Structural Analysis and Diffusional Behavior of Molecularly Imprinted Polymer Networks for Cholesterol Recognition

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Novel configurational biomimetic polymers for the recognition of cholesterol were prepared by molecular design of methacrylate-based structures containing poly(ethylene glycol) in moderately and highly cross-linked networks. Preparation in thermodynamically balanced solvents such as tetrahydrofuran and dimethyl sulfoxide led to recognitive systems with increased recognitive capacity after 30 min. Microporous and nanoporous networks were prepared. Their molecular structure was analyzed using a cross-linked network structure theory. It was determined that the recognitive capacity of these gels would be influenced by the diffusive resistance, as the molecular ratio of the template to the nanopore (mesh) size was in the range of 0.3−0.8. Use of porogens improved the porous structure, while at the same time significantly decreasing the time lag of recognition.

1. Introduction

Configurational biomimetic imprinting (CBIP) is a technique in which functional monomers are allowed to self-assemble around a template molecule and are subsequently cross-linked into place to form a polymeric network.[1−10] After removal of template molecules, specific recognition sites are created in the imprinted polymer[11−18] for the adsorption of template, as shown in Figure 1.

Thus, it has been possible to design several synthetic networks able to recognize and bind biological molecules such as carbohydrates,[19,20] proteins,[21−23] steroids,[24−28] and triglycerides, which may then have great importance and influence in a number of emerging technologies. These materials can be used as unique recognitive systems or can be incorporated into existing drug delivery devices that can aid in the delivery of therapeutic agents or the removal of biomolecules.[29−37]

In the present communication, we describe a new methology to achieve CBIP systems able to recognize steroids such as cholesterol. There is overwhelming evidence that hypercholesterolemia is the major risk factor for the early

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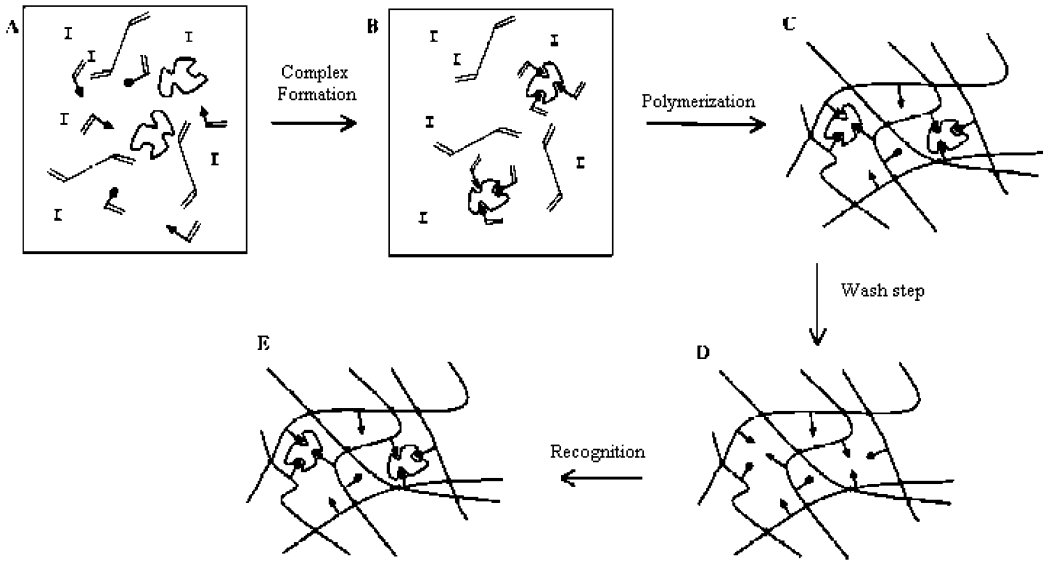

Figure 1. Configurational biomimetic process: (A) solution mixture of template, functional monomer(s) (Δ and O), cross-linking monomer, solvent, and initiator (I); (B) prepolymerization complex formed via covalent or noncovalent interactions; (C) network formation; (D) wash step where original template is removed; and (E) recognition of template.

development of atherosclerosis in man and, thus, the leading cause of coronary heart and peripheral atherosclerotic disease.[38] On the basis of results from various intervention studies, it is well-established that drastic lowering of blood cholesterol is followed by a reduction of clinical events and total mortality.

CBIP may represent one way of imparting recognitive properties to a material. Only a few approaches to imprint cholesterol have been described to date. Its relatively large size (molecular weight of 386.87 g/mol) and its amphipathic nature make imprinting of the cholesterol very difficult. Whitecombe et al.[24] conjugated cholesterol with 4-vinylphenol through a readily hydrolyzable carbonate ester linkage. After polymerization and removal of cholesterol by hydrolysis, rebinding was effected in *n*-hexane by hydrogen bonding between the hydroxyl group of cholesterol and the phenolic group on the polymer.

Sellergren et al.[25] synthesized polymerizable derivatives of cholesterol to be used as amphiphilic monomers in the imprinting of highly cross-linked methacrylates with cholesterol. Hwang and Lee[26] adapted a similar approach, wherein monomers such as cholesteryl(4-vinyl)phenyl carbonate was used for covalent imprinting and 4-vinyl pyridine and methacrylic acid were used for noncovalent imprinting of cholesterol in order to achieve materials useful in the chromatographic field for steroids separation. Asanuma et al.[27] described the cholesterol recognitive properties by polymers prepared by cross-linking β-cyclodextrin with diisocyanates in the presence of cholesterol.

Previous studies have been reported on molecularly imprinting polymers with cholesterol with varying chemical structure and have described mainly their binding capacity, their imprinting effect, and the mechanism of recognition. However, previous work did not provide information about the dynamics of recognition. The dynamics are particularly important if the system is designed for medical applications. Recognitive response within a medically acceptable time period is of utmost importance here.

