Discriminate Surface Molecular Recognition Sites on a Microporous Substrate: A New Approach

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ABSTRACT: A novel two-dimensional surface molecular imprinting method using water-in-oil emulsion photopolymerization on a microporous polypropylene substrate was developed and used to separate the bronchodilator, theophylline, from the mild stimulant, caffeine, both of similar chemical structure. Surface molecular recognition sites were generated that could discriminate between these two molecules with a concentration-dependent separation factor of 4.9 ± 0.8 for theophylline over caffeine at 0.2 mM (1:1 v/v) solution mixture. Besides demonstrating its proof of concept, the attractive features of this new imprinting method are the following: fewer imprint molecules are needed, it can be used to imprint from *aqueous* environments, postcrushing of the solid matrix is not needed, recognition sites are all near or at the surface reducing mass transfer limitations, and imprinting on synthetic microporous membranes (with convection and diffusion) instead of beads (with only diffusion) speeds up the process.

Introduction

Molecular imprinting, first introduced by the group of Wulff,[1] allows specific recognition sites for a desired target molecule (template) to be formed in synthetic polymers. Covalent interactions as well as noncovalent forces (self-assembly) such as hydrogen-bonding, electrostatic, charge-transfer, and hydrophobic interactions between the templates and functional monomers attached to the polymer matrix have been used to form molecular recognition sites.[2] The method has application in, among other areas, separations, catalysis, chemosensors, and biosensors and in the development of artificial antibodies.[3] It is one of several synthetic methods aimed at developing affinity systems capable of mimicking exquisite specific molecular recognition commonly found in biological systems. Other methods include using biocombinatorial methods such as phage display[4] and the use of biologically derived protein binding domains such as the maltose binding domain.[5]

The organizational process of the template in the polymer matrix has been mainly achieved by the following two methods. In the first method, a template molecule along with a functional monomer, a cross-linking agent, a radical initiator, and an organic solvent are polymerized thermally or photochemically.[6] We refer to this approach as *three-dimensional* molecular imprinting. The recognition sites, defined by their cavity size, shape, and chemical functionality, are formed in the bulk polymer matrix. Access to some or most of these sites or cavities is accomplished by crushing the solid polymer matrix and using the resultant particles in batch or column format. Several shortcomings of this approach include the need for substantial amounts of the template, the low capacity, the heterogeneity of the binding sites (diffusional mass transfer limitations), the restriction to imprinting small organic molecules, and the need for sufficiently apolar solvents.[1,7,8]

The second method and the one we report on here, *two-dimensional* (in-plane) molecular imprinting, involves the creation of template cavities on the surface of solid supports such as gold substrates[9,10] synthetic membranes,[11] and molecularly smooth mica.[12] Most studies with imprinted polymers have been carried out using the three-dimensional imprinting method and focused on selective binding, transport, and separation.[1,2,13] Thus, surface imprinting on synthetic commercial membranes may address the limitations listed above by requiring less imprint molecules (as none is needed in the polymer interior), increasing site capacity, access and speed of the imprint molecules for the imprint sites, reduce tailing with increased mass transfer, and possibly allowing imprinting with larger molecules of biological interest in aqueous environments.

In this paper, we describe a novel two-dimensional method for surface imprinting recognition sites onto a polypropylene microporous membrane using a water-in-oil emulsion with photoinduced polymerization. A fairly difficult separation of the bronchodilator, theophylline (THO), from the mild stimulant, caffeine (CAF), was used to test the novel surface emulsion polymerization method (Figure 1). Molecular recognition sites of THO-imprinted membranes were prepared. Attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR), contact angle, atomic force microscope (AFM), and NMR measurements were used to confirm and follow the surface modification and to analyze the efficacy of this process in *aqueous* medium. Following the results, the mechanism and other aspects of preparing discriminate molecular recognition sites are discussed.

