

NEW BIOMATERIALS FOR INTELLIGENT BIOSENSING, COGNITIVE DRUG DELIVERY AND THERAPEUTICS

Nicholas A. PEPPAS* and Mark E. BYRNE

Departments of Pharmaceutics, Chemical and Biomedical Engineering 1 University Station, C-0400 The University of Texas at Austin Austin, TX 78712-0231

Summary

Engineering the molecular design of intelligent biomaterials by controlling recognition and specificity is the first step in coordinating and duplicating complex biological and physiological processes. We address design and synthesis characteristics of artificial molecular structures capable of specific molecular recognition of biological molecules. Recent developments in protein delivery have been directed towards the preparation of targeted formulations for protein delivery to specific sites, use of environmentally-responsive polymers to achieve pH- or temperature-triggered delivery, usually in modulated mode, and improvement of the behavior of their mucoadhesive behavior and cell recognition. Molecular imprinting and microimprinting techniques, which create stereo-specific three-dimensional binding cavities based on a biological compound of interest can lead to preparation of biomimetic materials for intelligent drug delivery, drug targeting, and tissue engineering. We have been successful in synthesizing novel glucose-binding molecules based on non-covalent directed interactions formed via molecular imprinting techniques within aqueous media.

Keywords

*Tel: (512)-471-6644 ; Fax: (512)-471-3477 ; e-mail: peppas@che.utexas.edu

1. Introduction

Recognition in nature is a complex orchestration of numerous interactions between individual atoms and cumulative interactions between larger macromolecular secondary structures such as helices and sheets. The active sites of enzymes are composed of several amino acid residues, which bind ligand molecules in a very specific manner. However, the activity of the site is dependent on the stabilization of the three-dimensional structure by the interactions of hundreds of other residues.

Biomimetic polymeric networks can be prepared by designing interactions between the building blocks of a biocompatible network and the desired specific ligand and stabilizing these interactions by a three-dimensional structure. This structure is at the same time flexible enough to allow for diffusion of solvent and ligand into and out of the network. Synthetic networks that can be designed to recognize and bind biologically significant molecules are of great importance and influence a number of emerging technologies. These artificial materials can be used as unique systems or incorporated into existing drug delivery technologies that can aid in the removal or delivery of biomolecules so that the natural profiles of compounds in the body can be restored.

Such developments will have a major impact on diseases, such as diabetes and arteriosclerosis, which are caused by increased levels of certain compounds in the blood. The monitoring and removal of such detrimental compounds 'on-demand' by polymeric systems is highly desirable, and this can be achieved by biomimetic networks (Figure 1).

Biomimetic Approach

- Defined as "mimicking biological entities whether in structural or functional characteristics"
 - include a material containing a Biomolecular Component (e.g. Biologically Active Peptides, Antibodies)
- OR**
- ARTIFICIAL MATERIALS containing no active molecules
- OUR DEFINITION:
- Configurational Chemomimesis or Biochemomimesis — Chemical Group(s) Matching Functionality and Configurational Arrangement of Protein
 - Artificial Biological Functions Built into Materials
 - Less Antigenic
 - More Robust (Temp, Mechanical, Processing, etc.)

Figure 1: Biomimetic Approach to Material Design

The most important problems to be solved in the design of synthetic recognition-based networks are:

- (i) to obtain reproducible interactions between ligand molecule and network, and an ability to differentiate between the ligand and similar compounds;
- (ii) to create a network compatible with aqueous solutions;
- (iii) to reduce diffusional limitations of ligand into the bulk network; and
- (iv) to establish a platform by which recognition-based polymers can be used as biomimetic systems.

While many advances have been made in the discovery and delivery of drugs to cure chronic diseases, improving the quality of life of patients to a level where the control of their disease leads to completely natural metabolism is still far from reach. However, *the combination of intelligent material design with advances in nano-technology can provide a means to reach this goal*. Since many drugs act as protagonists or antagonists to different chemicals in the body, such as insulin and glucose, a delivery system that can respond to the concentrations of certain molecules in the body will be invaluable. For this purpose, we are one of a handful of groups to design the newest generation of sensitive materials based on molecular recognition.

Hydrogels, insoluble polymeric networks compatible with aqueous solutions, have been instrumental in the design of many biocompatible medical devices [1-3]. In particular, environmentally-sensitive hydrogels, which are molecularly designed to respond to changes in their chemical environment such as pH [4,5], temperature [6-10], and ionic strength, provide efficient ways to protect and deliver drugs. The network structure and the thermodynamic nature of the components of these networks play a key role in their molecular mesh size changes [2], in their diffusional behavior [1,2,] and in their associated molecular stability of the incorporated biomolecules. [11,12] Networks of this type have been used in dental systems, polymeric catheters,

and oral drug delivery devices for proteins and peptides in our laboratory.

2. Molecular recognition in polymeric systems

Natural processes such as enzymatic reactions, ligand-receptor interactions, antigen-antibody complexation, and the molecular mechanisms during the replication of genetic information through DNA/RNA enzyme, DNA/protein, and DNA/DNA interactions occur via molecular recognition. Through these recognition processes, molecules of interest are recognized from many structurally and/or functionally similar compounds and bound to an exact site on a receptor molecule. This receptor molecule can be an enzyme, which binds and chemically alters certain substrates in its active site composed of several amino acid residues specifically evolved for that purpose, or it can be a piece of DNA that can bind a complementary strand among many similar segments. Molecular recognition, in the context of this paper, can be defined as the ability of a polymer network to non-covalently complex with a designated target molecule amidst a range of other molecules.