In this communication, we report on the synthesis, by UV polymerization, of polymeric films able to recognize cholesterol. The materials were prepared from methacrylic acid (MAA) as a functional monomer and ethylene glycol dimethacrylate (EGDMA) or poly(ethylene glycol) dimethacrylate 400 (PEGDMA400) as comonomers/cross-linking agents. MAA is able to interact specifically with cholesterol, while cross-linking agents provide a structural integrity of the hydrogel after removal of the template. The polymerizations were carried out in solvent mixtures with varying polarity. Our studies allowed us to evaluate the effect of this parameter on the porosity of the biopolymers synthesized, as well as on the cholesterol uptake, the imprinting efficiency of the polymer, and, overall, the dynamic of recognition.

Our challenge was to prepare hydrogels able to recognize cholesterol rapidly. This was achieved by synthesis of the polymers in polar solvents such as dimethyl sulfoxide (DMSO) or water. Special reaction conditions leading to very porous materials rendered recognition sites easily accessible.

The hydrophobicity of cholesterol was the biggest problem to overcome in order to carry out the polymerization reactions in polar systems. Thus, we selected to carry out the polymerization in a mixture of solvents. One of the two solvents should render cholesterol soluble, while the other should guarantee a high polarity of the system. We selected tetrahydrofuran (THF) as a good solvent for the cholesterol and one that is completely miscible with polar solvents such as DMSO and water. Thus, we synthesized molecularly imprinted polymers (MIPs) and molecularly nonimprinted polymers (NIPs) in THF/water and THF/DMSO at varying volume ratios. We studied if the addition of a porogen, such as NaCl, could influence the morphology of the material achieved and the cholesterol recognition.

To evaluate the different porosities of materials, all polymeric films synthesized were analyzed by swelling studies according to the Peppas and Meadows theory.[39] Thus,

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Table 1. Preparation Conditions of Cholesterol-Imprinted MIPs and NIPs Prepared by UV Polymerization in THF and in Mixtures of THF/DMSO

cholesterol (mmol)	MAA (mmol)	EGDMA (mmol)	Irgacure184 (mg)	solvent (mL)		sample code
0.284	2.84	9.94	5.72	THF (8.0)	DMSO (0)	MT1
0	2.84	9.94	5.60	THF (8.0)	DMSO (0)	NT1
0.278	2.78	9.72	5.81	THF (4.0)	DMSO (4.0)	MD1
0	2.78	9.72	5.20	THF (4.0)	DMSO (4.0)	ND1
0.265	2.65	9.28	5.93	THF (2.0)	DMSO (6.0)	MD2
0	2.65	9.28	5.83	THF (2.0)	DMSO (6.0)	ND2
0.262	11.94	9.28	8.25	THF (6.0)	DMSO (2.0)	MD3
0	11.94	9.28	8.20	THF (6.0)	DMSO (2.0)	ND3

Table 2. Preparation Conditions of Nonporous Cholesterol-Imprinted MIPs and NIPs Prepared by UV Polymerization in THF and in Mixtures of THF/Water and Microporous MIPs and NIPs Prepared in a THF/NaCl Solution

cholestrol (mmol)	MAA (mmol)	PEGDMA400 (mmol)	Irgacure (mg)	solvent (mL)		sample code
0.260	2.60	9.10	5.63	THF (6.0)	H_2O (2.0)	MW1
0	2.60	9.10	5.74	THF (6.0)	H_2O (2.0)	MW1
0.262	2.62	9.18	5.39	THF (6.0)	NaCl 5.0 mM (2.0)	MW2
0	2.62	9.18	5.43	THF (6.0)	NaCl 5.0 mM (2.0)	NW2
0.262	2.62	9.18	5.27	THF (6.0)	NaCl 2.40 M (2.0)	MW3
0	2.62	9.18	5.43	THF (6.0)	NaCl 2.40 M (2.0)	NW3

we calculated the number-average molecular weight between cross-links $\overline{M_c}$, the network mesh size ξ, the cross-linking density ρ_x, and the equilibrium weight swelling ratio q. The recognition studies were done in a mixture of THF and water (6/5 vol/vol), and the amount of cholesterol recognized was evaluated by HPLC analysis.

2. Experimental Section

2.1. Synthesis of Recognitive Polymers. Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 1-hydroxycyclohexylphenyl ketone (Irgacure 184) were purchased from Sigma-Aldrich (Milwaukee, WI). Poly(ethylene glycol) dimethacrylate 400 (PEGDMA400) was received from Polysciences Inc. (Warrington, PA). Cholesterol was from Mallinckrodt Baker Inc. (Phillipsburg, NJ). THF, DMSO, chloroform, and acetonitrile were from Fischer (Pittsburgh, PA) and were all of HPLC grade.

For the synthesis of MIPs, a solution of cholesterol in different solvents was prepared at the desired concentration, as shown in Tables 1 and 2, and it was subsequently heated to 30 °C and continuously stirred for 15 min or until the cholesterol was dissolved completely. Next MAA, EGDMA or PEGDMA400, and Irgacure 184, as a photoinitiator, were added to the solution. For the polymerization reaction, two 20 × 20 cm glass plates were brought together using binder clips with a 0.7 mm Teflon spacer between the plates and were placed with the solution polymerization in a reaction vessel supply with a UV-light curing system (Dymax Corporation, Torrington, CT). The solution and the reaction vessel were then purged with nitrogen gas for 20 min. The solution was finally transferred in the glass plate assembly and UV polymerized (intensity 16−17 mW/cm^2) for 20 min. Finally, the polymeric film was washed with THF for 72 h and then dried for 12 h in the oven under vacuum. NIPs were prepared under the same conditions except that no template was used.

2.2. Swelling Studies. Investigation of the cross-linked structure of the prepared hydrogels was done by the method of Peppas and Meadows.[39] Specimens of ∼1 cm^2 were cut from each sample following polymerization and weighed in air and in a nonsolvent (*n*-heptane). The latter weight was obtained by placing the sample in a stainless-steel mesh basket suspended in *n*-heptane. The sample was then placed in a PBS solution (pH = 7.0) for 5 days, allowed to swell to equilibrium, and again weighed in air (after being blotted with a tissue to remove surface moisture) and in *n*-heptane.

