers were usually oppositely charged for the purpose of binding to each other by ionic interactions. Matuszew ska-Czerwik and Polowinski [78–80] investigated the kinetics of template polymerization by analyzing the polymerization of methacrylic acid in the presence of polyethylene glycol (PEG) derivatized with different end groups, or in the presence of poly(N-vinyl-2-pyrrolidone) (PVP). In the second case, it was found that templates which have strong interactions with the monomer PVP caused a significant decrease in the termination constant leading to an increase in overall reaction rates. Rate enhancement by the template is a well-known effect caused by templates, which have high degrees of polymerization (e.g., PEG and PVP). This phenomenon has been called the “chain effect” and has been linked to a large decrease in the termination constant for many macrotemplates [81].

Through observations of different types of monomers pairs, it was found that as the strength of attraction between monomers increased, the rate of propagation was enhanced [78–81]. This phenomenon was thought to be due to the rapid alignment of the free monomers along the polymer template, and thus, an increase in the rate of propagation. When the interactions were weak, free monomers tended to form oligomers by themselves in solution, then bind as a group to the template polymer. This decreased the effect of alignment on the polymerization rate [77].

Our efforts focus on creating polymers with recognition capabilities for low-molecular-weight templates of biological importance. The interactions between these template/guest molecules and selected monomers during polymerization create networks of selective recognition. Polymerization kinetics in the presence of the template reveal mechanisms of interaction as well as provide criteria with which other template–monomer systems can be chosen experimentally [82].

Polymer Micropatterning and Microfabrication

The dimensionality of biomedical devices has been reduced into micrometer and nanometer range with the assistance of emerging microfabrication techniques [21, 83–86]. Microfabrication techniques that were originally developed for manufacturing integrated circuits have been refined over the last few decades and are now being applied to create microstructures for additional applications, such as microelectromechanical systems (MEMS), bioMEMS, microfluidic devices, lab-on-a-chip, and other microdevices. For these technologies, polymers have been the focus of research due to their versatility as structural, sensing, and/or actuating elements. In addition, the ability of polymers, such as hydrogels, to be highly biocompatible has resulted in their consideration for medical applications. Furthermore, by controlling the functional groups along the polymer chain, hydrogels can be made sensitive to the conditions of the surrounding environment, such as temperature, pH, ionic strength, concentration, etc [9, 10, 87].

Recently, several research groups have begun utilizing hydrogels for application in microscale devices. For instance, Matsuda [88] and Ito [89] have micropatterned hydrogels to create surface regions with different physicochemical properties for the control of cell adhesion and behavior. In addition, Beebe and coworkers [90, 91] micropatterned hydrogels within a microchannel to create a valve that can sense a pH change and actuate for use in microdevice applications. These examples illustrate the applicability of hydrogels within microscale biomaterials and devices.

For implementation of the polymers into MEMS and other devices containing microelectronics, it is advantageous to use silicon substrates to enable simple integration with microelectronics. On the other hand, for a variety of other applications, all-polymer devices are valuable, because relative to silicon, polymers are inexpensive, have an array of properties that can be tailored, and can be highly biocompatible. In either application, spatial control of the polymer microstructures is crucial, and micropatterning via photolithography makes this control possible. In this work, microfabrication techniques have been developed, based on UV free-radical polymerizations, facilitating the creation of polymer micropatterns on silicon and polymer substrates for incorporation into microdevices.
These techniques develop a platform enabling the fabrication of novel microdevices and micro-diagnostic devices. Our scientific rationale is based on the use of initiators (initiator-transfer agent terminator) and organosilane coupling agents to create covalent bonds between polymer-polymer and silicon-polymer substrates, respectively.

EXPERIMENTAL

Materials

Methacrylic acid (MAA), acrylic acid (AA), acrylamide (Aam), 2-hydroxyethyl methacrylate (HEMA), tetraethylthiuram disulfide (TED), γ-methacryloxypropyl trimethoxysilane (γ-MPS), 2,2-dimethoxy-2-phenyl acetophenone (DMPA), dimethylsulfoxide (DMSO), and D-glucose were purchased from Aldrich (Milwaukee, WI). Poly(ethylene glycol) 200 methacrylate (PEG200MA), polyethylene glycol 200 dimethacrylate (PEG200DMA), and PEG 600 dimethacrylate (PEG600DMA), where the average molecular weight of the ethylene glycol chain is 200 and 600 respectively, were obtained from Polysciences, Inc. (Warrington, PA). Irgacure® 184, 1-hydroxy cyclohexyl phenyl ketone, was purchased from Ciba Specialty Chemicals (Tarrytown, NY). Fluorescent D-glucose analogue, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-glucose (2-NBDG), was purchased from Molecular Probes, Inc. All chemicals were analytical grade.

