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Artificial Receptor-Attached Amphiphilic Copolymer for Barbiturate Binding in Aqueous Media

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A water-soluble self-associating amphiphilic copolymer was employed to provide a microenvironment for the solvation of a hydrogen-bonding barbiturate artificial receptor, to facilitate molecular recognition in water. The receptor-attached amphiphilic polymer (RP) was synthesized through random copolymerization of 3% (mol) barbiturate receptor-monomer, 70% (mol) 3-sulfopropyl methacrylate, and 27% (mol) *n*-dodecyl acrylate. Difference UV spectra of pH 6.5 aqueous solutions of phenobarbital and receptor-polymer (RP) gave peaks and valleys at 272 and 301 nm respectively, consistent with binding characteristics of monomeric barbiturate receptors in chloroform. Specific association between phenobarbital and the receptor-polymer was further indicated based on investigations of a receptor-free control polymer (CP) of similar polar/nonpolar monomer ratio. Micellar electrokinetic chromatography was applied for studying polymer-phenobarbital association, by capillary electrophoresis.

Introduction

Artificial receptors based on noncovalent host−guest interactions have demonstrated great potential for applications in biomedicine,[1] facilitated membrane transport,[2] selective extraction and separations,[3] and sensor applications.[4−7] Among types of noncovalent interactions utilized for molecular recognition of artificial receptors, i.e., hydrogen-bonding,[8,9] electrostatic interactions,[10] $\pi-\pi$ stacking, and hydrophobic interactions,[11] hydrogen-bonding interaction is the most versatile as demonstrated by combinatorial synthesis of artificial receptors of diverse properties.[12−15]

However, wide applications of pure hydrogen-bonding based artificial receptors have been hampered due to incompatibility of aqueous or polar organic solvents.[16] To allow strong hydrogen-bonding interactions, a noncompeting solvent medium must be provided.[17,18] As a result, many hydrogen-bonding artificial receptors are not directly applicable to biomedical or bioanalytical problems that involve aqueous biological fluids. There has been a great interest in developing hydrogen-bonding artificial receptors for applications in aqueous or polar organic solvent media. One strategy commonly adopted was to achieve cooperative binding by assisting hydrogen-bonding based recognition with additional recognition elements that are not or little affected by polar solvents: e.g., $\pi-\pi$ stacking,[19] π-cation interactions,[16,20] ion-pairing,[21,22] metal-coordinations,[4,5,23] or substantial hydrophobic moieties.[12,24,25] To keep hydrogen-bonding as the major driving force of molecular recognition, these individual receptor-based synthetic approaches must balance various types of molecular forces involved in host−guest interactions.

In this work, we investigated the feasibility of utilizing the hydrophobic microenvironment of a micelle as a solvent media for facilitating receptor−substrate association in water. Nowick et al. demonstrated the feasibility of the approach by utilizing the sodium dodecyl sulfate (SDS) micellar microenvironment for facilitating adenine−thymine hydrogen-bonding recognition in aqueous solutions.[26,27] SDS micellar systems were also reported to solubilize a water-insoluble receptor, enabling molecular recognition.[28] However, success with the low-molecular weight surfactants is limited by two major drawbacks: the dynamic nature of monomeric micelles requires high surfactant concentration and the lack of partitioning[26] of free host molecules inside the micellar microenvironment. We attempted to tackle these issues by employing a polymeric surfactant capable of forming unimolecular micelles, and by covalently attaching the artificial receptor to the polymer to minimize entropic cost of partitioning.