Researchers have tried to influence recognition behavior with synthetic molecules and macromolecules. In the past few decades, biomaterials have evolved from non-medical, off-the-shelf materials to materials specifically designed for medical applications. To control the immunological response, efforts quickly focused on novel chemistry, macromolecular structure, and material design for the production of inert biomaterials. The earliest catheters and pacemakers were of this type. However, it is becoming more apparent that orchestrating interactions on a molecular level can lead to more natural biomaterials and biomolecular components. Cellular scaffolds, such as pieces of artificial arteries that can grow confluent epithelial tissue on them, or materials that can aid in long-term culture of hepatocytes or in the formation of new liver tissue, are results of this new strategy.

The next generation of biomaterials will not only act as stealth materials by manipulating

interactions on the molecular level, but will build on efforts in intelligent biomaterials and respond to changes in biomolecule concentration in the surrounding environment.

2.1 Methods to Achieve Molecular Recognition in Polymeric Systems

Creating synthetic polymers that can recognize and bind specific compounds involves polymerization in the presence of those specific compounds. Current research focuses on achieving equivalent specificity of interaction compared to protein-ligand systems by using synthetic monomers and macromers to create sequences of recognition.

Template polymerization began with the polymerization of one monomer using a previously prepared polymer as a pattern. [13] The monomer and macromer were usually oppositely charged for the purpose of non-covalent binding to each other via ionic interactions. For example, investigators [14-16] have studied the kinetics of template polymerization by considering the template polymerization of methacrylic acid in the presence of poly(N-vinyl-2-pyrrolidone) (PVP), or polyethylene glycol (PEG) with different end groups. In this case, it was found that the templates that have strong interactions with the macromer (PVP) caused a four order decrease in the termination constant leading to an increase in overall reaction rates. The decrease in the termination constant was explained by the reduced probability of radicals finding and reacting with each other due to the mobility-reducing effect of template. A two orders of magnitude decrease in the propagation constant was thought to have occurred because of several different phenomena possibly occurring. The template caused a disadvantageous spatial arrangement either by folding into a certain conformation upon itself or by sterically disabling the macroradicals' reaction. Also, the reduced mobility of the macroradicals made it more difficult in adding monomers.

Rate enhancement due to template is a well-known effect caused by templates that 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. [17]

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. [14-17] 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. [13]

2.2. Configurational Biomimetic Imprinting (CBIP)

The process of CBIP and the associated processes of molecular imprinting and nanopatterning call for the mixing of various functional monomers and the desired template molecule (i.e., the molecule to be recognized) (Figure 2).

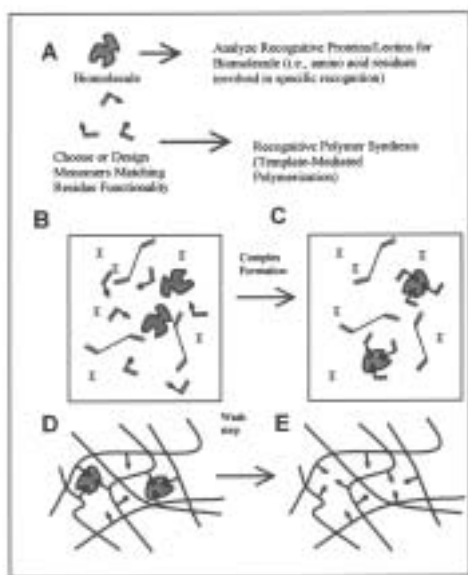


Figure 2: Configurational Biomimetic Imprinting (CBIP)

A: Mimic recognitive 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 pre-polymerization 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.

In the case of a desired catalytic reaction, it might be the substrate or a closely related molecule. The mixing of monomers and the template may or may not involve a solvent to dissolve the monomers and the template depending on the required polymer porosity and diffusional considerations.

Mixing allows the formation of interactions between the monomers and the template by covalent bonding [18], non-covalent bonding [19,20] or metal coordination [21]. With the addition of an initiator, polymerization takes place either 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.

The advantage of this procedure is that interactions are self-stabilized during the process, which produces vacuoles of desired selectivity and affinity. The limitations of this method involve optimizing capacity with strong complexation and mutual solubility of all components with reduced heterogeneity of binding. Moreover, the binding of the template to the monomers is kinetically controlled. In addition to the diffusional problems that might arise from the size and shape of the molecule or the preparation technique, this phenomenon might have a significant effect on selectivity. Therefore, the major problems associated with CBIP processes have been the heterogeneity of binding sites created with non-covalent bonding, the presence of water as a hydrogen-bonding solvent, and polymer morphology limitations to diffusion [22-25]. We address these questions in our work by choice of monomers, the polymerization medium, and polymerization conditions.

The network structure depends upon the type of monomer chemistry (anionic, cationic, neutral, amphiphilic), the association interactions between monomers and pendent groups, the solvent, and the relative amounts of comonomers in the feed from which the structure is formed. 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 clear advantage of the non-covalent technique is the ease of preparation of the polymers. The monomers and template are simply mixed together and allowed to interact based on “self-assembly”. In contrast to the covalent approach, the generality of the interactions allows application to many templates with many different kinds of monomers and environments.

2.3. Imprinting with Biological Templates, Bioconjugation, and Surface Imprinting

Research on macromolecular networks with recognition capabilities by complexation has included polymer-biomolecule immobilization (protein, enzyme, lectin bio-conjugation techniques) [26-34] and molecular imprinting methods (surface or bulk template-directed polymerization). [35-38] 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] By complexation of template molecules with fixed distance between functional groups and monomers, Shi et al. [44] were able to create surfaces with considerable selectivity towards fibrinogen, albumin and lysozyme. Another type of surface imprinting was developed by Uezu and coworkers [45], which used the surface of an oil-in-water emulsion to fix the template-monomer complex.