2.3. Recognitive Studies. In typically experiments MIPs and NIPs (∼15−30 mg) were placed, under stirring, in 15.0 mL of 0.4−0.5 mg/mL cholesterol solution in THF/water (6/5 vol/vol). At predetermined times, 10.0 mL of the solution were extracted with chloroform (3 × 10 mL) and, after evaporation of the solvent, the residual was redissolved in 10.0 mL of acetonitrile and analyzed by high performance liquid chromatography (HPLC) (Shimadzu (Kyoto, Japan) with LC-10 AT VP pump, with SIL-10AD VP auto injector, SCL-10VP system controller and a SPD-10A VP detector set at 208 nm). An Alltech Macrosphere 300 C18 5U (Deerfield, IL) column was employed. The mobile phase was acetonitrile and the flow rate was 0.5 mL/min.

3. Results and Discussion

3.1. Synthesis of the Polymers. Medical importance of lowering cholesterol in the blood is well established.[38] In view of the problems associated with the administration of cholesterol lowering agents such as statins, there is an increasing emphasis on the use of polymeric adsorbents as sequestrants for cholesterol. Since cholesterol contains no ionizable groups, the only recognitive interactions possible are hydrogen bonding and hydrophobic interactions.

In particular, our attention in this work was focused on the synthesis of a system able to recognize the cholesterol rapidly. Molecular imprinting using a covalent approach is reported to be more efficient than the noncovalent approach.[17,23,28,40] Nevertheless, imprinting using a non covalent approach presents the advantage that guest binding and guest release are very fast.

Cholesterol will be able to interact with such structures both because of the existence of a hydrophilic moiety and a hydrophobic structure (see Figure 2).

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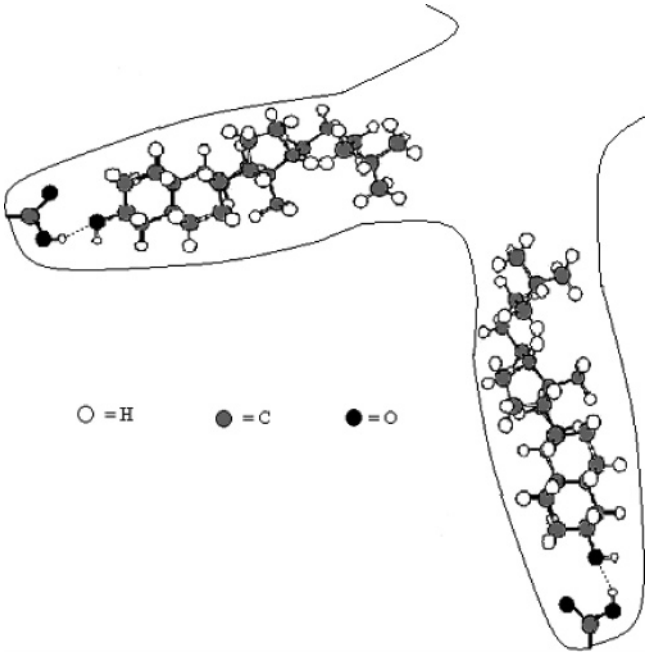

Figure 2. Recognitive interactions of cholesterol with polymer network.

To achieve a system able of rapid cholesterol recognition we carried out the polymerization in a polar solvent. These reactions conditions could ensure the achievement of a porous material which renders the recognition sites easily accessible.

Cholesterol-imprinted MIPs and NIPs were prepared, in a mixture of a cholesterol-dissolving and a polar solvent, by UV polymerization using Irgacure 184 as an initiator. MAA was the main functional monomer, whereas EGDMA or PEGDMA400 acted as comonomers and cross-linking agents. The polymerizations were carried out in THF or in THF/DMSO and THF/water mixtures. To achieve highly porous materials NaCl was used as a porogen.

The polymers synthesized in THF (labeled MT1 and NT1; *nota bene*: in all code numbers the first letter indicates an imprinted (M) or nonimprinted (N) structure) were obtained using MAA as a functional monomer and EGDMA as a comonomer and cross-linking agent. To achieve a recognitive polymer judicious selection of the molar ratios between cholesterol and MAA and between cholesterol and EGDMA was of utmost importance. Reaction optimization showed that the right molar ratio of cholesterol/MAA was 1/10, whereas cholesterol/EGDMA was 1/35.

These reaction conditions were also used to prepare polymers in THF/DMSO mixtures. We synthesized polymeric films employing volume ratios THF/DMSO equal to 1 (samples labeled MD1 and ND1) and 1/3 (samples labeled MD2 and ND2). Besides, polymeric films MD3 and ND3 were prepared using the same conditions for samples MD2 and ND2 respectively, except that the molar ratio of cholesterol/MAA was 1/45.

Finally, we synthesized cholesterol-imprinted MIPs and NIPs (labeled MW1 and NW1) using a mixture of THF/water where the volume ratio was equal to 3. These polymers were prepared by using water soluble PEGDMA400 instead of EGDMA. We also prepared polymeric films using NaCl as a porogen (samples MW2 an NW2 with NaCl solution 5.0 mM and MW3 and NW3 with NaCl solution 2.40 M).

Dynamic and equilibrium swelling studies on the polymeric films were used to analyze the morphology of systems following specific reaction conditions.

Recognitive studies were used to obtain information about the influence of the different reaction conditions on the recognitive capacity, RC, of the system, on the imprinting efficiency (ratio between recognitive capacity of MIPs and recognitive capacity of NIPs) and on the speed of recognition.