Methods: D-Glucose Recognitive Network Synthesis

Generally, copolymer films of differing composition of template, crosslinking monomer, and functional monomer(s) were synthesized in an appropriate amount of solvent via UV free-radical polymerization in a nitrogen atmosphere. Specific examples of D-glucose recognize polymers will be presented, mainly three copolymers consisting of HEMA-PEG600DMA, Aam-PEG200 DMA, and AA-PEG200DMA. Monomers were selected to match corresponding glucose binding protein residues of aspartate, glutamate, asparagine, and serine. However, these techniques are applicable to other biologically significant molecules, in which hydrogen bonding, hydrophobic, or ionic contributions will direct recognition.

Our experimental procedure follows the molecular imprinting technique in that we mixed the model template compound, D-glucose, with the monomers and solvent before polymerization and allowed complexation to occur. In a typical experiment involving HEMA functional monomer, 0.4 g of vacuum-distilled HEMA, 1.9 g of PEG600DMA were allowed to complex with 0.1 g of D-glucose. This mixture was diluted with deionized water to 50 wt%. After mutual solubilities were achieved, Irgacure® 184 initiator was added in the amount of 1 wt%. In a typical experiment involving Aam or AA as functional monomer, D-glucose, Aam or AA, and 2.0 ml of DMSO were mixed together. Then PEG200DMA and 1.5 wt% Irgacure® 184 were added to the mixture. Feed monomer compositions of this type varied from 20 to 80 mole% Aam per monomer molecules (or 50 to 85 mole% AA) as well as from 4 to 15 mole% D-glucose. Control polymers were made with exactly the same composition except D-glucose was not added.

After preparation, the solution was placed in a nitrogen atmosphere and nitrogen was bubbled for 30 minutes. Polymerizations occurred between glass microscope slides (75 x 50 x 1 mm, Fisher Scientific, Pittsburgh, PA) using 0.5 mm, 0.7 mm, or 0.035 mm Teflon® spacers in a nitrogen atmosphere at a UV intensity of 10.0–15.0 mW/cm² for 15 min (UltraCure 100, EFOSS Inc., Ontario, Canada). Polymers were placed in deionized water for 24 hr, then carefully separated from the slides, and were cut into various diameter disks using a cork borer. Disks were then placed in 50 ml conical tubes and placed on a rotating mixer (25 rpm, 70 degree angle, Glas-Col, Terre Haute, IN) and resuspended within multiple 24-hr wash steps (two washes in acetic acid/deionized water (1:5 ratio); eight washes in deionized water) to remove template and excess monomer. The resulting disks were then dried in air at ambient conditions and placed in a vacuum oven (T = 26°C, 28 mm Hg vacuum) until a constant weight was obtained (less than 0.1 wt% difference). The disks were then stored in a desiccator until testing.

Methods: Recognition, Selectivity, and Dynamic Swelling Studies

In a typical binding study, a known amount of the template solution (i.e., D-glucose and D-galactose in competitive binding studies) was added to an aqueous solution containing a known amount of polymer disks within a 50 ml conical tube. Samples were placed on a rotating mixer (70 degree angle; 25 rpm) and supernatant was sampled at equilibrium. Blank solutions (i.e., no disks or particles) also were placed in the procedure to check for microbial degradation. The time for equilibrium to occur in each system was predetermined by separate kinetic binding studies, where supernatant samples were taken at different time points. Equilibrium and kinetic-binding results were quantitatively calculated by HPLC measurements of the resulting supernatant (Phenomenex RPM monosaccharide column (300 x 7.8 mm), DI water mobile phase, 0.6 ml/min flow rate, temperature 80°C, Shimadzu RID-10A refractive index detector). Competitive binding results were visualized using a fluorescent glucose analogue, 2-NBDG (Fig. 2). The analogue was added to vials containing a known amount of polymer (maximum absorption 466 nm; maximum emission 542 nm). A Nikon Labophot fluorescent microscope with a FITC filter set was used and images were acquired with an Optronics 470T CCD camera and captured using MetaMorph software from Universal Imaging. By analyzing a large amount of pixels (N = 10000)
within these images, a histogram of intensity values was obtained (Adobe Photoshop). Confocal analysis was performed using a Bio-Rad MRC 1024 Confocal Microscope with an MRC 1024 system. Images, z-sections, etc. were collected using Laser-Sharp software and image analysis was conducted using Confocal Assistant software.