With the polymer serving as a solvation scaffold for an artificial receptor, the strategy may have general applicability to a diversity of hydrogen-bonding based artificial receptors. For the purpose of testing the concept, we have chosen a well-documented barbiturate artificial receptor[3,7,9,18,29−31] for covalent attachment to an amphiphilic copolymer **1**. The original low-molecular weight barbiturate artificial receptor was developed by Hamilton et al.[9,29] This class of receptor forms 1:1 complexes with phenobarbital **2**, through formation of six hydrogen bonds in noncompeting solvents, e.g., $CHCl_3$ or CH_2Cl_2 with K_f in the range 10^4-10^5 M^{-1}. Our objective was to test whether specific association between phenobarbital and receptor−polymer can be achieved in aqueous

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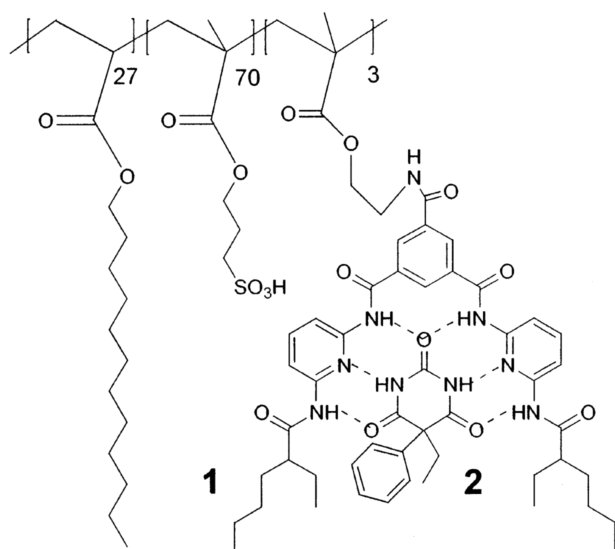
Scheme 1. Barbiturate Receptor-Modified Amphiphilic Copolymer (RP) **1**, Phenobarbital **2**[a]



[a] Hydrogen Bonds between Phenobarbital and Receptor Are Indicated by Dotted Lines

media. The random linear copolymer **1** was synthesized through radical copolymerization of 3% (mol) barbiturate receptor monomer, 70% (mol) 3-sulfopropyl methacrylate, and 27% (mol) *n*-dodecyl acrylate. A receptor-free reference polymer of similar polar/nonpolar monomer ratio was also synthesized for comparison.

Acrylate copolymers with linear hydrophobic side chains and sulfonate hydrophilic groups are known to self-associate in water, forming microdomains of hydrophobic interiors.[32-39] The organic solvent-like microenvironment has found many applications for solubilizing organic species in water for extractions[39] and for chemical analysis.[38,40] Considering the hydrophobic nature of the barbiturate artificial receptor, we hypothesized that the hydrophobic microenvironment would play a dual role of solubilizing the barbiturate artificial receptor and shielding the hydrogen-bonding binding sites from bulk water. Thus, we anticipated phenobarbital-receptor association inside polymer microdomains.

In this communication, we report preliminary investigations of the RP by difference UV spectroscopy[30] and capillary electrophoresis in micellar electrophoretic chromatography mode.[38,40,41]

Experimental Section

Materials. 2,6-Diaminopyridine, 2-ethylhexanoyl chloride, benzene tricarbonyl chloride, *n*-dodecyl acrylate, 2,2′-azobis(isobutyronitrile) (AIBN, recrystallized from methanol), and phenobarbital, **2**, were purchased from Aldrich. 3-Sulfopropyl methacrylate and 2-aminoethyl methacrylate hydrochloride salt were purchased from Polysciences Inc. All reagents were used without further purification, unless otherwise noted. All aqueous solutions were prepared in 18.3 MΩ cm^{-1} water. All sample and buffer solutions were filtered with 0.2 μm pore nylon membranes before studies.

Synthesis and Characterization of Barbiturate Receptor Attached Polymer (RP). The procedures for synthesis and characterization of barbiturate receptor−monomer are described in the Supporting Information section. The RP **1** was prepared according to a procedure adapted from a previous report.[40] Radical initiated copolymerization was carried out with three monomers at desired feed-ratio: 3-sulfopropyl methacrylate (0.23 g, 0.93 mmol, 70%), *n*-dodecyl acrylate (0.09 g, 0.36 mmol, 27%) and barbiturate receptor monomer (0.03 g, 0.04 mmol, 3%), in the presence of 0.01 mmol AIBN and 20 mL DMF at 60 °C under N_2 atmosphere, for 24 h with continuous stirring. The polymer was purified by reprecipitation from DMF into excess diethyl ether and dialyzed against 18.3 MΩ cm^{-1} pure water for a week. In the same manner, a control polymer (CP) consisting of 72% (mol) 3-sulfopropyl methacrylate and 28% *n*-dodecyl acrylate was synthesized and purified.