3.2. Swelling Studies. Characterization of the cross-linked structure of the MIPs and NIPs samples was achieved by equilibrium swelling studies in a PBS solution (pH = 7.0) at 37°C as described by Peppas and Meadows[39] and Peppas and Barr-Howell.[41] The polymer volume fraction in the gel immediately after preparation (relaxed state), $\upsilon_{2,r}$, and the polymer volume fraction of the swollen gels (swollen state), $\upsilon_{2,s}$, were determined using eqs 1 and 2:

$$\upsilon_{2,r} = \frac{V_p}{V_{g,r}} \tag{1}$$

$$\upsilon_{2,s} = \frac{V_p}{V_{g,s}} \tag{2}$$

Here, $V_{g,r}$ is the volume of polymer immediately after the polymerization and V_p and $V_{g,s}$ are the gel sample volumes before and after equilibrium swelling, respectively. These volumes were determined from the weights of the polymer in air, $W_{a,r}$, and in *n*-heptane, $W_{h,r}$, before swelling and the weights in air, $W_{a,s}$, and in *n*-heptane, $W_{h,s}$, after swelling. They were calculated using eqs 3 and 4, where ρ_h is the density of *n*-heptane.

$$V_p = \frac{W_{a,r} - W_{h,r}}{\rho_h} \tag{3}$$

$$V_{g,s} = \frac{W_{a,s} - W_{h,s}}{\rho_h} \tag{4}$$

The equilibrium polymer volume fractions in equilibrium swollen gels are presented in Table 3 for hydrogels of varying polymerization conditions. The data showed that, as the polarity of the reaction mixture increased, the polymer volume fraction of the swollen gels decreased. This reduction was more evident in macroporous MIPs, when a porogen such as NaCl was used. The results also demonstrated that, under the same polymerization conditions, the MIPs exhibited lower $\upsilon_{2,s}$ values than the NIPs, indicating that the presence of cholesterol led to the formation of a more nanoporous structure.

The equilibrium swelling data were used to evaluate the cross-linked structure of the polymers. Typically, the number-average molecular weight between cross-links, $\overline{M}_c$, was calculated. This parameter is an indication of the cross-linked nature of the hydrogel, as high values of $\overline{M}_c$ imply loosely cross-linked hydrogel. Experimental values of $\overline{M}_c$ were calculated using an expression developed by Peppas and

(41) Peppas, N. A.; Barr-Howell, B. D. *Hydrogels in Medicine and Pharmacy*; CRC Press: Boca Raton, FL, 1979; p 27.

Table 3. Molecular Parameters of Networks of Cholesterol MIPs and NIPs Prepared According to the Conditions of Samples in Tables 1 and 2

polymer	polymer components[a]	equilibrium polymer vol fraction, $v_{2,s}$	molecular weight between cross-links, $\overline{M_c}$(g·mol^{-1})	mesh size, ξ (Å)	cross-linking density, ρ_x x 10^4 (mol/cm^3)	equilibrium weight swelling ratio, q
MT1	cholesterol/MAA = 1:10 (mol/mol) cholesterol/EGDMA = 1:35 (mol/mol)	0.341	585	15.4	2.93	11.1
NT1	THF	0.346	490	14.0	2.9	13.1
MD1	cholesterol/MAA = 1:10 (mol/mol) cholesterol/EGDMA = 1:35 (mol/mol)	0.325	760	17.8	3.1	9.1
ND1	THF/DMSO = 1:1 (vol/vol)	0.335	700	16.8	3.0	10.3
MD2	cholesterol/MAA = 1:10 (mol/mol) cholesterol/EGDMA = 1:35 (mol/mol)	0.299	1250	23.5	3.3	6.4
ND2	THF/DMSO = 1:3 (vol/vol)	0.302	1100	22.0	3.3	6.7
MD3	cholesterol/MAA = 1:45 (mol/mol) cholesterol/EGDMA = 1:35 (mol/mol)	0.275	1880	29.6	3.6	4.5
ND3	THF/DMSO = 1:3 (vol/vol)	0.282	1670	27.6	3.5	5.0
MW1	cholesterol/MAA = 1:10 (mol/mol) cholesterol/PEGDMA400 = 1:35 (mol/mol)	0.306	1100	16.1	3.3	7.1
NW1	THF/H_2O = 3:1 (vol/vol)	0.312	1100	15.8	3.2	7.7
MW2	cholesterol/MAA = 1:10 (mol/mol) cholesterol/PEGDMA400 = 1:35 (mol/mol)	0.262	2100	23.3	3.8	3.6
NW2	THF/NaCl 5.0 mM = 3:1 (vol/vol)	0.267	1910	22.0	3.7	3.8
MW3	cholesterol/MAA = 1:10 (mol/mol) cholesterol/PEGDMA400 = 1:35 (mol/mol)	0.225	5050	37.9	4.4	1.7
NW3	THF/NaCl H_2O 2.45 M = 3:1 (vol/vol)	0.236	3645	31.7	4.2	2.2

[a] The ratios of monomers or cholesterol to solvent(s) are reported in Tables 1 and 2.

Merrill[42] for swollen networks produced by cross-linking in solution and are presented in Table 3:

$$\frac{1}{\overline{M_c}} = \frac{2}{\overline{M_n}} - \frac{\frac{\bar{v}}{V_1}[\ln(1 - v_{2,s}) + v_{2,s} + \chi v_{2,s}^2]}{v_{2,r}\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \frac{1}{2}\left(\frac{v_{2,s}}{v_{2,r}}\right)\right]} \quad (5)$$

Here, V_1 is the molar volume of water (18.1 cm^3/mol); $\overline{M_n}$ (~76.000 g·mol^{-1}) is the number-average molecular weight of the linear polymer produced under the same conditions of polymerization but without cross-linking agent and $\bar{v}$ is the specific volume of the polymer defined as the ratio between the weight of the polymer, $W_{a,r}$, in air before swelling and the volume, V_p. The Flory polymer−solvent interaction parameter, χ, was calculated as a weighted average of the values for poly(methacrylic acid) (PMAA; χ = 0.5987) and poly(ethylene glycol) (PEG; χ = 0.55) in water.[43] Such averaging procedures are not usually acceptable in polymer thermodynamics but are very appropriate here because of the similarity of the parameter values under the conditions of operation.