The equilibrium swelling behavior of the imprinted gels was studied by weighing dry samples and placing them in a known volume of solution with and without template. The gels were weighed by removing the gels at specific periods of time and blotting with filter paper to remove excess surface solvent. The equilibrium weight ratio can be calculated as the ratio of the equilibrium weight of the swollen sample to the weight of the dry sample.

Methods: Template Concentration on Extent of Polymerization

To investigate the effect of template concentration on the extent of polymerization, non-imprinted PHEMA and PHEMA imprinted with D-glucose with a HEMA/glucose ratio of 6 (crosslinking ratio = 0.8) were prepared. The initiator was 0.75 wt% tetraethylthiuram disulfide (TED).

An FTIR cell was purged with dry nitrogen for 45 min to 1 hr until the carbon dioxide stretching vibrations at 2200 and 2300 cm⁻¹ were relatively small compared to the background noise. Before every sample, a background spectrum was collected. The monomer mixture, which was purged with dry nitrogen for 45 min, was pipetted between a glass slide and the ATR crystal separated by 0.7 mm Teflon® spacers. The ATR crystal was exposed to ultraviolet radiation of 10 mW/cm² intensity for 60 min. Spectra were recorded every 5 min.

Conversion was calculated by measuring the area under the =C=H vibration at 1323 cm⁻¹. The area under this peak at time zero was taken as zero conversion corresponding to the total number of C≡C bonds in the system.

Methods: Micropatterning on Polymer Substrates

For the initial polymer layer, a sample of monomer (PEG200DMA), 1 wt% DMPA, and 1 wt% TED were mixed and bubbled with nitrogen. Then the solution was pipetted between two glass slides separated by 1 mm Teflon® spacers. Next, the sample was irradiated with UV light in a nitrogen atmosphere for 8 min at an intensity of approximately 20 mW/cm². The polymer sample was then washed with deionized water for 4 hr to remove any unreacted monomer, followed by drying overnight in a vacuum oven. After the sample had dried, it was covered with the second monomer (50 wt% PEG200MA and 50 wt% PEG200DMA) by spin-coating or by pipetting the solution onto the polymer. A mask was then placed atop the polymer and monomer sample, ensuring contact in order to prevent oxygen from inhibiting the reaction. Next, the sample was irradiated with UV light through a collimating lens for 30 minutes. Finally, the exposed sample was washed with ethanol to remove the remaining unreacted monomer. A schematic of the micropatterning procedure with inverter is included as Fig. 3.

Methods: Micropatterning on Silicon

Silicon wafers were cleaved into square pieces (approximately 2 cm by 2 cm) and were cleaned utilizing a standard industry piranha wafer clean. The wafers were first soaked in deionized distilled water for 2 min to remove dust particles. Next, the pieces were soaked in Piranha solution (H₂O₂:H₂SO₄, 1:1) for 15 minutes to remove any
organic residues. This step also created a thin oxide film on the surface, which was utilized in the surface modification steps. Lastly, the pieces were soaked again in deionized distilled water.

To achieve adhesion between the silicon surface and the polymer, an organosilane coupling agent was utilized. The silicon pieces were soaked in a 10 wt% solution of γ-MPS in acetone for more than 2 hr. Then, these were rinsed in acetone and ethanol. The organosilane coupling agent formed a self-assembled monolayer on the silicon dioxide surface and presented methacrylate pendant groups that reacted and bonded with the polymer film.

The monomer mixtures were made with a mole ratio of 80:20 MAA:PEG2000DMA and contained between 1 and 10 wt% DMPA. The monomer mixture was either spray-coated or spin-coated onto the silicon pieces. Next, polymer micropatterns were created by UV free-radical polymerization using a Karl Suss MJBS mask aligner. After bringing the sample into contact with the mask, the sample was exposed to UV light with intensity of 23.0 mW/cm² for exposure times of 1 to 20 minutes. The pieces were then removed and allowed to soak in deionized distilled water for greater than 24 hr to remove any unreacted monomer. A schematic of the organosilane treatment and procedure for micropatterning on silicon is included as Fig. 4.