Molecular weight of polymers were determined by gel permeation chromatography (GPC), with poly(styrene sulfonate, sodium salt) (4.6, 8, 35, 100, and 780 K) as calibration standards (Polysciences Co.). A Waters Ultrahydrogel linear column (7.8 × 300 mm) was used with mobile phase containing 30/70 (v/v) acetonitrile/10 mM pH 7.0 phosphate buffer, 40 °C, 254 nm UV detection. The RP showed a bimodal molecular weight distribution, with weight average molecular weight $\bar{M}_w$ centered at 10 and 100 kDa, respectively. The two polymer species, 10 and 100 kDa, were not separated and binding studies were performed using the crude polymer mixture. The molecular weight of CP was 100 kDa. ^{1}H NMR spectra were obtained on a Varian INOVA 500 spectrometer using 5 mm O. D. tubes. Polymer samples were prepared in D_2O and filtered with 0.2 μm pore nylon membrane filtration cartridges to give polymer concentration of approximately 5 mg/mL. The actual monomer composition in the RP was estimated based on NMR: 2% (mol) receptor, 34% (mol) *n*-dodecyl acrylate, and 64% (mol) 3-sulfopropyl methacrylate. The CP was estimated to consist of 33% (mol) n-dodecyl acrylate and 67% (mol) 3-sulfopropyl methacrylate.

Difference UV−Spectroscopy. The peak-valley absorbance difference relates quantitatively to complex concentration and has been applied for binding studies of low-molecular weight barbiturate receptor in chloroform.[30] In this work, qualitative characterizations of both the RP in aqueous solutions and barbiturate receptor monomer in chloroform were carried out by difference UV spectroscopy. UV spectra were obtained with an Agilent 8453 UV−visible diode array spectrophotometer, 1 nm slit width, room temperature, 1 cm quartz cuvette. In a typical difference spectroscopic experiment, a reference solution and a sample solution were prepared. The reference solution contained 0.8 mg/mL RP to give an equivalence of 64 μM barbiturate receptor concentration, based on the assumption of 2% (mol) receptor-composition. The sample solution contained the same concentration of the RP plus 884 μM phenobarbital. This gives substrate/receptor concentration ratio of 14. Both solutions were prepared in 50 mM pH 6.5 phosphate buffer and filtered with 0.2 μm pore nylon membrane cartridges before UV-spectra were taken. Subtraction of the UV spectrum of the reference from that of the sample gives the difference UV spectrum.

Capillary Electrophoresis. Electropherograms were obtained using a Beckman P/ACE 5510 system with a diode array detector and a liquid-cooling system. UV spectra were recorded every 0.5 s. 50 μm I. D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) were used in all experiments. Typical capillary lengths are 29 cm inlet detector (L_{det}) and 37 cm total length (L_{tot}). Experiments were carried out at 25 °C, with detection wavelength in range 200−400 nm. Samples were typically introduced to the capillary by pressure-injection at 50 mPa for 2−5 s. All polymer solutions were prepared at 5 mg/mL in 50 mM pH 4.0 acetate buffer. Solutions were sonicated followed by filtration with 0.2 μm-pore nylon membrane filters. Phenobarbital sample solution was prepared at 0.01 M in 50/50 (v/v) acetone and 50 mM pH 4.0 acetate aqueous buffer.