Experimental Section

Materials. Toluene, mesitylene (1,3,5-trimethylbenzene), oleic acid, 2-(trifluoromethyl)acrylic acid (TFMAA), theophylline (THO = 1,3-dimethylxanthine), and caffeine (CAF = 1,3,7-

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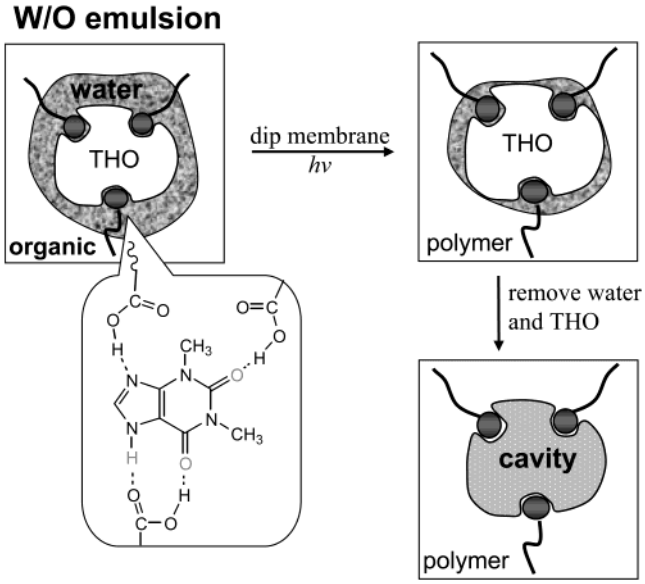


Figure 1. Schematic representation of a novel two-dimensional method for surface imprinting recognition sites onto a microporous substrate (Celgard 2500 microporous flat sheet polypropylene membrane) using water-in-oil emulsion photoinduced polymerization.

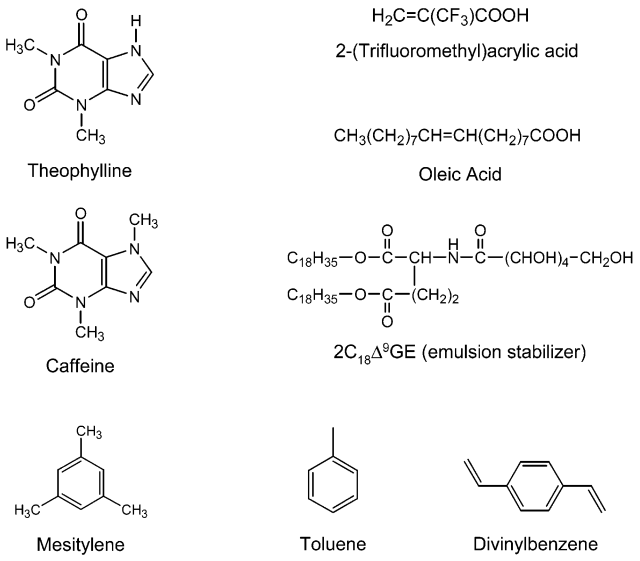
trimethylxanthine) were purchased from Sigma-Aldrich Co. Divinylbenzene (DVB, Aldrich Chemical Co.) was used after treatment with silica gel to remove an inhibitor (Chart 1). Polypropylene (PP) membranes (Celgard 2500 microporous flat sheet polypropylene membrane; thickness 25 μm; porosity 55%; pore size 0.05−0.2 μm wide, 0.2−0.5 μm long; Celgard Inc., Charlotte, NC) were used as microporous substrates.

Preparation of Molecularly Imprinted Polymer. 0.56 g (2.0×10^{-3} mol) of oleic acid, 0.28 g (2.0×10^{-3} mol) of 2-(trifluoromethyl)acrylic acid (TFMAA), and 0.23 g (3.0×10^{-4} mol) of *N*-ribitol-L-glutamic acid dioleyl diester ($2C_{18}\Delta^9$GE, emulsion stabilizer[14]) were dissolved in 60 mL of toluene/DVB (1:2 (v/v)), which was mixed with 30 mL of aqueous solution containing 0.14 g (8×10^{-4} mol) of theophylline. Although methacrylic acid (MAA, pK_a = 4.6) and TFMAA (pK_a = 2.3) have been used as functional monomers for various template molecules, we chose TFMAA since it was more acidic and could increase hydrogen bonding with the template.[6a,c,15] Using only oleic acid (without TFMAA) as a functional monomer, selectivity for THO over CAF was hardly noticeable. The mixture was sonicated for 5 min to give a water-in-oil (w/o) emulsion. After adding 0.36 g of 2,2′-azobis(isobutyronitrile) (AIBN), the mixture (w/o emulsion) was sonicated for 3 min. After the mixture was kept at room temperature for 5 min, 24 mL of upper organic layer was removed to concentrate the emulsion.