Both covalent and non-covalent approaches have been used to imprint various carbohydrates (galactose derivatives [46-48], glucose derivatives [46], fucose derivatives [46], fructose derivatives [48], and mannose derivatives [49]). The covalent approach involved the formation of reversible boronate esters between pairs of hydroxy groups on the sugar molecule and 4-vinylphenylboronic acid [50], which has been shown to work for glycosides [50] and free sugars. [48,49] However, favorable recognition depended upon two pairs of cis diols on the sugar ring, which could limit use for biological saccharides.

Although very selective sites were obtained using this technique, polymerization was not performed in aqueous solution and required establishing covalent bonds upon rebinding. Therefore, *in vivo* use would not be possible.

3. Drug Delivery Technologies

There is a large potential for recognitive structures to influence mechanisms of intelligent drug delivery and our work is progressing on two main fronts. Our laboratory is focused on creating the next generation of biomolecule-sensitive intelligent drug delivery systems using recognitive structures in design [51]. Also, these structures are being combined with sensor nanotechnologies to create novel, robust diagnostic devices [52]. Coupling these diagnostic devices with controlled reservoirs can lead to a wide range of therapeutic devices. However, a future goal is to use *in vivo* macromolecular networks that respond to a specific biomolecule with a modulated release of drug or peptide-based drug. Of equal importance, is that recognitive theory and associated technology will aid in the optimization of existing drug delivery associated with controlled release mechanisms of delivery.

The most important problems to be solved in the design of the next generation biomolecule-sensitive networks are:

- (i) to overcome kinetic binding limitations between ligand molecule and network functionality resulting in quick actuation;
- (ii) to overcome the diffusional limitations of ligand into the bulk network; and
- (iii) to prevent unwanted diffusion of drug from the macromolecular reservoir.

3.1. Sensors and Disease Management

There is a wide-range of medical conditions that require the sensing/control of a particular molecule. For example, the insulin-dependent diabetes mellitus market has a wide array of sensors available that do a variety of things in the management of the disease (e.g., small sample sizes, multiple day averages, event markers,

diabetes management software, data ports/access to transfer readings). However, there are many diseases that require a clinic visit or a blood sample to properly monitor the disease or adequately manage medication levels. The technology either does not exist for these sensors or cannot be technically or economically scaled down for point-of-care use. ***With nanotechnology and novel material design, the future of medical management will involve small, point-of-care, and possibly disposable sensors that monitor virtually any biological molecule of interest.*** This will translate into an informed patient with better overall hands on control for disease management. Also, it may alert and aid patients in determining when a low-risk environment exists. Conversely, it may alert patients to a high-risk situation and offer certain measures to control the situation. As such, an increased quality of life and term of life of a person with a chronic disease can be achieved. With the increase in wireless technology, data can be processed and transmitted to a variety of systems, as well as a family or specialist physician. Recognitive structures have the capability to mimic nature, but provide a robustness and low cost to provide materials for this platform. Thus, first generation, robust sensor technologies for certain diseases are being developed now.

3.2. Optimizing Loading of Controlled Release Systems

Hydrogels have been well documented in the areas of controlled release [1-3]. The hydrophilic and hydrophobic balance of a gel carrier can be altered to provide tunable contributions with different solvent diffusion characteristics, which in turn influence the diffusive release of drug contained within the gel matrix. The technology developed in the early seventies has evolved to achieve sustained delivery of large molecules such as high molecular weight peptides for long periods of time (e.g., days and months). Current systems incorporate the drug or peptide in two main ways. The drug is introduced into the polymer network via imbibition, which is equilibrium partitioning after the network is formed, or the drug is included during the

polymerization of the network. With drugs sensitive to ultraviolet light or exothermic activity, the choice has been equilibrium partitioning. For other molecules, recognitive structures can add a chemical framework to greatly enhance the equilibrium partitioning of drug within the macromolecular confinement. The result may not be a binding cavity, but an association of functionality to enhance the partitioning within the network (Figure 3). With peptide-based drugs, even though inclusion during polymerization might denature the peptide, effective sites can be made during polymerization, which can enhance the partition factor. Thus, for protein systems there would be a reloading of peptide by imbibition. Therefore, proper understanding and optimization of this effect along with a detailed analysis of polymerization kinetics, can lead to a substantial increase in the loading of hydrogels in controlled release systems.

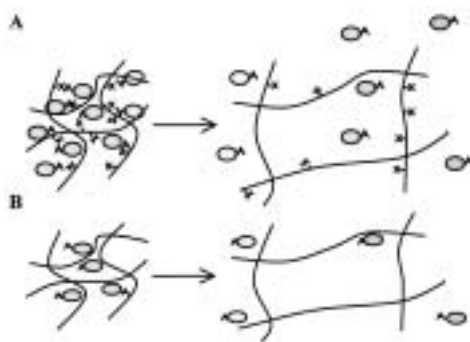


Figure 3: Optimization of Drug Loading Within Hydrogels.
A. Recognitive network with pendant residues, X, which have affinity for drug chemistry, designated by the letter A.
B. Typical hydrogel network without enhanced partition factor. In both cases the diffusion of water into the network and/or the relaxation of the polymer network influences the diffusive release of drug from the matrix.