Capillary zone electrophoresis experiments were conducted to obtain electrophoretic mobilities of phenobarbital and the polymers. The capillary was filled with 50 mM pH 4.0 acetate buffer and test solutes were injected. Upon application of 10 kV across the capillary, charged solutes migrate in electrical field. The electrophoretic mobility μ was obtained according to eq 1

$$\mu = \frac{L_{det}L_{tot}}{V} \cdot \left(\frac{1}{t} - \frac{1}{t_0}\right) \quad (1)$$

where V is the voltage applied across the capillary, t is the migration time of the test solute, and t_0 is the migration time of acetone serving as a neutral marker. Experiments were also operated in micellar electrokinetic chromatography (MEKC) mode,[41] allowing the investigation of phenobarbital-polymer association. In this case, the capillary was filled with a polymer solution, and acetone was used as an unretained neutral marker for electroosmotic velocity. The observed apparent electrophoretic mobility μ_{app} of phenobarbital in a polymer solution is quantitatively related to retention factor k', according to eq 2[41,42]

$$k' = \frac{\mu_{app} - \mu_0}{\mu_m - \mu_{app}} \quad (2)$$

where μ_m and μ_0 are electrophoretic mobilities of polymer and phenobarbital respectively in buffer, obtained by capillary zone electrophoresis. As k' is the product of binding constant K and the volume ratio Φ of the polymer pseudo phase and bulk liquid, i.e., $k' = \Phi K$, its value allows quantitative evaluation of binding between phenobarbital and the polymers.

Results and Discussion

Difference UV Spectroscopy of the RP. The effectiveness of difference UV spectrometry for studying binding between phenobarbital and barbiturate artificial receptor relies on the fact that the complex has a maximum absorption band near 305 nm. Due to potential interference of the free barbiturate receptors that absorb at near 302 nm, the spectrum of a receptor−barbiturate mixture is subtracted by the spectrum of a receptor solution at the same concentration. Because phenobarbital has negligible absorbance beyond 240 nm, a flat profile of difference UV spectrum would be observed if no binding had occurred. When binding does occur, the magnitude of peaks and valleys in difference spectrum is proportional to the concentration of the complex.[30]

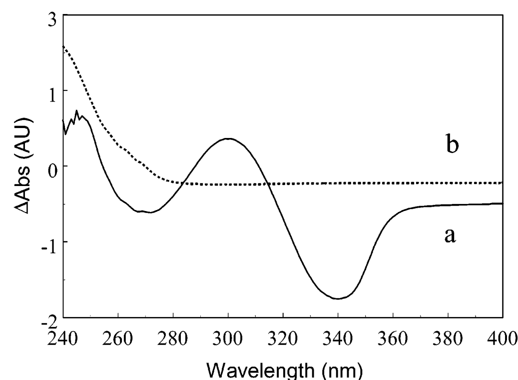


Figure 1. UV difference spectra, room temperature. (a) Solid line: receptor−polymer (RP) (0.8 mg/mL) and phenobarbital (884 μM) in 50 mM pH 6.5 aqueous phosphate buffer, [phen.]/[rec.] = 14. (b) Dashed line: control polymer (CP) (0.8 mg/mL) and phenobarbital (884 μM) in 50 mM pH 6.5 aqueous phosphate buffer.

First, we obtained a difference UV spectrum of a monomeric barbiturate receptor in chloroform, at concentrations of 884 μM phenobarbital and 64 μM receptor monomer. The spectrum shows a valley and a peak at 271 and 306 nm, respectively, characteristic of phenobarbital−barbiturate receptor association as reported in the literature.[30] The same investigation was carried out for a solution containing 0.8 mg/mL RP dissolved in 50 mM pH 6.5-phosphate buffer, with an estimated receptor concentration of 64 μM. Sample solution contained 884 μM phenobarbital in addition to 0.8 mg/mL RP, to give [phenobarbital]/[receptor] ratio of 14. The spectrum in Figure 1a shows valley and peak at 272 and 301 nm, respectively, which are in excellent agreement with that observed for phenobarbital binding of monomeric receptors.

For comparison, the same experiment was carried out for CP of similar 3-sulfopropyl methacrylate/*n*-dodecyl acrylate molar ratio as the receptor−polymer: 67/33 for the CP and 64/34 for the RP. The difference UV spectrum of 884 μM phenobarbital with 0.8 mg/mL CP is shown in Figure 1b. In contrast to the spectrum corresponding to the RP, a flat spectrum profile was observed for the CP. This result allows us to exclude the likelihood that the valley and the peak of the difference spectrum is due to nonspecific phenobarbital−polymer associations involving partitioning or surface adsorption. As will be mentioned later, the micellar properties of the polymer and the partitioning of phenobarbital were confirmed by capillary electrophoresis.