The polypropylene membrane (5 cm × 8.8 cm) was dipped into the emulsion for 5−10 s. The membrane was fixed to the polypropylene holder and placed in the quartz vessel. After a 5 min nitrogen purge, the polypropylene membrane was modified using a UV-induced polymerization procedure. A Rayonet photochemical chamber reactor system (model RPR-100, Southern New England, Ultraviolet Co., Branford, CT) with 16 300 nm UV lamps (∼15% of the energy was at <280 nm) was used. This was the same UV reactor used previously by our group.[16] After UV irradiation for 10 min, the membrane was washed with 5 wt % aqueous acetic acid (1 h, 3 times) at room temperature to remove the absorbed THO.[17] The imprinted membrane was dried under vacuum. The nonimprinted polypropylene membrane was prepared using the same procedure as THO-imprinted one in the absence of theophylline.

Characterization. Attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR) (Magna-IR 550 series II, Nicolet Instruments, Madison, WI) was used to confirm polymerization and to measure the degree of grafting onto the polypropylene membrane under UV irradiation. Using

Chart 1. Chemical Structure of the Materials Used for Imprinting THO on a Microporous Polypropylene Membrane Using a Water-in-Oil Emulsion Photopolymerization Method



an incident angle of 45°, the penetration of IR sample depth was approximately 0.1−1.0 μm.[18] Each spectrum was recorded at a resolution of 4.0 cm^{-1}. The absorbance peak heights at 1376 and 1458 cm^{-1} were due to C−H bending of polypropylene membrane.[19] The degree of grafting is defined as

$$\mathrm{DG} = [I_{1600}/I_{1376}]_t - [I_{1600}/I_{1376}]_{\text{unmodified PP}} \qquad (1)$$

where $[I_{1600}/I_{1376}]_t$ and $[I_{1600}/I_{1376}]_{\text{unmodified PP}}$ are the ratio of the peak height of the benzene carbon−carbon double (C=C) bond at 1600 cm^{-1} to that of C−H bending of the polypropylene membrane at 1376 cm^{-1} at the irradiation time (t) and initially for the unmodified polypropylene membrane, respectively.

Sessile contact angles, θ, of water drops on unmodified and modified polypropylene membrane surfaces were measured using a video camera (SIT66, Dage-MTI Inc., Michigan City, IL) connected to a video screen and tape recorder.[16] Our group has extensive experience in measuring contact angles on microporous surfaces and has developed a protocol for correcting for capillary forces and roughness.[20] Unfortunately, the standard captive bubble in the water method could not be used here because of the solubility of THO and CAF in water. Hence, we had to use the standard sessile water drop method with many measurements (at least 10 for each sample) and very fast after the drop had been deposited onto the substrate (within 20−30 s) in order to minimize capillary effects. The standard error in θ was approximately ±2°.

Topographical AFM images of unmodified and imprinted polypropylene membranes were made in contact mode using silicon nitride cantilevers (Veeco Instruments Inc., Sunnyvale, CA) with an atomic force microscope (AFM, Auto Probe PC, Veeco Instruments Inc.) and surface analysis and data acquisition software (Pro Scan Version 1.5, Veeco Instruments Inc.). Taniguchi et al.[20] have used AFM to estimate the roughness of a surface (mean vertical, δ_V, and horizontal, δ_H, length scales and mean roughness angle, α) and correct the measured contact angles to obtain an intrinsic (or corrected) contact angle for a rough or porous surface. More than 300 measurements of the depth (mean vertical distance of top of peak to bottom of groove) and the width (mean horizontal peak to peak) for each membrane surface were obtained. Other common measures of roughness that will be used include the average roughness, R_{ave} ($= \sum_{n=1}^{N} |Z_n - \tilde{Z}|/N$, where $\tilde{Z}$ = mean height), and the roughness factor, γ (ratio of the actual surface area to the projected surface area).

^{1}H NMR spectra were obtained using a Varian 500 MHz spectrometer (Varian Associates Inc.) at room temperature.