Also, proper design can lead to the inclusion of certain wanted molecules into the gel and the exclusion of other molecules that impose side effects (e.g., racemic drugs that have intended and non-intended chiral twins). Loading and separation of intended drug would occur simultaneously and release could be tailored by the properties of the hydrogel. Depending on the nature of the macromolecular network in terms of flexibility and chemistry and the constraints

of the separation, the time of release for molecules from less flexible gels can be on the order of 5 to 6 hours [53]. However, due to novel designs on conventional techniques, this can lead to longer, sustained release profiles.

4. Biomimetic Recognitive Gels for Glucose

Our goal, in general, is to produce stereospecific, three-dimensional binding cavities for biomolecules 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 recognitive proteins and enzymes by analyzing the amino acids involved in binding a particular molecule and to duplicate the complexation interactions involved. 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 and can be configured into the network, the network formation will proceed with effective recognitive domains that function in aqueous environments. For example, many binding proteins contain non-covalent binding mechanisms (e.g., hydrogen bonding, hydrophobic interactions) that bind specific molecules quite well in water, which is a polar, protic solvent. [54] Our scientific rationale is based on the hypothesis that effectively designed recognitive 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, [55] including strong hydrogen bond donors and acceptors, [56] or including strong ionic directed recognition sites [57]).

4.1 Materials and Methods

Methacrylic acid (MAA), acrylic acid (AA), acrylamide (Aam), dimethylsulfoxide (DMSO), and D-glucose were purchased from Aldrich (Milwaukee, WI). Poly(ethylene glycol) 200

dimethacrylate (PEG200DMA) was obtained from Polysciences, Inc. (Warrington, PA). Irgacure[®] 184, 1-hydroxycyclohexyl 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.

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 recognitive polymers will be presented, mainly two copolymers consisting of Aam-PEG200 DMA, and AA-PEG200DMA. Monomers were selected to match corresponding glucose binding protein residues of aspartate, glutamate, and asparagine (Figure 4).

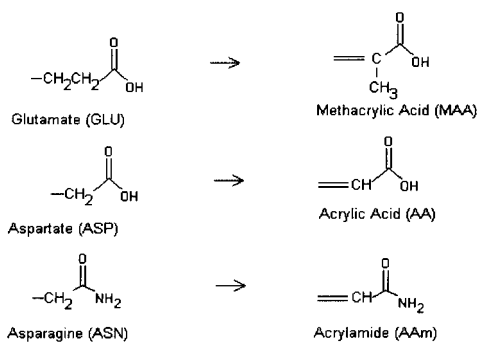


Figure 4: Gluco-recognitive Material Design. Monomers (right, methacrylic acid, acrylic acid, and acrylamide) were selected to match corresponding glucose binding protein residues of aspartate, glutamate, and asparagine.

In a typical experiment involving Aam or AA as functional monomer, D-glucose, Aam and/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 mole monomers (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 (75x50x1 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 minutes (UltraCure 100, EFOS Inc., Ontario, Canada). Polymers were placed in deionized water for 24 hours, then carefully separated from the slides, and were cut into various diameter discs using a cork borer. Discs 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 hour wash steps (2 washes in acetic acid/deionized water (1:5 ratio); 8 washes in deionized water) to remove template and excess monomer. The resulting discs 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 discs were then stored in a dessicator until testing.

4.2 Recognition, Selectivity, and Dynamic Swelling Studies

In a typical binding study, a known amount of the template solution (e.g., D-glucose and D-galactose in competitive binding studies) was added to an aqueous solution containing a known amount of polymer discs 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 discs 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, 0.6ml/min flow rate, temperature 80°C, Shimadzu RID-10A refractive index detector). Competitive binding results were visualized using a fluorescent glucose analogue, 2-NBDG (Figure 5). The analogue was added to vials containing a known amount of polymer

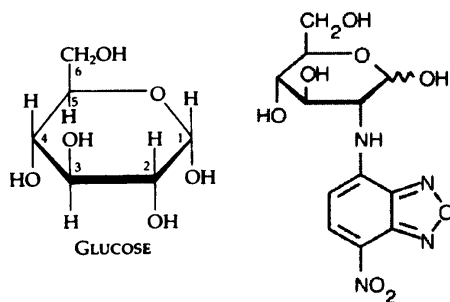


Figure 5: D-Glucose - Template and Fluorescent Analogue. Competitive binding results were visualized using a fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) -2-deoxy-glucose (2-NBDG, Molecular Probes, Inc.). In solution, glucose mutarotates between two conformations: alpha (as shown, 30-35%) and beta (65-70%) position of the carbon 1 hydroxyl group at equilibrium. D-Galactose only differs by the carbon 4 hydroxyl position.

(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=10,000) 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 LaserSharp 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.

4.3 Results And Discussion

For AA and Aam functional networks, a bound ratio (amount D-glucose bound recognitive network/control network) greater than one indicates that glucose was memorized 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 (DMSO is aprotic and does not have the ability to be a hydrogen bond donor and interfere with complex formation).

Figure 6 provides an aqueous equilibrium binding analysis of glucose-imprinted Aam-PEG200DMA copolymers prepared in DMSO. The recognitive to non-imprinted (control) bound ratio is 3.4, demonstrating the memorization of glucose within the network. Also, the equilibrium binding data was well represented by a linear isotherm at low concentration ranges (i.e., < 1 mg/ml D-glucose).

Typically, the difference between recognitive and non-imprinted polymer binding values increased as the cross-linking ratio (mole crosslinking monomer/mole total monomers) increased.