The results of these studies indicate that, by increasing the polar solvent content in the reaction mixture, the $\overline{M_c}$ value of the imprinted polymers increased from 585 g·mol^{-1} for polymer MT1 (corresponding to ~7 methacrylic acid units) to 1250 g·mol^{-1} for polymer MD2 (corresponding to ~15 methacrylic acid units). Clearly, this led to a doubling in the number of repeating units between consecutive tie-points (junctions). Considering that the actual end-to-end distance between consecutive cross-links in networks with Gaussian distribution is proportional to the square-root of $\overline{M_c}$, the linear molecular distance of the recognitive nanopores (in **Å**) increased by 41% simply because of the presence of the polar agent DMSO. The advantages of DMSO in imprinting have been already discussed by Byrne and Peppas[2] and Byrne and co-workers.[32]

A comparison of the results for MD2 and MD3 indicates that, by increasing the amount of MAA in the networks, the $\overline{M_c}$ value increased to 1880 g·mol^{-1} (corresponding to ~22 methacrylic acid units). Again, this unexpected increase in the number of repeating units between junctions (~32% in the presence of MAA) can be attributed to the synergistic effect of DMSO on the network structure. The same behavior was observed when using water as the polar solvent. In particular, polymer MW1 exhibited a value of $\overline{M_c}$ equal to 1110 g·mol^{-1}(corresponding to ~13 methacrylic acid units). Production of the recognitive networks in the presence of a porogen led to a microporous network structure, evidently with rather high values of $\overline{M_c}$. In particular, MIPs such as MW2 had a value of 2100 g·mol^{-1}, whereas a value of 5050 g·mol^{-1} was observed for polymer MW3. These values corresponded to ~25 and to 60 methacrylic acid units, respectively. A similar trend was observed for the $\overline{M_c}$ values of all NIP polymers.

3.3. Mesh Size and Micro- and Nanoporous Structural Analysis. The primary mechanism of recognition of many drugs or other biological molecules in hydrogels is a binding interaction of specific functional groups. However, diffusion plays a major role in the overall process as it occurs through the space available between macromolecular chains. This space is often regarded as the "pore". Depending upon the size of these pores, hydrogels can be macroporous, microporous, or nonporous (also often named nanoporous). A structural parameter that can be used to describe the size of the recognitive pores is the correlation length, ξ, which is

(42) Peppas, N. A.; Merrill, E. W. *J. Polym. Sci., Polym. Chem.* **1976**, *14*, 441.
(43) Lowman, A. Ph.D. Thesis, Dept. Chem. Eng., Purdue University, West Lafayette, IN, 1997.

defined as the linear distance between two adjacent cross-links and can be calculated using the following equation:

$$\xi = \alpha(\bar{r}_o^{\,2})^{1/2} \tag{6}$$

Here, α is the elongation ratio of the polymer chains in any direction and $(\bar{r}_o^{\,2})^{1/2}$ is the root-mean-square, unperturbed, end-to-end distance of the polymer chains between two neighboring cross-links.[44]

For isotropically swollen hydrogel, the elongation ratio, α, can be related to the swollen polymer volume fraction, $v_{2,s}$, using eq 7.

$$\alpha = v_{2,s}^{\,-1/3} \tag{7}$$

The unperturbed end-to-end distance of the polymer chain between two adjacent cross-links can be calculated using eq 8, where C_n is the Flory characteristic ratio, l is the length of the bond along the polymer backbone (for methacrylates polymers, 1.54 Å), and N is the number of links per chain that can be calculated by eq 9.

$$(\bar{r}_o^{\,2})^{1/2} = l(C_n N)^{1/2} \tag{8}$$

$$N = \frac{2\bar{M}_c}{M_r} \tag{9}$$

In eq 9, M_r is the molecular weight of the repeating units from which the polymer chain is composed. Finally, when one combines eqs 6−9, the correlation distance between two adjacent cross-links in a swollen hydrogel can be obtained:

$$\xi = v_{2,s}^{\,-1/3}\left(\frac{2C_n\bar{M}_c}{M_r}\right)^{1/2} l \tag{10}$$

A detailed theoretical characterization of the network structure of the MIP carrier in terms of the correlation length or mesh size, ξ, in combination with diffusion studies of templates provides an invaluable insight into the very complex structure of polymer networks and aids in the design of better recognitive networks.[45]

To investigate the importance of diffusional limitations in the recognitive process, the network mesh size, ξ, was next calculated using eq 10. In this expression, C_n, the Flory characteristic ratio, was $C_{n,PMAA} = 14.6$ and $C_{n,PEG} = 3.8$ for the two polymers used.[43]

The results of these calculations are presented in Table 3 and are quite revealing of the performance of the new imprinted hydrogels. The values of ξ vary from 15.4 to 37.9 Å**.** It must be noted that the radius of gyration of cholesterol is reported[46] as 5.8 Å, indicating an average molecular diameter of 11.6 Å. As shown in Figure 2, for most imprinted systems, there will be a very tight molecular configuration. Meadows and Peppas[39] indicate that a molecular ratio of radii of template and "pores" in the order of 0.6−0.3 would already reduce the solute diffusion coefficient by 1 order of magnitude.

The swelling results showed that the polarity of the polymerization mixture influences and eventually controls the morphology of the materials. In particular, when the imprinting reaction was performed in a very polar mixture of solvents, both values of $\bar{M}_c$ and ξ increased. These results demonstrate that, by modifying the polarity of the solvent of polymerization, it was possible to change the morphology of the material.

Previous studies have ignored the importance of the diffusional limitations of template transport on the recognitive behavior of a system. The diffusional effects on the recognitive behavior of these networks were further analyzed here using free-volume-based and hydrodynamic theories developed by us and others.

Recognition of an active agent by a polymeric hydrogel first requires the movement of the agent through the bulk of the polymer. This phenomenon can be described by Fick's law of diffusion:

$$\frac{\partial c_i}{\partial t} = D_{ip}\frac{\partial^2 c_i}{\partial x^2} \tag{11}$$

Here, c_i is the concentration of a template molecule i, D_{ip} is the diffusion coefficient in the recognitive polymer, and x and t are position and time, respectively. It should also be emphasized that, in this form of Fick's law, the diffusion coefficient is assumed to be independent of concentration. This assumption, while not conceptually correct, has been largely accepted because of the computational simplicity. A summary of the various forms of the diffusion coefficient is provided in Table 4.