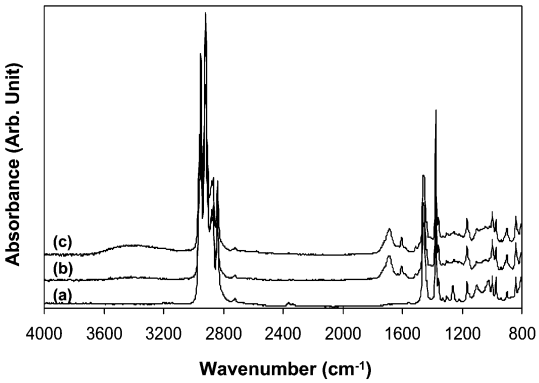


Figure 2. ATR/FT-IR spectra of (a) unmodified polypropylene membrane, (b) THO-imprinted polypropylene membrane, and (c) after dipping the imprinted membrane into 1 mM THO solution for 1 day.

Mesitylene (1,3,5-trimethylbenzene) (bp 162−164 °C/760 mmHg) was used as an internal standard because tetramethylsilane (bp 26−28 °C) was susceptible to evaporation during the sonication before the NMR measurements (Chart 1).

Results

ATR/FT-IR confirmed that photochemical polymerization occurred on the polypropylene membrane surface (Figure 2). The most significant change in the spectra of the imprinted polypropylene membranes was the appearance of absorption bands in the range 1450−1750 cm^{-1}, signifying the carbonyl stretch of oleic acid, TFMAA, *N*-ribitol-L-glutamic acid dioleyl diester[14] ($2C_{18}\Delta^9$GE), and THO and the C═C stretch of the phenyl group of divinylbenzene (Chart 1). An intermediate degree of grafting (DG = 0.069) for the 300 nm lamps was achieved even at a short irradiation time of 10 min (exposure energy = 39 J/cm^2), which is very short compared to that for thermal polymerization[14,15] (DG vs time of irradiation data not shown).

Corrected sessile contact angle measurements gave useful supporting information on the surface modification. As expected, the unmodified polypropylene membrane (102 ± 2°) was more hydrophobic than the THO-imprinted (*sans* THO template) (88 ± 2°) and the imprinted with THO bound (78 ± 2°; after dipping the THO-imprinted membrane into 1 mM THO solution for 1 day) polypropylene membrane (Table 1). Modifying the polypropylene membrane made the surface more hydrophilic. The contact angle of the imprinted polypropylene membrane decreased by about 14° from that of the original untreated polypropylene membrane. The contact angle of the THO-imprinted membrane, after binding THO from solution, decreased by about another 10°. These changes in the values of the contact angles are clearly significant.[16,21,22] Furthermore, after dipping the imprinted membrane into a 1.0 mM THO solution, a broad absorption band around 3600−3000 cm^{-1} appeared in IR spectra, which is likely due to formation of hydrogen bonds (Figure 2) between the template, functional monomers, and residual water on the dried membrane.[19] These changes in contact angle and IR spectra indirectly confirm the formation of THO cavities onto the coated polypropylene membrane and the binding of THO to this imprinted membrane.

With respect to the AFM images of the same three membranes—the unmodified, the THO-imprinted *sans* THO, and the imprinted with bound THO—several differences can be seen in Figure 3. The topographical

Table 1. Roughness-Corrected Sessile Contact Angle Measurements of Water Drops on the Unmodified, the THO-Imprinted (*sans* THO Template), and the Imprinted Membrane with Bound Template

membrane[a]	R_{ave}[b]	γ [c]	α[d]	θ_M[e]	θ[f]
unmodified[g]	232 ± 40	1.5 ± 0.1	13.3 ± 1.7	115 ± 2	102 ± 2
imprinted[h]	220 ± 20	1.6 ± 0.2	17.6 ± 1.2	106 ± 2	88 ± 2
imprinted-THO[i]	330 ± 50	2.7 ± 0.2	29.9 ± 2.1	108 ± 2	78 ± 2

[a] The substrate was a microporous polypropylene membrane (Celgard 2500). [b] The average roughness, $R_{ave} = \sum_{n=1}^{N}|Z_n - \bar{Z}|/N$, where $\bar{Z}$ = mean height. [c] The roughness factor, γ (= ratio of the actual surface area to the projected surface area). [d] Mean roughness angle, α [= $\tan^{-1}(\delta_V/\delta_H)$, where δ_V and δ_H are the mean vertical and horizontal characteristic lengths of the rough surface as measured by AFM]. [e] The measured sessile contact angle, θ_M. Contact angles were measured at least 10 times for each sample. [f] The corrected sessile contact angle, θ (= $\theta_M - \alpha$). [g] Unmodified means original polypropylene membrane without treatment. [h] THO-imprinted membrane from which the THO template has been removed (extracted). [i] Imprinted-THO means a THO-imprinted membrane that has been exposed to a 1 mM THO solution for 1 day.