For improved analysis of fluorescent intensities, thin polymer discs (diameter, 5.5 mm; thickness, 35 μ m) were prepared. By analyzing fluorescent intensity values from polymer discs of equal thickness, a histogram of intensity values was obtained, which provided quantitative analysis of binding (Table I). This data contains fluorescent profiles from competitive binding analysis of Aam-PEG200DMA copolymers with varying amounts of D-glucose added to a given amount of fluorescent analogue (FITC filter cube, 1/8 integration time, 4x 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. As glucose concentration is increased and fluorescent analogue is held at constant concentration, the fluorescent intensity of the gel decreases. Thus, glucose competed and filled binding sites once occupied by the fluorescent analogue.

The highest glucose ratio (3,000x F_g) drops the fluorescent intensity to level consistent with control polymer. A gel consisting of PEG200DMA only (i.e., no functionality in feed)

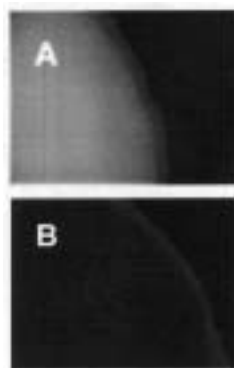
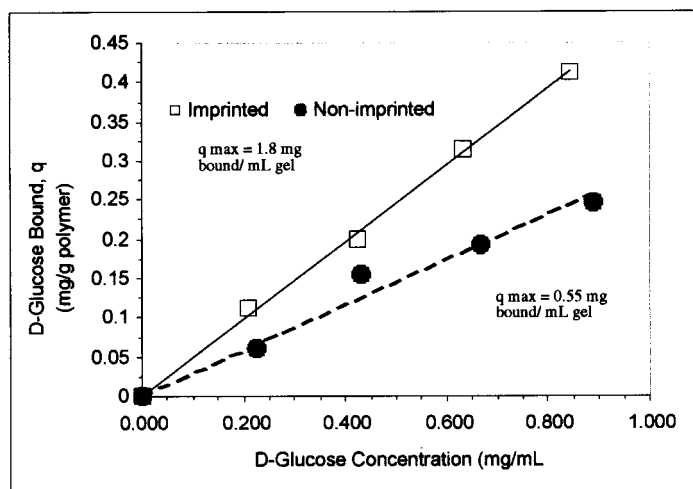


Figure 6: D-Glucose Binding Isotherm in Water. Acrylamide-PEG200DMA Copolymers of 67% Crosslinking Ratio Prepared in DMSO ($T=24^{\circ}\text{C}$). Fluorescent D-Glucose Analogue Binding in Water. Acrylic Acid-PEG200DMA Copolymers with 67% Crosslinking Ratio Prepared in DMSO. A: Recognitive Polymer ($I=208.84\pm/6.48$ (10,000 pixels)). B: Control Polymer ($I=36.28\pm/1.26$ (10,000 pixels)).

Table I. Competitive D-Glucose Substrate Binding in Water: Acrylamide-PEG200DMA Copolymers with 67% Crosslinking Ratio (prepared in DMSO).

Competitive Substrate	Recognitive Intensity	Control Intensity
Fluorescent Analogue Only (Concentration = F_g)	223 +/- 11.81	50.97 +/- 0.77
F. Analogue and Glucose (100 x F_g)	99.44 +/- 4.36	80.77 +/- 3.95
F. Analogue and Glucose (3,000 x F_g)	49.81 +/- 1.09	48.85 +/- 0.88
F. Analogue and Galactose (100 x F_g)	220 +/- 10.10	na
F. Analogue and Galactose (3,000 x F_g)	209.06 +/- 10.46	na
Fluorescent Analogue Only (PEG only network)	na	56.57 +/- 0.90

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 2-NBDG fluorophore. AA functionalized networks exhibited similar behavior (Figure 6 A, B).

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 I). The results suggest polymers selective to D-glucose since the fluorescent intensity remained approximately unchanged.

Figure 7 shows dynamic swelling data (without template present) from Aam-PEG200DMA copolymers. No significant differences occurred when template was present. The imprinted gels swelled at a faster rate than the non-imprinted gels displaying non-Fickian swelling behavior (i.e., anomalous transport since swelling process is not dominated by the polymer viscoelastic relaxation time or water diffusion) and exhibiting a higher degree of equilibrium swelling. As both systems adsorbed water, the discs became increasingly opaque, with the non-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. Due to the presence of template, the imprinting process resulted in a more porous structure as exhibited by these studies and confocal microscopy/FE-SEM (Figure 7 A, B, C, D).

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, crosslinking monomers with similar reactivities to the chain building monomer are selected to produce a homogeneous network with spatially even crosslinking density. Differences in the reactivity of the monomers can lead to structural heterogeneity. With imprinting, the template functional monomer complex influences 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. 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.

5. Conclusions

The studies presented in this paper aid in further understanding and optimizing template-mediated polymerization processes for the production of novel recognitive networks for biologically significant molecules. Based on a biomimetic approach, we have synthesized novel imprinted gel structures for glucose. Equally important in this endeavor is the potential to produce recognitive networks for a wide range of biomolecules providing hydrogen bonding, hydrophobic, or ionic contributions direct recognition. Thus, these techniques and the resulting polymers can be tailored to recognize a broad range of molecules in