The patterned polymer final samples were examined several ways. Microscopic photographs were taken at 200×, 400× and 1000× magnification. A profilometer (alpha-step 200, Tencor Instruments, San Jose, CA) was used to determine the

FIGURE 3. Micropatterned block copolymer. (A) Synthesis of block copolymers in the presence of initiator. (B) Schematic of the micropatterning process when utilizing an initiator.
height and profile of the pattern. Also, a JSM 35 CF scanning electron microscope was used to examine the micropatterns.

RESULTS AND DISCUSSION

Biomimetic Recognitive Networks for d-Glucose

We have been successful in synthesizing novel glucose-binding gels based on non-covalent interactions (hydrogen bonding, hydrophobic interactions) formed via molecular imprinting techniques within polar media (both protic (water) and aprotic solvent (DMSO)). Our results qualitatively and quantitatively demonstrate effective glucose-binding polymers in aqueous solvent. Glucose has a molecular weight of 180 g/mol, and in solution glucose mutarotates between two solution conformations: alpha (30–35%) and beta (65–70%) position of the carbon-1-hydroxyl group at equilibrium.
Recognizable networks were characterized by single and competitive equilibrium and kinetic binding studies, single and competitive fluorescent and confocal microscopy studies, dynamic network swelling studies, ATR-FTIR, and other methods.

Figure 5 shows that HEMA networks cross-linked with PEG600DMA and imprinted with d-glucose have 30% more uptake of glucose than non-imprinted controls. The difference between recognizable and non-imprinted polymer binding values increased as the crosslinking ratio (mole crosslinking monomer/mole total monomers) increased from a value of 0.045 to 0.80. These results suggest that the use of PEGDMA instead of EGDMA can be beneficial to the imprinted networks, which are not as rigid and still demonstrate memorized recognition toward template molecules. The network formed with longer crosslinking agent will be more flexible, but will have better diffusional characteristics. The results also demonstrate the func-

![Figure 5](image-url)  
**FIGURE 5.** The effect of increasing the crosslinking ratio on uptake of d-glucose in water. Non-imprinted (■) and imprinted (◆) poly(2-hydroxyethyl methacrylate) (PHMA) polymers crosslinked with PEG600DMA.

![Figure 6](image-url)  
**FIGURE 6.** Kinetic d-glucose binding study in water. Acrylamide–PEG200DMA copolymers of 67% crosslinking ratio prepared in DMSO (T = 24°C).
TABLE 1. Competitive $\alpha$-Glucose Substrate Binding in Water: Acrylamide-PEG200DMA Copolymers with 67% Crosslinking Ratio (Mole/Mole Monomers). Polymer Prepared in DMSO

<table>
<thead>
<tr>
<th>Competitive substrate</th>
<th>Imprinted intensity</th>
<th>Non-imprinted intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent analogue only (concentration = $F_0$)</td>
<td>223 ± 11.81</td>
<td>50.97 ± 0.77</td>
</tr>
<tr>
<td>Fluorescent analogue and glucose (100 × $F_0$)</td>
<td>99.44 ± 4.36</td>
<td>80.77 ± 3.95</td>
</tr>
<tr>
<td>Fluorescent analogue and glucose (3000 × $F_0$)</td>
<td>49.81 ± 1.09</td>
<td>48.85 ± 0.88</td>
</tr>
<tr>
<td>Fluorescent analogue and galactose (100 × $F_0$)</td>
<td>220 ± 10.10</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fluorescent analogue and galactose (3000 × $F_0$)</td>
<td>209.06 ± 10.46</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fluorescent analogue only (PEG only network)</td>
<td>n.a.</td>
<td>56.57 ± 0.90</td>
</tr>
</tbody>
</table>

For AA and Aam functional networks, a bound ratio (amount $\alpha$-glucose bound recognitiowork/control network) greater than unity indicates that glucose was imprinted within the gel compared to a randomly polymerized network (bound ratios in water were 3.4 and 5.0 for Aam-PEG200DMA and AA-PEG200DMA networks, respectively). It is important to note that the control polymer will bind some amount of template (i.e., will contain some randomly introduced, properly positioned functional groups). For AA and Aam networks, the choice of DMSO as solvent during polymerization increased the bound ratio compared to aqueous solvents during polymerization. Since DMSO is a polar, aprotic solvent it does not have the ability to be a hydrogen bond donor. Therefore, it does not interfere with the formation of the functional monomer-template complex.