One of the earliest approaches of estimating the diffusion coefficient through an MIP polymer carrier is that of Eyring.[47] Template diffusion through a medium is presented as a series of diffusional jumps instead of a continuous process. Therefore, in eq 12 in Table 4, which comes from the Eyring analysis, κ is the diffusional jump of the drug in the polymer and ν is the frequency of jumping.

Fujita[48] utilized the idea of free volume in polymers to estimate the drug diffusion coefficient and arrived at an exponential dependence of the drug diffusion coefficient on the free volume, v_f, which is given by eq 13 in Table 4. Yasuda and Lamaze[49] refined Fujita's theory and presented a molecularly based theory, which predicts the diffusion coefficients of templates through a recognitive MIP matrix rather accurately (eq 14). In their treatment, the normalized diffusion coefficient, i.e., the ratio of the diffusion coefficient of the solute or template in the polymer, $D_{2,13}$, to the diffusion coefficient of the template in water, $D_{2,1}$, is related to the degree of hydration, H, and the free volume occupied by the swelling medium, $V_{f,1}$. In addition, φ is a sieving factor which provides a limiting mesh size impermeable to drugs with cross-sectional area q_s, and B is a parameter characteristic of the polymer. In eq 14, the subscripts 1, 2, and 3 refer to the solvent, template, and MIP, respectively.

(44) Canal, T.; Peppas, N. A. *J. Biomed. Mater. Res.* **1989**, *23*, 1183.
(45) Narasimhan, B.; Peppas, N. A. *J. Pharm. Sci.* **1997**, *86*, 297.
(46) Kim, H.; Kang, S.; Jung, S. *Bull. Korean Chem. Soc.* **2001**, *22* (9), 979.
(47) Eyring, H. *J. Chem. Phys.* **1936**, *4*, 283.
(48) Fujita, H. *Fortschr. Hochpolym.-Forsch.* **1961**, *3*, 1.
(49) Yasuda, H.; Lamaze, C. E. *J. Macromol. Sci. Phys.* **1971**, *B5*, 111.

Table 4. Template Diffusion Coefficients in Relation to Hydrogel Structural Parameters

type of recognitive hydrogel (network)	eq. no.	eq for D_{ip}	ref.
all systems	12	$D_{ip} = \frac{\kappa^2 \upsilon}{6}$	47
nonporous or nanoporous	13	$D_{ip} = D_o \exp\left\{-\frac{k}{\upsilon_f}\right\}$	48
nonporous or nanoporous	14	$\frac{D_{2,13}}{D_{2,1}} = \varphi(q_s) \exp\left[-B\left(\frac{q_s}{V_{f,1}}\right)\left(\frac{1}{H}-1\right)\right]$	49
nonporous or nanoporous	15	$\frac{D_{2,13}}{D_{2,1}} = k_1\left(\frac{\bar{M}_c - \bar{M}_c^*}{\bar{M}_n - \bar{M}_c^*}\right)\exp\left(-\frac{k_2 r_s^2}{Q-1}\right)$	50
microporous	16	$D_{eff} = D_{iw} K_p K_r \frac{\epsilon}{\tau}$	51
microporous	17	$\frac{D_{ip}}{D_w} = (1-\lambda)^2(1 - 2.104\lambda + 2.09\lambda^3 - 0.95\lambda^5)$	51

Peppas and Reinhart[50] also developed a theoretical model based on a free volume of the polymer matrix, here, the MIP. In their theory, they assumed the free volume of the polymer to be the same as the free volume of the solvent, and they arrived at eq 15 in Table 4. They related the normalized diffusion coefficient to the degree of swelling, Q, the solute radius, r_s, and the molecular weight of the polymer chains. All the other parameters, $\bar{M}_c$ and $\bar{M}_n$, are defined as before, and $\bar{M}_c$* is the critical molecular weight between cross-links, below which a drug of size r_s could not diffuse through the polymer network. In addition, k_1 and k_2 are constants related to the polymer structure. This theory is applicable to template transport in swollen, nanoporous hydrogels.

Yet another approach for the prediction of the diffusion coefficient of a template in a recognitive network is adopted here. In these MIPs, the diffusion and recognition are assumed to be taking place predominantly through the water or physiological fluids that fill the pores. The template diffusion coefficient in an MIP, D_{ip}, in eq 11 is replaced by an effective diffusive coefficient, D_{eff}, which is defined by eq 16 in Table 4. Here, ϵ is the porosity, or void fraction, of the polymer, which is a measure of the volume of the pores available for diffusion and τ is the tortuosity, which describes the geometric characteristics of the pores. The term K_p is the equilibrium-partitioning coefficient, which is an important parameter when the template is soluble in the polymer matrix and is the ratio of the concentration inside of the pore to the concentration outside of the pore. The term K_r describes the fractional reduction in diffusivity within the pore when the template diameter, d_s, is comparable in size to the nanopore diameter d_r.

Equation 17 in Table 4 is a semiempirical relation proposed by Peppas and Meadows[51] for diffusion of solutes through porous media. In this equation, λ is the ratio of the template radius, r_s, to the average pore radius, r_p, while D_{ip} and D_w are the diffusion coefficients of the template through the pore and in water, respectively. It is clear that, as the size of the template gets smaller with respect to the size of the nanopore, the ratio of D_{ip}/D_w approaches the limit of 1.

It is then quite clear that, as shown in Table 3 for all imprinted systems studied here, there is a very tight molecular configuration. The molecular ratio of radii of solute and pores in the order of 0.3−0.8, which would already reduce (according to eq 17) the template diffusion coefficient by 1 order of magnitude. Figure 3 presents the normalized cholesterol diffusion coefficient through cholesterol-imprinted MIPs prepared under the conditions described in this contribution. The molecular restrictions provided by mesh size, ξ, as calculated above, was quantified by the Peppas and Meadows analysis.[51] Clearly, a polynomial expression of importance for diffusional and recognitive processes is established here. Because of the values of the restriction parameter, λ, being between 0.3 and 0.8, the corresponding reduction in the template (cholesterol) diffusional coefficient is significant. Indeed, for samples MT1 with a characteristic equilibrium mesh size $\xi = 15.4$ Å, the reduced diffusion coefficient is $D_{ip}/D_w = 5 \times 10^{-3}$.