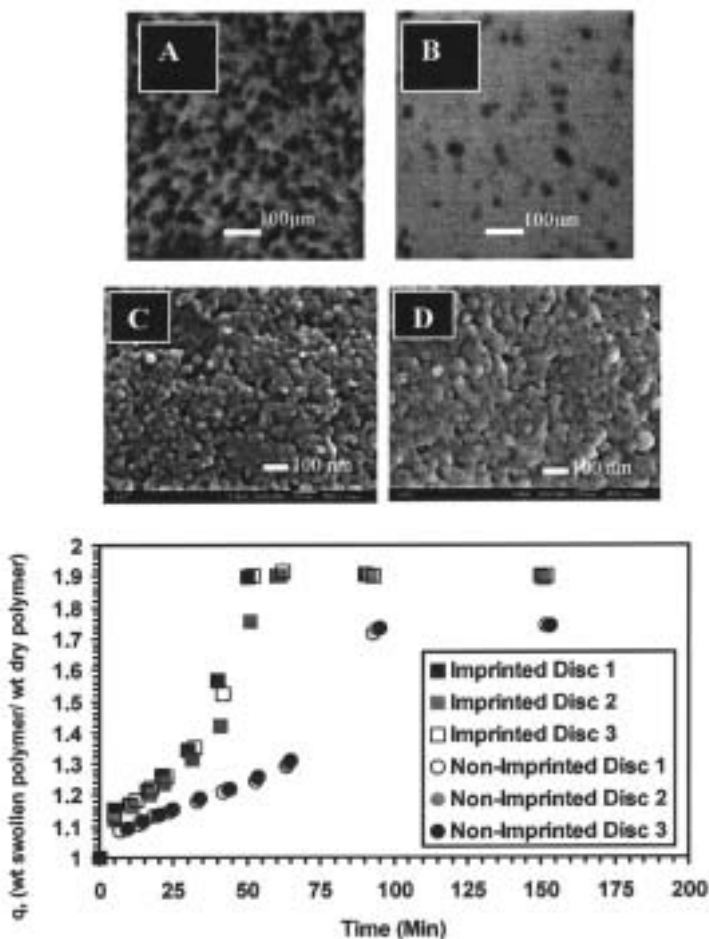


Figure 7: Dynamic Network Swelling Study in Water and Polymer Morphology via Confocal Microscopy Transmission /FE-SEM. Acrylamide-PEG200DMA Copolymers with 67% Crosslinking Ratio Prepared in DMSO. A. Recognitive Network. B. Control Network. C. Recognitive FE-SEM D. Control FE-SEM

aqueous environment. Currently, using similar techniques, our group is producing and optimizing recognitive networks for a variety of drugs and biological molecules, including proteins.

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, nano-scale patterning (Figure 8) 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.

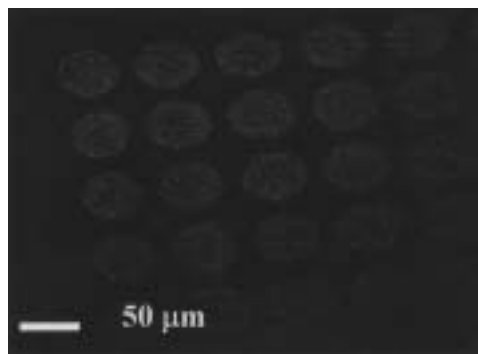


Figure 8: Recognitive Network Patterned on Silicon. Acrylamide-PEG200DMA Copolymers with 67% Crosslinking Ratio Prepared in DMSO.