AFM image of the unmodified polypropylene membrane (Figure 3a) is similar to the scanning electron micrograph image available from Celgard Inc.[23] Also, we see that the unmodified membrane has strands (Figure 3a) that are covered with photopolymerized coating (Figure 3b: THO-imprinted membrane *sans* THO). On binding of the THO template, the identity of the strands have disappeared (Figure 3c) although small pores are still clearly noticeable. Referring to Table 1, two roughness parameters, γ and α (= $\tan^{-1}\delta_V/\delta_H$), increased in value for the three membranes listed above. For R_{ave}, there was no significant difference between the unmodified and the THO-imprinted (*sans* THO) membranes. On binding THO to the imprinted membrane, R_{ave} increased significantly. Clearly, coating the membrane with the photopolymerized imprinted surface and binding the THO template result in a modest and significant increase in surface roughness, respectively. The presence or absence of the template in the affinity cavity makes a difference to the condition of the coating layer.

To evaluate competitive binding between THO and CAF, THO-imprinted and nonimprinted polypropylene membranes (5 cm × 8.8 cm) were dipped into various concentrations of mixtures of THO and CAF (1:1) in 4 mL of water:ethanol (1:1 (v/v)) solution[24] for 1 day. The membranes were removed, and the remaining solution in the test tube was evaporated and dried under vacuum for 3 h. An abundance of chloroform-*d* was added to the test tube, and the sample tube was sealed with Parafilm and allowed to sonicate for more than 4 h. Mesitylene in chloroform-*d* as an internal standard (with typical proton peaks at 2.26 and 6.78 ppm) was added to the sample tube and sonicated for 30 min, before NMR measurements. The separation factor for THO over CAF is defined as[25]

$$\alpha = \frac{K_{THO}}{K_{CAF}} = \frac{([THO]_s^0 - [THO]_s^t)/[THO]_s^t}{([CAF]_s^0 - [CAF]_s^t)/[CAF]_s^t} \qquad (2)$$

where K_{THO} and K_{CAF} are the equilibrium distribution constants for THO and CAF between the coated polypropylene membrane and the solution. $[THO]_s^0$ and $[CAF]_s^0$ are the initial concentrations of THO and CAF in the solution mixture. $[THO]_s^t$ and $[CAF]_s^t$ represent