The implications of this analysis are profound. From a recognitive point of view, systems such as the ones developed here will have a response (or recognition) time on the order of 3−4 h. Of course, a faster response can be achieved by decreasing the size of the recognitive system, because the characteristic diffusion time is proportional to the length squared and inversely proportional to the template diffusion coefficient. It is also interesting to note that a significant reduction of the diffusional limitations can be achieved only in very "open" recognitive networks. Indeed, according to Figure 3, the extrapolated value of the reduced diffusion coefficient at $\lambda = 0.2$ would be $D_{ip}/D_w = 0.381$. Clearly, such values would correspond to recognitive networks with mesh sizes >58 Å, which are attainable only with addition of a very large amount of porogen.

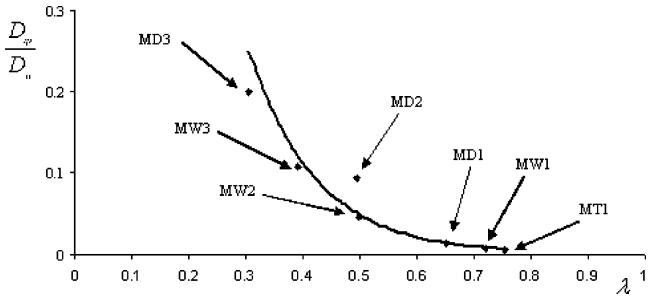

Figure 3. Normalized cholesterol diffusion coefficient as a function of restriction parameter λ (cholesterol diameter/network mesh size) for various recognitive networks, according to Peppas and Meadows.[51]

(50) Peppas, N. A.; Reinhart, C. T. *J. Membr. Sci.* **1983**, *15*, 275.
(51) Peppas, N. A.; Meadows D. L. *J. Membr. Sci.* **1983**, *16*, 361.

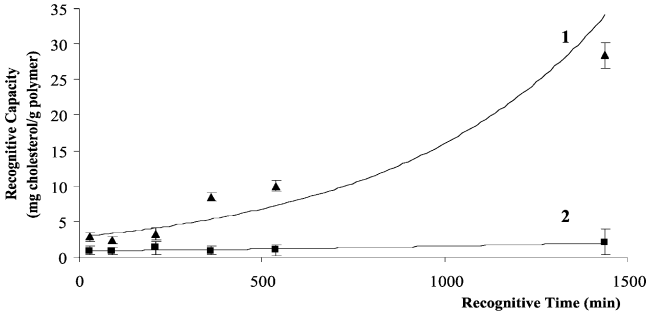

Figure 4. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MT1(s) and NT1(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MT1 (curve 1) and NT1 (curve 2).

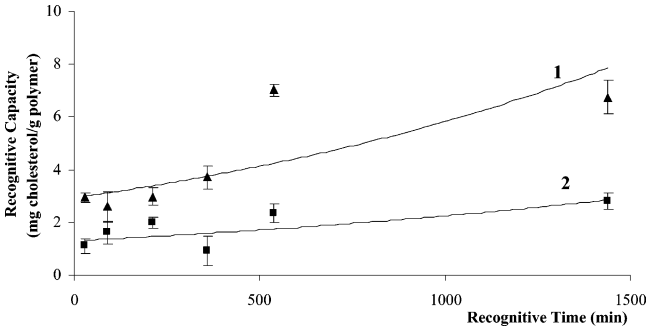

Figure 5. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MW1(s) and NW1(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MW1 (curve 1) and NW1 (curve 2).

Finally, the cross-linking density, ρ_x, (eq 18) and the equilibrium weight swelling ratio q (eq 19) were calculated.

$$\rho_x = \frac{1}{v \cdot \overline{M}_c} \tag{18}$$

$$q = \frac{1}{v_{2,s}} \tag{19}$$

The results are reported in Table 3 and demonstrate the dependence of ρ_x and q on the polarity of polymerization solvent for all polymers synthesized.

3.4. Evaluation of Polymers Synthesized. Recognitive studies allowed an understanding of the influence of different reaction conditions on the ability of the systems to absorb cholesterol. These studies permitted us to determine the imprinting efficiency, IE, defined by eq 20.

$$\mathrm{IE} = \frac{\mathrm{RC_{MIP}}}{\mathrm{RC_{NIP}}} \tag{20}$$

Evaluation of the capacity of the polymers to recognize and bind the cholesterol was performed in a mixture of THF/water (6/5 vol/vol). Typically, MIPs and NIPs were placed in a solution of ~0.5 mg/mL of cholesterol and the cholesterol was determined at different times.

The data showed clearly that recognitive capacity, RC, and imprinting efficiency depended on the reaction conditions. Clearly, these parameters influenced the recognition dynamics. For example, the MIP polymer MT1 showed a RC of 28.43 mg/g and an IE of 13.35 after 24 h. Nevertheless, the recognitive capacity was evident only after 6−9 h, as shown in Figure 4. Inspection of the data in Figure 4 indicates that there is a significant delay in cholesterol recognition. In fact, a fitting of the recognitive data of sample MT1 indicated that recognition is substantial (>5 mg/g) only after ~4 h. A "time lag of recognition" indicates that diffusional limitations are significant early in the recognition process.

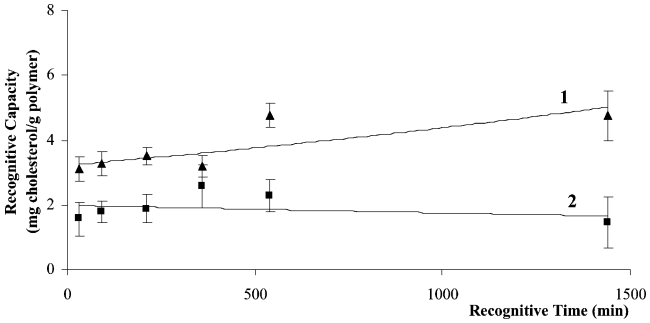

Figure 6. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MW2(s) and NW2(n) as a function of recognition time in THF/water solution (6/5 volume ratio). Exponential curve fitting is also shown for MW2 (curve 1) and NW2 (curve 2).