Solvent and pH Studies. To test whether phenobarbital−receptor polymer association is dependent on the structural integrity of the micelle, as well as the partitioning of phenobarbital, we investigated solution conditions less favorable to association. First, based on solvatochromic studies,[43] we expected that addition of 50% (v/v) ethanol to aqueous polymer solution would disrupt micellar structure. In this case, hydrogen-bonding based binding would attenuate if the receptors were exposed to a more polar solvent environment.

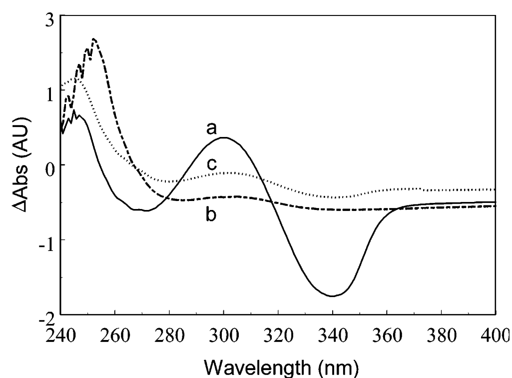


Figure 2. Comparison of receptor−polymer/phenobarbital UV-difference spectra in different solvents. [phen.]/[rec.] = 14, all other experimental conditions are the same as those in Figure 1, except for (a) in 50 mM pH 6.5 phosphate buffer (solid line); (b) in 50% (v/v) ethanol and 50 mM pH 6.5 phosphate buffer (dashed line); and (c) in 50 mM pH 8.3 phosphate buffer (dotted line).

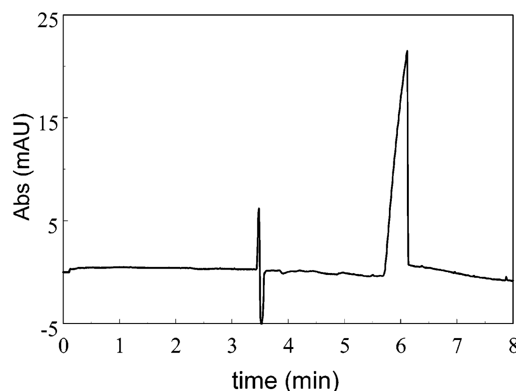


Figure 3. Electropherogram of phenobarbital in the presence of 5 mg/mL receptor−polymer (RP) in 50 mM pH 4 acetate buffer. Voltage 10 kV, total capillary length 37 cm, length to the detector 29 cm, detection wavelength 200−400 nm (diode array), temperature 25 °C. 3 s injection of phenobarbital at 0.01 M.

Addition of ethanol would also decrease the extent of partitioning of phenobarbital into the microenvironment. Second, we anticipated that the partitioning of phenobarbital would decrease upon increasing the solution pH from 6.5 to 8.3, as it has a pK_a of 7.3.

These hypotheses were studied by difference UV spectroscopy as shown in Figure 2. Indeed, both the addition of 50% (v/v) ethanol and the increase of solution pH to 8.3 have resulted in decreased valley−peak absorbances, indicating decreases in the concentration of phenobarbital/receptor polymer complexes under these conditions. The results support the argument that the association is dependent on the partitioning of phenobarbital and the integrity of the micellar structure. Considering the fact that the barbiturate receptor is rather hydrophobic, it is reasonable to anticipate its location in a low polarity solvent media; therefore, partitioning is required for complexation.

