Colloid & Polymer Science Colloid Polym Sci 272:1627–1632 (1994)

Interaction of an anticancer drug, methotrexate with amphiphilic copolymers of methyl quaternized 2-dimethylaminopropyl acrylamide and styrene

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Abstract: The copolymers of methyl quaternized 2-dimethylaminopropyl acrylamide and styrene have been prepared. Studies were made of the binding of an anticancer drug, methotrexate by the copolymers, bovine serum albumin, and polyvinylpyrrolidone in aqueous solution. The first binding constants and the thermodynamic parameters in the course of the binding were evaluated and compared with those of a "binding probe", methyl orange and its homologs in order to gain insight into the nature of drug binding and to apply the drug-polymer complex to a drug-delivery system. The nature and phenomena of drug binding with the polymers are discussed.

Key words: Amphiphilic copolymer – methotrexate – methyl orange – drug binding – dye binding

Introduction

In a previous paper [1], the binding of small substrates, a "binding probe," methyl orange, and a "hydrophobic fluorescent probe," 2-*p*-toluidinylnaphthalene-6-sulfonate by the copolymers of methyl quaternized 2-dimethylaminoethyl acrylate and styrene, 2-vinylnaphthalene, acrylic acid *iso*-octyl ester, or acrylic acid *n*-butyl ester was examined in connection with hydrophobic interactions in the small organic substrate-polymer complex formation. The exceptionally strong binding capacity of the matrices for the small molecules has been shown. To our knowledge, no polymer with a higher binding ability than that of the polymers exists at the present time.

In the investigation described here studies were made of the binding of an anticancer drug, methotrexate by serum albumin of a naturally occurring carrier protein, polyvinylpyrrolidone with properties similar to the albumin, and the copolymers of methyl quaternized 2-dimethylaminopropyl acrylamide and styrene, in order to obtain fundamental information concerning application of the polymers to a drug-delivery system, in particular, controlled release of the drug from the polymer complex. For this purpose the binding behaviors of the drug to the polymers were studied and compared with those of a "binding probe," methyl orange. We are now trying to prepare functional microcapsules involving polymers with high binding affinity for a drug and to release the small substrate loaded in the inner phase of the capsule. The capsule can be expected to entrap a large amount of substrate inside and show very slow release.

Experimental

Crystallized bovine serum albumin (BSA, Lot. No. 50H9300) was purchased from Sigma Corp. The moisture content was determined by drying a separate portion in vacuo over P_2O_5 at 100 °C. Polyvinylpyrrolidone (PVP, K30) was obtained from Tokyo Kasei Kogyo Corp. According to the manufacture, this type of polymer has a molecular weight of about 40000. Methyl quaternized 2-dimethylaminopropyl acrylamide (DMAPAA-Q, 80% aqueous solution, Kohjin Co.) and styrene

(ST, Kishida Chemical Co.) were copolymerized according to the procedure outlined in a previous paper [1]. To fabricate polymers of different DMAPAA-Q contents the concentration ratio of the two species, DMAPAA-Q and ST, was varied in the synthesis. The composition in the copolymers was determined by elemental analysis (see Table 1). These copolymers prepared were designated P-I, P-II, and P-III. P-I and P-II were water-soluble and P-III water-insoluble.

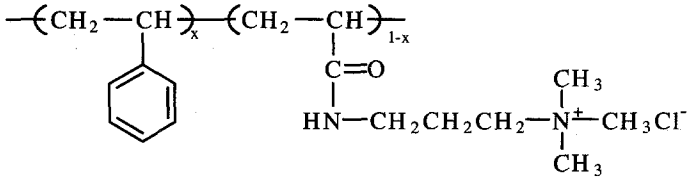
The small organic substrates used in the binding experiment were an anticancer drug, methotrexate (MTX, L-amethopterin trihydrate, 98%, Aldrich Chemical Co., lot. no. 05472JV) and a "binding probe," methyl orange. Also, a homologous series of methyl orange derivatives, ethyl orange, propyl orange, and butyl orange were employed for comparison. MTX was used without further purification. Methyl orange and its homologs were the same materials described elsewhere [2].