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References

- [1] Lowman, A. M.; Peppas, N. A. Hydrogels. In *Encyclopedia of Controlled Drug Delivery*; Mathiowitz, E., Ed.; Wiley and Sons: New York, 1999; Vol. 1, pp 397-418.
- [2] Peppas, N. A. *Hydrogels in Medicine and Pharmacy*; Vol.1, CRC Press: Boca Raton, 1986.
- [3] Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. Hydrogels in pharmaceutical formulations *Eur. J. of Pharm. Biopharm.* 2000, 50, pp. 27-46.
- [4] Lowman, A. M.; Morishita, M.; Kajita, M.; Nagai, T.; Peppas, N. A. Oral delivery of insulin using pH-responsive complexation gels *J. Pharm. Sci.* 1998, 88(9).
- [5] Peppas, N. A.; Huang, Y.; Torres-Lugo, M.; Ward, J. H.; Zhang, J. Physicochemical foundations and structural design of hydrogels in medicine and biology *Annu. Rev. Biomed. Eng.* 2000, 2, pp. 9-29.
- [6] Brazel, C. S.; Peppas, N. A. Synthesis and characterization of thermo- and chemomechanically responsive poly(N-isopropylacrylamide-co-methacrylic acid) hydrogels *Macromolecules* 1995, 28, pp. 8016-8020.
- [7] Leobandung, W. L.; Peppas, N. A. In *AICHE*: Los Angeles, CA, 2000, p 199h.
- [8] Peppas, N. A.; Zhang, J. Diffusional behavior in pH- and temperature-sensitive interpenetrating networks used in drug delivery. In *Biomaterials and Drug Delivery systems towards the new millennium*; Park, K. D., Kwon, I. C., N. Yui, Yeong, S. Y., Park, K., Eds.; Seoul, Korea, 2000, pp 87-96.
- [9] Vakkalanka, S. K.; Brazel, C. S.; Peppas, N. A. Temperature and pH-sensitive terpolymers for modulated delivery of streptokinase *J. Biomed. Materials Sci., Polym. Ed.* 1996, 8, pp. 119-129.
- [10] Zhang, J.; Peppas, N. A. Molecular interactions in poly(methacrylic acid)/poly(N-isopropyl acrylamide) interpenetrating polymer networks *J. Appl. Polym. Sci.* 2001, 82, pp. 1077-1082.
- [11] Madsen F., Peppas N. A. Complexation graft copolymer networks: swelling properties, calcium binding and proteolytic enzyme inhibition. *Biomaterials* 1999, 20, pp. 1701.
- [12] Lehr C. M. Bioadhesion technologies for the delivery of peptide and protein drugs to the gastrointestinal tract. *Crit. Rev. Ther. Drug* 1994, 11, pp. 119.
- [13] Odian, G. *Principles of polymerization*; Vol. 1, Wiley: New York, 1991.
- [14] Matuszewska-Czerwik, J.; Polowinski, S. Template photopolymerization of methacrylic acid-III. Determination of the rate constants k_t/k_p for elementary processes *Eur. Polym. J.* 1991, 27(8), pp. 743-746.
- [15] Matuszewska-Czerwik, J.; Polowinski, S. Template photopolymerization of methacrylic acid in water-IV. Determination of rate constants of elementary processes *Eur. Polym. J.* 1991, 27(12), pp. 1335-1337.
- [16] Matuszewska-Czerwik, J.; Polowinski, S. Template photopolymerization of methacrylic acid in water-V. Low molecular weight templates. *Eur. Polym. J.* 1992, 28(12), pp. 1481-1483.
- [17] Tan, Y. Y.; Alberda Von Ekenstein, G. O. R. A generalized kinetic model for radical-initiated template polymerizations in dilute template systems *Macromolecules* 1991, 24, pp. 1641-1647.
- [18] Whitcombe, M.; Rodriguez, M. E.; P. Villar; Vulfson, E. N. A new method for the introduction of recognition site functionality into polymers prepared by molecular imprinting: Synthesis and characterization of polymeric receptors for cholesterol *J. Am. Chem. Soc.* 1995, 117, pp. 7105-7111.
- [19] Sellegren, B. Noncovalent molecular imprinting: antibody-like molecular recognition in polymeric network materials *Trends Anal. Chem.* 1997, 16(6), pp. 310-320.
- [20] Sellegren, B. Important considerations in the design of receptor sites using noncovalent molecular imprinting. In *Molecular and Ionic Recognition with Imprinted Polymers*; Bartsch, R. A., Maeda, M., Eds.; American Chemical Society: Washington, D.C., 1998; Vol. 703, pp 49-81.
- [21] Dhal, P. K.; Arnold, F. H. Metal-coordinated interactions in the template-mediated synthesis of substrate-selective polymers: Recognition bis(imidazole) substrates by copper(II) iminodiacetate containing polymers. *Macromolecules* 1992, 25, pp. 7051-7059.
- [22] Mosbach, K.; Haupt, K.; Liu, X.-C.; Cormack, P. A. G.; Ramstrom, O. Molecular imprinting: Status artis et quo vadere? In *Molecular and Ionic Recognition with Imprinted Polymers*; Bartsch, R. A., Maeda, M., Eds.; American Chemical Society: Washington, D.C., 1998; Vol. 703, pp 29-48.
- [23] Sellegren, B.; Shea, K. J. Influence of polymer morphology on the ability of imprinted network polymers to resolve enantiomers *J. Chromatogr.* 1993, 635, pp. 31-49.
- [24] Chen, C.; Chen, G.; Guan, Z.; Lee, D.; Arnold, F. H. Polymeric sensor materials for glucose *Polym. Prepr.* 1996, 37(2), pp. 216-217.
- [25] Lele, B. S.; Kulkarni, M. G.; Mashelkar, R. A. Productive and non-productive binding in enzyme mimics *Polymer* 1999, 40, pp. 4063-4070
- [26] Hoffman A. S. Molecular bioengineering of biomaterials in the 1990s and beyond: A growing liaison of polymers with molecular biology. *Artif. Organs* 1992, 16, pp 43.
- [27] Ding Z., Fong R. B., Long C. J., Stayton P. S., Hoffman A. S. Size-dependent control of the binding of biotinylated proteins to streptavidin using a polymer shield. *Nature* 2001, 411, pp. 59.
- [28] Bulmus V., Ding Z., Long C. J., Stayton P. S., Hoffman A. S. Site-specific polymer-streptavidin bioconjugate for pH-controlled binding and triggered release of biotin. *Bioconjugate Chem.* 2000, 11, pp. 78.
- [29] Hoffman A. S., Stayton P. S., Bulmus V., Chen G., Chen J., Cheung C., Chilkoti A., Ding Z., Dong L., Fong R., Lackey C. A., Long C. J., Miura M., Morris J. E., Murthy N., Nabeshima Y., Park T. G., Press O. W., Shimoboji T., Shoemaker S., Yang H. J., Monji N., Nowinski R. C., Cole C. A., Priest J. H., Harris J. M., Nakamae K., Nishino T., Miyata T. Really smart bioconjugates of smart polymers and receptor proteins. *J. Biomed. Mater. Res.* 2000, 52, pp.577.
- [30] Hubbell J. A. Bioactive biomaterials. *Curr. Opin. Biotech.* 1999, 10, pp.123.
- [31] Kokufuta E., Zhang Y. Q., Tanaka T. Saccharide-sensitive phase transition of lectin-loaded gel. *Nature* 1991, 351, pp.302.
- [32] Obaidat A. A., Park K. Characterization of glucose dependent gel-sol phase transition of polymeric glucose-concanavalin A hydrogel system. *Pharmaceut. Res.* 1996, 13, pp.989.
- [33] Podual K., Doyle F. J., Peppas N. A. Glucose-sensitivity of glucose oxidase-containing cationic copolymer hydrogels having poly(ethylene glycol) grafts. *J. Control. Release* 2000, 67, pp.9.