Binding of phenobarbital to the receptor−polymer can be considered as a two-step equilibrium process: partitioning and specific host−guest binding. In the first step, phenobarbital partitions into microenvironment where receptors are located, with a partitioning coefficient K_p. In the second step, specific binding between phenobarbital and the barbiturate receptor occurs in the microenvironment, with a formation constant K_f. Therefore, the concentration of complex observed in an experiment depends on the product K_pK_f. Analogous to receptor-assisted liquid−liquid two-phase extractions,[30] to observe a high concentration of phenobarbital and receptor−polymer complex, a solvent must favor both partitioning and hydrogen-bonding interactions.[18]

Micellar Electrokinetic Chromatography. Further studies of phenobarbital−receptor polymer association were carried out by capillary electrophoresis, for investigating the mobility of phenobarbital in the RP and the CP, respectively. Due to the relationship between apparent mobility of a test solute in a micellar solution with its binding to polymer, micellar electrokinetic chromatography has proven to be powerful for obtaining both qualitative and quantitative information on solute−micelle bindings.[41] From capillary zone electrophoresis, the electrophoretic mobilities of both the RP and the CP were determined to be -4×10^{-8} m^2/s V at pH 4 in a 50 mM acetate buffer. The negatively charged polymers migrate in the opposite direction of electroosmotic flow, with a mobility proportional to the charge/size ratio. We confirmed micellar properties of both the RP and the CP at concentrations of study, by using naphthalene as a test solute. A linear trend of apparent time of naphthalene with polymer concentration was observed.

Nearly neutral phenobarbital at pH 4 would have a very small electrophoretic mobility unless it associates with the negatively charged polymer. Upon association, phenobarbital migrates with the polymer, and an increase in apparent mobility can be detected. The stronger the association, the more negative the apparent mobility. We carried out experiments with 5 mg/mL RP and CP solutions in 50 mM pH 4 acetate-buffer. A typical electropherogram of phenobarbital in RP is shown in Figure 3. The peak at near 6 min corresponds to phenobarbital, and the spike at near 3.5 min corresponds to acetone as the neutral marker. The large difference in migration time between phenobarbital and acetone clearly indicates phenobarbital/receptor-polymer association. 3D electropherograms of phenobarbital recorded with a diode array detector are shown in Figure 4. In CP, phenobarbital elutes from the capillary at time very close to acetone, indicating weak nonspecific association (partitioning and adsorption). In the presence of RP, however, the migration time of phenobarbital increased dramatically, suggesting its strong specific association with the RP.

Values of apparent electrophoretic mobility of phenobarbital were obtained under the following conditions: $\mu_{Buf} = -0.9 \times 10^{-9}$ m^2/s V, in 50 mM pH 4 acetate buffer; $\mu_{Ref} = -2.0 \times 10^{-9}$ m^2/s V, in 5 mg/mL CP buffer solution; and $\mu_{Rec} = -22 \times 10^{-9}$ m^2/s V, in 5 mg/mL RP buffer solution. The data shows that the near-neutral phenobarbital has very small mobility at pH 4 buffer. Upon addition of 5 mg/mL CP, a slight increase in mobility was observed, indicating very weak nonspecific association between phenobarbital and the CP. In 5 mg/mL RP, mobility of phenobarbital increased 10-fold compared to the CP at the same concentration.

According to eq 2, retention factor k' of phenobarbital can be estimated for quantitative evaluation of partitioning and specific binding to the barbiturate receptor. Results show that phenobarbital has k' values of 1.2 and 0.03 in RP and CP solutions, respectively. Assuming the polymeric micelle