For improved analysis of fluorescent intensities, thin polymer disks (diameter, 5.5 mm; thickness, 35 μm) were prepared. By analyzing fluorescent intensity values from polymer disks of equal thickness, a histogram of intensity values was obtained, which provided quantitative analysis of binding (Table 1). This data contains fluorescent profiles from competitive binding analysis of Aam-PEG200DMA copolymers with varying amounts of $\alpha$-glucose added to a given amount of fluorescent analogue (FITC filter cube, 1/8 integration time, 4× objective). These results were analyzed carefully by matching as many parameters as possible during analysis (excitation time, objective and field of view, camera integration time, etc.). The results demonstrate the effectiveness of fluorescent tracing in relation to a competitive analogue molecule. By comparing imprinted intensity from each polymer, a comparison can be made on competitive binding behavior. As glucose concentration is increased and fluorescent analogue is held at constant concentration, the fluorescent intensity of the gel decreased. Thus, glucose competed and filled binding sites.
once occupied by the fluorescent analogue. The highest glucose ratio drops the fluorescent intensity to level consistent with non-imprinted polymer. A gel consisting of PEG200DMA only (i.e., no functionality in feed) shows that there is a low level of interaction between the PEG network and the fluorescent analogue. Competitive binding studies between d-glucose and 2-NBDG yielded conclusive results that d-glucose is bound to this polymer system and not the additional fluorophore present in 2-NBDG. AA functionalized networks exhibited similar behavior.

The selectivity of the Aam-PEG200DMA polymers was determined by varying concentrations of d-galactose added to a constant concentration of d-glucose fluorescent analogue (Table 1). The results suggest polymers selective to d-glucose since the fluorescent intensity remained approximately un-

![Figure 8](image-url)

**FIGURE 8.** Dynamic network swelling study in water. Acrylamide–PEG200DMA copolymers with 67% crosslinking ratio prepared in DMSO. Owing to the presence of template, the imprinting process resulted in a more macroporous structure. Swelling studies (without template present) indicated that the absorption of water occurs via non-Fickian diffusion at a faster rate with a higher equilibrium value corresponding to imprinted networks \((T = 24^\circ\text{C})\).

![Figure 9](image-url)

**FIGURE 9.** Polymer morphology via confocal microscopy transmission middle-sections. Acrylamide–PEG200DMA copolymers with 67% crosslinking ratio prepared in DMSO. (A) Control network. (B) Recognitive network. Gross differences in macroporous structure are evident from confocal microscopy analysis of a middle section of each network.
changed. Figure 7 highlights a fluorescent photograph of an AA-PEG200DMA copolymer disk.

Figure 8 shows dynamic swelling data from Aam-PEG200DMA copolymers. The imprinted gels swelled at a faster rate than the non-imprinted gels displaying non-Fickian behavior and exhibiting a higher degree of equilibrium swelling. As both systems adsorbed water, the disks became increasingly opaque, with the imprinted gels displaying a higher degree of translucence. The water swelling tests correlate the properties of the network (homogeneity and porosity) to the imprinting process. Owing to the presence of template, the imprinting process resulted in a more macroporous structure as exhibited by these studies and confocal microscopy (Fig. 9).

The effect of the template on the rate of polymerization can be seen in Fig. 10 for a PHEMA system. Although the imprinted and non-imprinted PHEMA networks eventually reach the same conversion, the initial rate of polymerization is quite different (0.021 min⁻¹ for the imprinted, and 0.033 min⁻¹ for non-imprinted). This result suggests that the template has more than a dilution effect on the polymerization; the amount of template accounts for less than 5% of the monomer and cross-linking agent, yet the decrease in the initial rate of polymerization is around 50%. Although contrary to previous results with small molecular compounds [78–82], this decreased rate of polymerization can be attributed to the fact that an iniferter (initiator-transfer agent terminator) increases the rate of termination. Therefore, the decrease in termination rates, caused by the template in previous cases without the use of iniferter, is not observed. Hence, the effect of the presence of template is related strongly to the rate of propagation.

An iniferter is a type of initiator molecule, which takes an active part in propagation and termination of free-radical polymerizations. The presence of an iniferter results in a reversible termination reaction allowing for more control of the polymerization. This reversible termination can be used for creating block copolymers and monodisperse polymers. It is typically a disulfide (e.g., TED) or a dithiocarbamate molecule. The sulfur radicals, formed by the absorption of ultraviolet radiation by the molecule, can transfer free-radicals to monomers or can react with a free-radical to terminate the reaction. The reactions where iniferter participate are called “controlled” radical polymerizations because of the large effect of termination, hence the reduction in the rate of polymerization.