The extent of binding of the small substrates by the water-soluble macromolecules was measured by the equilibrium dialysis technique at 5°, 15°, 25°, and 35°C in 0.1 M Tris-acetate buffer, pH 7.0. The experimental method used in this work was

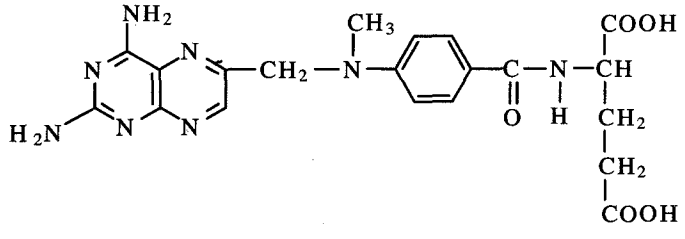
Table 1. First binding constants and thermodynamic parameters for the binding of MTX and methyl orange and its homologs by polymers

Small substrate	Polymer	x[a]	nk[b,c]	ΔF^{0}[d] (cal/mol)	ΔH^{0}[d] (cal/mol)	ΔS^{0}[d] (eu)
Methyl orange	PVP[e]		1.79×10^4	− 5780	− 4070	5.7
Methyl orange	BSA[f]		5.40×10^4	− 6580	− 3600	10.0
Methyl orange	P-I	0.33	1.15×10^6	− 8260	− 5370	9.7
Methyl orange	P-II	0.74	1.19×10^7	− 9650	− 3670	20.1
Methyl orange	P-III	0.90	8.26×10^6	− 9430	4480	46.7
Ethyl orange	P-I	0.33	3.85×10^6	− 8940	− 5390	11.9
Propyl orange	P-I	0.33	8.06×10^6	− 9420	− 3710	19.2
MTX[g]	PVP		1.44×10^3	− 4310	− 5130	− 2.8
MTX[g]	BSA		5.57×10^3	− 5110	− 4480	2.1
MTX[g]	P-I	0.33	1.39×10^5	− 7010	− 5810	4.0
MTX[g]	P-II	0.74	5.71×10^5	− 7850	− 5030	9.5
MTX[g]	P-III	0.90	1.85×10^5	− 7180	2150	31.3

[a] In structure:



[b] Calculated from r values computed for 10^5 g of polymer.
[c] Measurements in 0.1M Tris-acetate buffer, pH 7.0.
[d] Measurements at 25 °C.
[e] Taken from ref. [2].
[f] Taken from ref. [6].
[g] In structure:



essentially the same as that used previously [2]. A 10-ml portion of the polymer solution inside a dialysis bag was equilibrated for about 48 h with 15 ml of the small molecule solution. Control tubes contained only buffer inside the dialysis bag. Equilibrium small molecule concentrations were determined from absorbance measurements. With the water-insoluble polymer the procedure for the binding experiments was essentially the same as that described in ref. [3]. Equilibrium data were obtained by mixing the water-insoluble polymer, the amount of the polymer depending on the affinity of the polymer for the small molecule, with 10 ml of buffered solution (0.1 M Tris-acetate, pH 7.0) and varying amounts of small molecule. Test tubes that contained the solutions were gently shaken in a circulating water bath at 5°, 15°, 25°, and 35°C. After equilibrium was attained the water-insoluble polymer was separated by centrifugation (at more than 10000 rpm) and the concentrations of the small molecules in the supernatant liquid were determined spectrophotometrically. The amount of small molecules in the supernatant subtracted from that initially added gave the quantity of small molecules absorbed by the polymer. Since MTX is very sensitive to light, the binding experiment was carried out in a dark room.

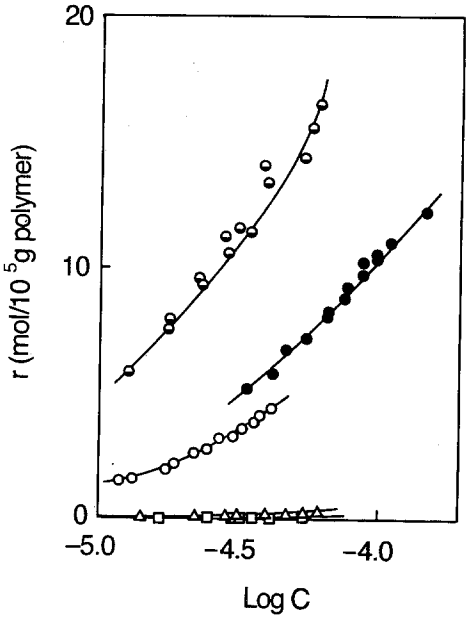


Fig. 1. Extent of binding of MTX by polymers at 25 °C in 0.1M Tris-acetate buffer at pH 7.0: (○) P-I, (◒) P-II, (●) P-III, (△) BSA, (□) PVP