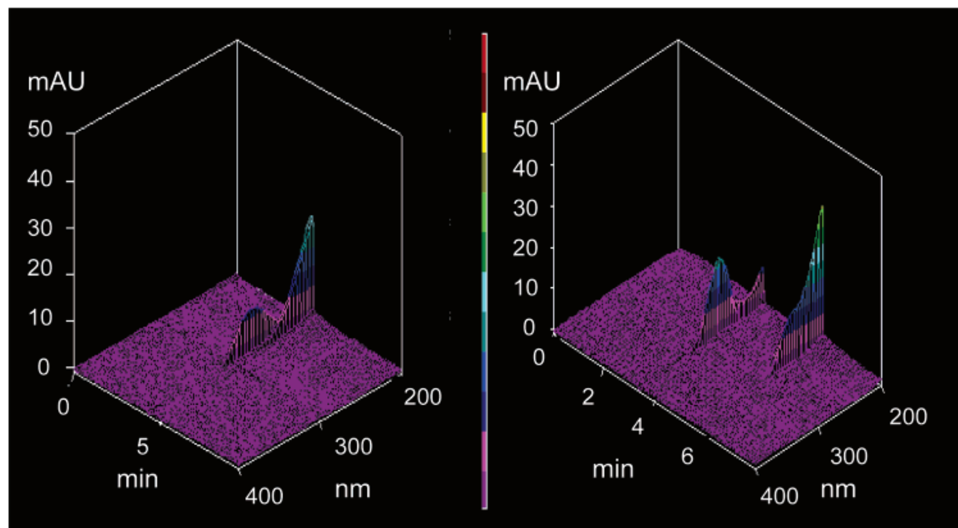


Figure 4. 3D electropherograms of phenobarbital in 5 mg/mL control polymer (CP) (left) and in 5 mg/mL (w/v) receptor-polymer (RP) (right). Both polymer solutions were prepared in 50 mM pH 4 acetate buffer. Experimental conditions are the same as those in Figure 3.

has a density of 1.0 g/mL, a polymer concentration of 5 mg/mL gives volume ratio $\Phi = 0.005$ of polymer vs bulk liquid phase. Based on the relationship $k' = K\Phi$, equilibrium constant K can be obtained. The partition coefficient for the CP K_p is 6, whereas K_pK_f for the RP is 240 M^{-1}. Because the RP and the CP have a very similar structure and electrophoretic mobility, a similar partitioning coefficient K_p can be assumed for both polymers. Therefore, the ratio of the two retention factors allows estimation of K_f, the value of which shows that the binding constant of phenobarbital to the polymer-bound artificial receptor is approximately 40 M^{-1} in pH 4 acetate aqueous buffer. This additional piece of evidence supports the finding of specific binding in the UV-spectroscopic studies in pH 6.5 phosphate buffer.

Based on these experiments, K_f seems to be the major contributor to K in this system while K_p is less important. Phenobarbital contains six hydrogen-bond accepting sites and two hydrogen-bond donating sites that can interact with the bulk water molecules. Based on MEKC experiments in our lab, solutes with hydrogen-bond donating or accepting functionalities demonstrated weak partitioning in micellar systems of these type.[44] The observation that K_f is the major contributor to binding, is valid only for this system, with phenobarbital as the substrate. It is highly likely that the magnitude of K_f and K_p vary with the barbiturate structure. In fact, the barbiturate receptor demonstrated selectivity in organic solvents and we wish to examine the case with the receptor-polymer in aqueous media.

Both the CE and UV experiments prove that specific binding occurs between the receptor in the polymer and phenobarbital. However, the nature of the specific binding cannot be determined as yet. It is probable other forces become important in addition to hydrogen bonding. The specific role of hydrogen bonding will be clarified by examining the association of the receptor−polymer with barbiturates bearing different hydrogen bonding sites.

Conclusions

In this study, we covalently attached a purely hydrogen bonding based barbiturate receptor to an amphiphilic copolymer and examined molecular recognition in aqueous media. Due to its amphiphilic nature, the copolymer self-associates in water creating hydrophobic microdomains. We anticipated the receptor to preferentially solubilize in the hydrophobic core and the water-free environment to encourage hydrogen bonding based recognition. A simple two-step binding model was adopted: partitioning of phenobarbital from aqueous solution into the micellar phase and specific binding within the micelle. UV difference spectroscopy provided strong indications for specific binding in aqueous media. Binding was pH dependent presumably due to the acidic nature of phenobarbital. Furthermore, capillary electrophoresis was applied for studying the effect of partitioning. An estimate of K_p was determined, based on experiments of a receptor-free control polymer and subsequently an estimate of K_f. Near-future work involves optimization of K_f by manipulating experimental conditions such as solution pH and polymer concentration. In addition, we wish to investigate the selectivity of the receptor−polymer toward different barbiturates.

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Supporting Information Available. Reaction scheme for receptor−polymer synthesis, ^{1}H NMR spectra, UV-spectroscopy of receptor−polymer in water, synthesis of receptor monomer. These materials are available free of charge via the Internet at http://pubs.acs.org

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