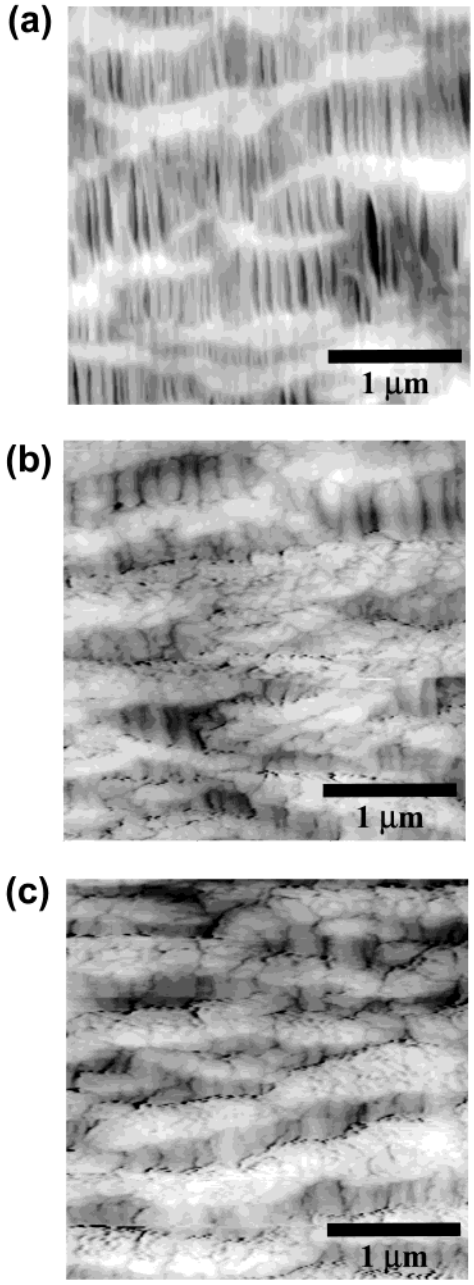


Figure 3. Topographical AFM images of (a) the unmodified polypropylene membrane, (b) the THO-imprinted polypropylene membrane (without template present), and (c) the THO-imprinted polypropylene membrane with THO in the recognition sites (after dipping the imprinted membrane into 1 mM THO solution for 1 day). The average roughness, R_{ave}, for a 3 $\times$ 3 μm^2 area of (a), (b), and (c) above was 232 $\pm$ 40, 220 $\pm$ 20, and 330 $\pm$ 50 Å, respectively (Table 1).

the concentrations of THO and CAF in the remaining solution mixture after time *t*, respectively. These concentrations were evaluated from the changes in proton peak areas at 3.64 and 3.44 ppm for THO, 3.59 and 3.41 ppm for CAF, and 6.78 and 2.26 ppm for the internal standard, mesitylene.

As shown in Figure 4a, which was constructed from the NMR data, the THO-imprinted membrane exhibited selectivity for THO over CAF. Unlike the nonimprinted membrane, selectivity of the imprinted membrane for THO over CAF rose with decreasing concentration of the mixture. For example, the ratio of [THO] over [CAF] in remaining solution after dipping a sheet of imprinted

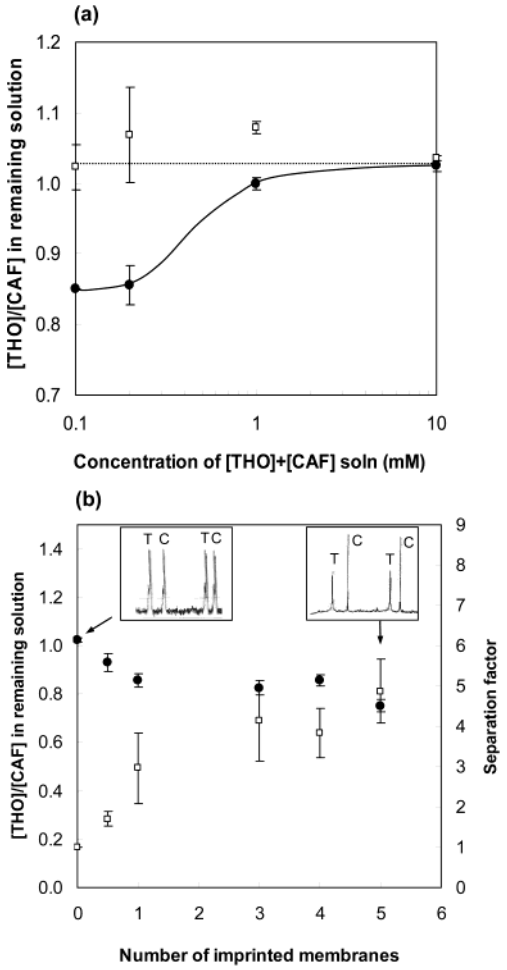


Figure 4. (a) Competitive THO binding to the imprinted (filled circle) and to the nonimprinted (open square) polypropylene membrane as a function of the concentration of THO and CAF (1:1) in a water:ethanol = 1:1 (v/v)) solution. (b) Competition of THO binding to the imprinted polypropylene membrane as measured by $[THO]_s^t/[CAF]_s^t$ remaining in solution (filled circle) a function of the number of imprinted polypropylene membranes (i.e., number of recognition sites) dipped into 0.2 mM THO and CAF (1:1) in a water:ethanol (1:1 (v/v)) solution). The separation factor (open square), calculated according to eq 2 in the text, is also plotted against number of imprinted membranes. Insets: NMR spectra (ranging from 3.7 to 3.4 ppm) of initial 0.2 mM THO and CAF (1:1) solution (left) and of remaining solution after dipping five sheets of imprinted membrane into 0.2 mM THO and CAF (1:1) solution for 1 day (right).