- [34] Miyata T., Jikihara A., Nakamae K., Hoffman A. S. Preparation of poly(2-glucosyloxyethyl methacrylate)-concanavalin A complex hydrogel and its glucose-sensitivity. *Macromol. Chem. Physic.* 1996, 197, pp.1147.
- [35] Shi H., Ratner B. D. Template recognition of protein-imprinted polymer surfaces. *J. Biomed. Mater. Res.* 2000, 49, pp.1.
- [36] Shi H., Tsai W., Garrison M. D., Ferrari S., Ratner B. D. Template-imprinted nanostructured surfaces for protein recognition. *Nature* 1999, 398, pp.593.
- [37] Wulff G. Molecular imprinting in cross-linked materials with the aid of molecular templates-a way towards artificial antibodies. *Angew. Chem. Int. Edit.* 1995, 34, pp.1812.
- [38] Sellergren B. Noncovalent molecular imprinting: antibody-like molecular recognition in polymeric network materials. *Trends Anal. Chem.* 1997, 16, pp.310.
- [39] Enoki T., Tanaka K., Watanabe T., Oya T., Sakiyama T., Takeoka Y., Ito K., Wang G., Annaka M., Hara K., Du R., Chuang J., Wasserman K., Grosberg A. Y., Masamune S., Tanaka T. Frustrations in polymer conformation in gels and their minimization through molecular imprinting. *Phys. Rev. Lett.* 2000, 85, pp.5000.
- [40] Alvarez-Lorenzo C., Guney O., Oya T., Sakai Y., Kobayashi M., Enoki T., Takeoka Y., Ishibashi T., Kuroda K., Tanaka K., Wang G., Grosberg A. Y., Masamune S., Tanaka T. Polymer gels that memorize elements of molecular conformation. *Macromolecules* 2000, 33, pp.8693.
- [41] Alvarez-Lorenzo C., Guney O., Oya T., Sakai Y., Kobayashi M., Enoki T., Takeoka Y., Ishibashi T., Kuroda K., Tanaka K., Wang G., Grosberg A. Y., Masamune S., Tanaka T. Reversible adsorption of calcium ions by imprinted temperature sensitive gels. *J. Chem. Phys.* 2001, 114, pp.2812.
- [42] Alvarez-Lorenzo C., Hiratani H., Tanaka K., Stancil K., Grosberg A. Y., Tanaka T. Simultaneous multiple-point adsorption of aluminum ions and charged molecules by a polyampholyte thermosensitive gel: controlling frustrations in a heteropolymer gel. *Langmuir* 2001, 17, pp.3616.
- [43] Hiratani H., Alvarez-Lorenzo C., Chuang J., Guney O., Grosberg A. Y., Tanaka T. Effect of reversible cross-linker, n,n-bis(acryloyl)cystamine, on calcium ion adsorption by imprinted gels. *Langmuir* 2001, 17, pp.4431.
- [44] Shi, H. Q.; Ratner, B. D. Template recognition of protein-imprinted polymer surfaces *J. Biomed. Mater. Res.* 2000, 49(1), pp. 1-11.
- [45] Uezu, K.; Nakamura, H.; Kanno, J.; Sugo, T.; Goto, M.; Nakashio, F. Metal ion-imprinted polymer prepared by the combination of surface template polymerization with postirradiation by gamma-rays, *Macromolecules* 1997, 30(13), pp. 3888-3891.
- [46] Mayes A.G., Andersson L. I., Mosbach K. Sugar binding polymers showing high anomeric and epimeric discrimination by non-covalent molecular imprinting. *Analytical Biochemistry* 1994, 222, pp.483-488.
- [47] Nilsson K.G., Sakaguchi K., Gemeiner P., Mosbach K. Molecular imprinting of acetylated carbohydrate derivatives into methacrylic polymers. *Journal of Chromatography* 1995, 707, pp.199-203.
- [48] Wulff G., Haarer J. 1991. Enzyme-analogue built polymers. The preparation of defined chiral cavities for the racemic resolution of free sugars. *Macromolecular Chemistry* 1991, 192, pp.1329-1338.
- [49] Wulff G., Oberkobusch D., Minarik M. Enzyme-analogue Built Polymers. Chiral cavities in polymer layers coated on wide-pore silica. *Reactive Polymers* 1985, 3, pp.261-275.
- [50] Wulff G., Minarik M. Template imprinted polymers for HPLC separation of racemates. *Journal of Liquid Chromatography* 1990, 13(15), pp.2987-3000.
- [51] Byrne M. E., Park K., Peppas, N. A. Molecular imprinting within hydrogels, *Adv. Drug Deliv. Rev.* 2002, 54, pp.149.
- [52] Byrne M. E., Hilt J. Z., Bashir R., Park K., Peppas N. A. *Trans. Soc. Biomater.* 2002, 28.
- [53] Scott, R. A., Peppas N. A. Highly crosslinked, PEG-containing copolymers for sustained solute delivery *Biomaterials* 1999, 20, pp. 1371-1380.
- [54] Li T., Lee H., Park K. Comparative stereochemical analysis of glucose-binding proteins for rational design of glucose specific agents. *J. Biomater. Sci. Polym. Edn.* 1998, 9, pp.327.
- [55] Yu C., Ramstrom O., Mosbach K. Enantiomeric recognition by molecularly imprinted polymers using hydrophobic interactions. *Anal. Lett.* 1997, 30, pp. 2123.
- [56] Yu C., Mosbach K. Molecular imprinting utilizing an amide functional group for hydrogen bonding leading to highly efficient polymers. *J. Org. Chem.* 1997, 62, pp.4057.
- [57] Haupt K. Noncovalent molecular imprinting of a synthetic polymer with the herbicide 2,4-Dichlorophenoxyacetic acid in the presence of polar protic solvents. In *Molecular and Ionic Recognition with Imprinted Polymers*, ACS Symposium Series 703, Bartsch RA, Maeda M (eds). ACS: Washington, DC, 1998; pp.135-142.



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