In our work, better understanding and more control of template-mediated polymerizations is a major focus and will lead to optimization of recognition polymers. Specifically, the homogeneous or heterogeneous nature of the imprinted
network correlating to high binding affinity and specificity is currently being studied.

The formation of a three-dimensional network is a kinetically controlled process that depends on
the functionality, reactivity, and concentration of the monomeric components. Traditionally, cross-
linking monomers with similar reactivities to the chain-building monomer are selected to produce a
homogeneous network with spatially even cross-linking density. Differences in the reactivity of the
monomers can lead to structural heterogeneity. With imprinting, the template functional monomer
complex will influence the polymerization conditions and the resulting polymer network. The
influence of the complex formation on the resulting network has not been explored and is currently
being studied by our group.

Other important studies within our laboratory are focusing on fundamental experiments of the
monomer chain flexibility and size, the number and type of monomer functional groups (e.g., a mixture
of effective functional monomers), and the chemical reactivity and solvent interaction parameter of
the functional groups on the specific recognition event needed to produce a spatially defined
recognition site.

**Micropatterned Polymers on Polymer Substrates Using Initiators**

Polymer microstructures have been patterned on polymer substrates using UV photolithography
techniques. A photosensitive polymer substrate was first created through incorporation of dithio-
carbamate groups from initiators. A second polymer that could have different properties was
patterned on the substrate by applying monomer and selectively polymerizing through photomasking.
Figure 11 is an example of micropatterns where the substrate was formed with PEG200DMA (a
hard, strong, highly crosslinked polymer) and the micropatterns consisted of 50 wt% PEG200MA and
50 wt% PEG200DMA (more flexible than the substrate polymer).

**Micropatterned Environmentally Sensitive Polymer on Silicon**

Polymer microstructures have been patterned on silicon substrates using UV photolithography tech-
niques. To gain covalent adhesion between the polymer network and the silicon surface, an
organosilane coupling agent, γ-MPS, was used. An organosilane coupling agent contains chemical
functional groups that can react with silanol groups and form covalent bonds, and then have functional
groups that are exposed and can react to become included in the polymer network [92]. The γ-MPS
molecules formed a self-assembled monolayer on the silicon surface and presented pendant metha-
crylated groups reacted and bonded with the polymerizing network. Examples of the micropat-
terns using 80 mole% MAA and 20 mole% PEGDMA are included as Figs 12 and 13. Since
ionic hydrogels such as MAA have been shown to exhibit pH-dependent behavior, the technique for

![Figure 11](image-url)
FIGURE 12. Micropatterning on silicon. (A) Environmentally sensitive hydrogel patterned in a spiral shape onto a silicon substrate. Image captured with an optical microscope in Nomarski mode. (B) Profilometry data for the path shown in (A). Height of pattern is shown to be 450 nm.

Micropatterning this network onto silicon substrates is currently being extended within our laboratory for the fabrication of a micro-diagnostic pH sensor.

CONCLUSIONS

The studies presented in this paper aid in further understanding and optimizing template-mediated polymerization processes for the production of novel recognizable networks for biologically significant molecules. Based on a biomimetic approach, we have synthesized novel imprinted gel structures for biomolecule recognition. In particular, we have been successful in producing and characterizing recognizable networks for the recognition of D-glucose. Equally important in this endeavor is the potential to produce recognizable networks for a wide range of biomolecules providing hydrogen bonding, hydrophobic, or ionic contributions direct recognition.

In related work, polymer micropatterns have been fabricated using UV free-radical polymerizations. Utilizing photolithography techniques, polymer micropatterns were created with strict spatial control for integration into microdevices. These results demonstrate the feasibility of integrating polymers patterns into silicon devices and all-polymer devices.

Therefore, future developments will integrate our work in both these fields to create novel materials and devices. Developments of particular interest are expected to be wide and far-reaching.
and cover a broad range of materials, such as intelligent biomolecule-modulated drug and protein delivery, nanoscale patterning and recognition of biological molecules for diagnostic and therapeutic devices, site or ligand-specific interaction with cells and tissues for targeting applications and tissue engineering, and biosensors.

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