membrane (5 cm $\times$ 8.8 cm) into THO and CAF mixture solution was about 0.85 at the lower concentration of 0.2 mM compared with 1.02 (no significant selectivity) at the high concentration of 10 mM. To check whether the number of molecular recognition sites was limiting at the high concentrations, additional imprinted polypropylene membranes were added to a solution containing 0.2 mM THO and CAF (1:1) in a water:ethanol (1:1 (v/v) solution). Figure 4b shows the relationship between separation factor and the number of imprinted polypropylene membranes (proportional to the number of THO-recognition sites). As expected, selective affinity for THO increased with the number of THO-recognition sites, and a high separation factor of 4.9 $\pm$ 0.8 was obtained when five sheets of imprinted membrane were dipped into the solution. The NMR data confirmed the formation of THO-recognition sites on the imprinted membrane, and their selective affinity for THO over CAF

was consistent with the changes in contact angle and IR spectra. Repeated measurements at room temperature with the same THO-imprinted membrane after 6 months exhibited the same high value of the selectivity (~5) for THO over CAF.

Discussion

With respect to the preparation of discriminate surface molecular recognition sites on a microporous substrate, the two-step procedure described in Figure 1 illustrates the proposed mechanism. The fact that omitting the functional monomers (TFMAA and/or oleic acid) from the mixture results in zero selectivity between theophylline (THO) and caffeine (CAF) suggests their critical importance. The contact angle measurements also confirm that the polypropylene surface has been modified. The roughness-corrected contact angles for the unmodified, the imprinted *sans* THO, and imprinted with bound THO membranes were 102 ± 2°, 88 ± 2°, and 78 ± 2°, respectively (Table 1). Clearly, the difference between these values of θ are significant and indicate that the photopolymerization imprinted coating is hydrophilic and that binding the template into the affinity cavities increases the hydrophilicity of the surface even more. Goto and co-workers have optimized a similar process for imprinting metals.[14]

Since we have demonstrated that THO distributes favorably into the water as opposed to the oil phase, it is thus likely that the THO template interacted with the functional monomers as described in Figure 1.

Although THO is a stronger base than CAF (pK_a of 5.2 and 3.6, respectively[26]), we were unable to demonstrate selectivity with nonimprinted polypropylene membranes. Clearly, imprinting was needed to effect selectivity, and the difference in pK_a values did not have a major effect.

With regard to the transfer of the water drops (containing template) in oil to the substrate, we observed a color change from white (unreacted) to yellow (coated) on both faces of the imprinted polypropylene membrane, suggesting that they were coated with the polymerized imprinted material. We estimate from observation and the literature that the water drops were in the size range from 0.1 to 1 μm.[27] Since the pores (not contiguous and produced by stretching the bulk material) of the polypropylene microporous membrane had a size range from 0.05 to 0.2 μm in width and 0.2−0.5 μm in length (see Figure 3a), it was possible for the smaller water drops to enter the pores. The rest of the droplets likely coated the outside faces of the membrane (Figure 3b). Clearly, larger pore size membranes will be needed to increase the number of the imprinted cavities and the capacity of the membranes.

Other issues are the importance of fixing the coating to the support and the stability of the composite imprinted membranes. A cross-linked coating on a porous membrane is the process used for the production of some commercial membranes (Durapore line, Millipore Corp. Bedford, MA), a durable and popular membrane. Their coating (cross-linked poly(acrylate)) is not covalently linked to the microporous poly(vinylidene fluoride) substrate.[28] This example illustrates that, for a stable membrane, all one needs is a good stable cross-linked coating, which we believe we have with the imprinted membranes. Direct covalent bonding to the substrate is not required. The imprinted membranes were stable and robust and could be handled without loss of the coating. Also, as mentioned above, the imprinted membranes have now been retested after a 6 month period and give virtually the same selectivities as originally reported.

Conclusions

A novel, simple, and inexpensive two-dimensional surface molecular imprinting method using water-in-oil emulsion polymerization on a microporous polypropylene substrate was developed. To test the method, we imprinted theophylline on a synthetic polypropylene membrane and demonstrated a preferential selectivity of 4.9 ± 0.8 for theophylline over caffeine in *aqueous* medium. Doing this in *aqueous* medium rather than organic solvents may offer the possibility of imprinting larger molecules of biological interest. Further work with biological molecules and convective flow to improve mass transfer is being pursued. Moreover, since the surface molecular imprinting method presented here uses a relatively simple technique with *flexible* porous membranes and overcomes several major limitations associated with previous three- and two-dimensional imprinting methods, new opportunities for applications and scale-up of molecular imprinting are now possible.[29]

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