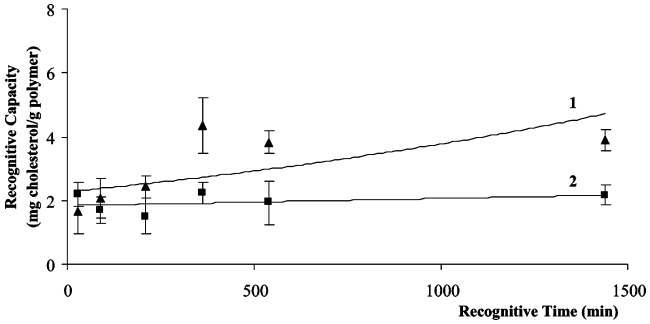

Figure 7. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MW3(s) and NW3(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MW3 (curve 1) and NW3 (curve 2).

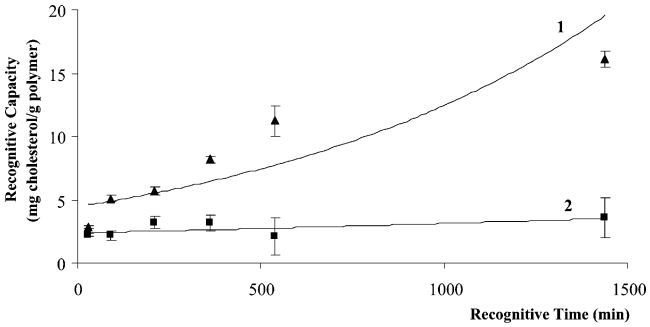

Figure 8. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MD1(s) and ND1(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MD1 (curve 1) and ND1 (curve 2).

The RC and IE of sample MW1 were smaller than for MT1, as shown in Figure 5. Introducing a porogen led to a decrease of the IE (Figure 6). This behavior is clearer as we studied the effect of increasing NaCl concentration on the imprinting process (Figure 7). The results suggest that, although very porous materials were obtained, the water in the reaction mixture did not permit the interaction between the template and the functional monomer in the first step of polymerization. This problem was associated with the disruption of the hydrogen bond at high water concentrations.

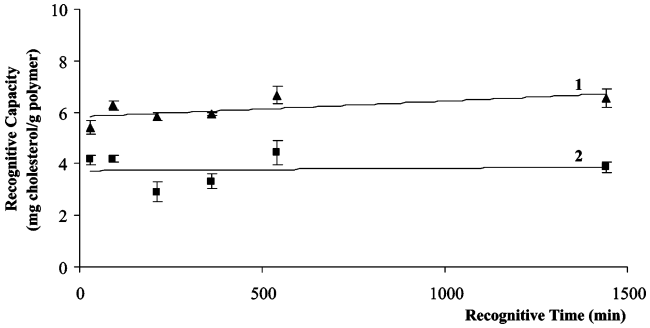

Figure 9. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MD2(s) and ND2(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MD2 (curve 1) and ND2 (curve 2).

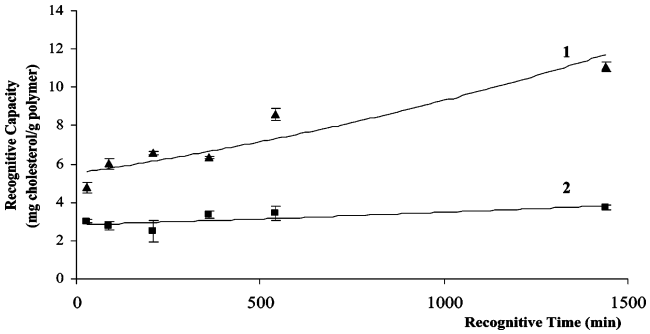

Figure 10. Cholesterol recognitive capacity (mg of cholesterol per g of dry polymer) of cholesterol-imprinted polymers MD3(s) and ND3(n) as a function of recognition time in THF/water (6/5 vol/vol). Exponential curve fitting is also shown for MD3 (curve 1) and ND3 (curve 2).

By introducing DMSO as a reaction solvent, interesting results were obtained. As shown in Figure 8, sample MD1 exhibited RC = 5.10 mg/g already after 90 min, approximately double the value of sample MT1 (2.45 mg/g). However, the IE and RC after 24 h were lower for sample MT1.

These results indicate the importance of the nature of the reaction solvent in the MIP network formation. Because DMSO is an aprotic and polar solvent, it does not compete in the formation of the hydrogen bond host−guest and at once permits the achievement of porous materials.

As shown in Figure 9, the sample MD2 exhibited RC = 5.41 mg/g after 30 min but low IE (<2). This polymer was not able to recognize high amounts of cholesterol (6.56 mg/g after 24 h).

By increasing the MAA content in the network, it was possible to achieve a high RC after 30 min and at the same time increase the IE and the amount of cholesterol detected after 24 h.

Sample MD3, as shown in Figure 10, exhibited a RC of 4.67 mg/g after 30 min and 11.07 mg/g after 24 h and an IE of nearly 3.

4. Conclusions

In conclusion, the design of a precise macromolecular chemical architecture that can recognize target molecules such as cholesterol from an ensemble of closely related molecules has a large number of potential applications. The main thrust of this research was focused upon three-dimensional imprinting, a method of recognition within a bulk polymer matrix.

Thus, polymer structures in contact with biological fluids, cells, or cellular components can be tailored to provide specific recognition properties or to resist binding, depending on the intended application and environment. Engineering the molecular design of biomaterials by controlling recognition and specificity is the first step in coordinating and duplicating complex biological and physiological processes.

We have shown that the choice of reacting monomers and reaction solvents has a major effect on the recognitive capabilities for cholesterol, and we have been able to analyze the three-dimensional network structure of these systems and identify the importance of a diffusional versus recognitive action.

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