Results and discussion

BSA, PVP, and the water-soluble and -insoluble copolymers have been examined for their binding ability toward MTX and methyl orange and its homologs. The typical binding results for MTX at 25 °C are shown in Fig. 1, in which the binding isotherm is expressed as r, the number of moles of small substrate bound per 10^5 g of the polymer, as a function of C, free substrate concentration, in 0.1M Tris-acetate buffer, pH 7.0. Evidently, the binding affinities of these polymers for MTX increase in the following order: PVP, BSA, P-I, P-III, P-II. A tremendously greater extent of binding by P-II can be seen as compared to the other polymers, in particular PVP and BSA. Extensive studies have been made of the binding of small organic molecules and ions by BSA and PVP, not only because of their pharmaceutical significance, but also as a model of biomacromolecular interactions with substrates. It is well known that BSA stands far above any of the other naturally occurring polymers in binding. Also, PVP reversibly forms complexes with many types of small molecules, particularly organic anions, although with only about one-third of the affinity shown by BSA. With the copolymers the amount of binding increases with increasing content of ST and then decreases with a further increase of the content. Similar plots of the extent of binding for methyl orange are shown in Fig. 2. The situation of the binding behavior is the same as that of MTX. Methyl orange is bound to these polymer matrices much more strongly compared to MTX because the value of r for methyl orange exceeds that for MTX at a fixed concentration of free small substrate C.

For purposes of quantitative comparison of affinities the first binding constants nk, where k represents the intrinsic binding constant and n is the number of binding sites per 10^5 g of polymer, were calculated from the slopes of the double reciprocal plots (Klotz plot, $1/r$ versus $1/C$) shown in Fig. 3 as a typical example [4]. It is apparent that the plots are essentially linear at each temperature measured. Thus, the intercept on the ordinate axis is $1/n$, so k can be easily calculated. Nevertheless, since the numerical value of this

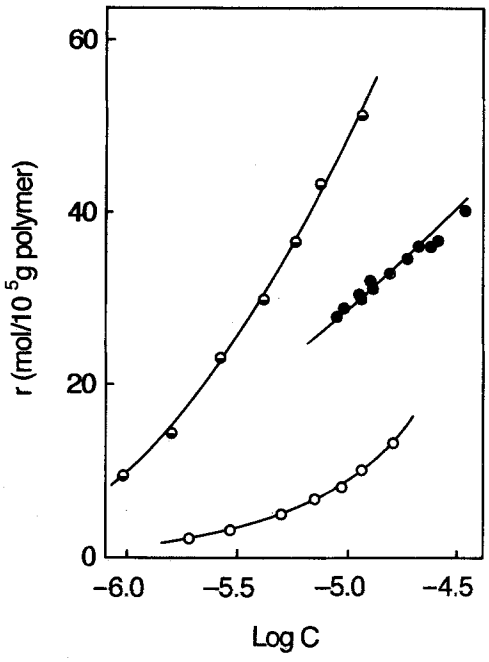


Fig. 2. Extent of binding of methyl orange by polymers at 25 °C in 0.1M Tris-acetate buffer at pH 7.0: (○) P-I, (◒) P-II, (●) P-III

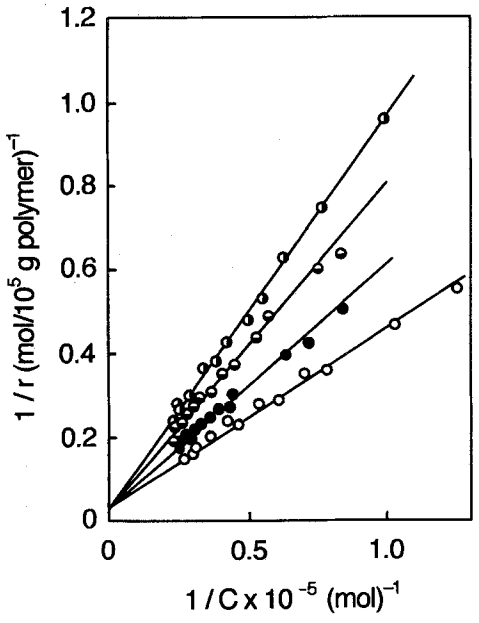


Fig. 3. Relationship between $1/r$ and $1/C$ for binding of MTX by P-I in 0.1M Tris-acetate buffer at pH 7.0: (○) 5 °C, (●) 15 °C, (◒) 25 °C, (◑) 35 °C

intercept on the ordinate is generally near zero, a very small error in the extrapolation to $1/n$ may be reflected in a large uncertainty in n. Strictly speaking, compounds should be compared with respect to their intrinsic binding constants k rather than their first binding constants nk. Since the uncertainty of variable n for different small molecules and polymers still exists, however, it seems preferable to use nk, which can be evaluated more precisely without a knowledge of n. The value of nk is comparable to the extent of binding of polymers for small molecules.

The values of nk and thermodynamic quantities associated with the binding of MTX and methyl orange and its derivatives by the polymers in 0.1 M Tris-acetate, pH 7.0 are summarized in Table 1. As is evident from the results listed in Table 1, the affinity of MTX for the polymer is about one-tenth the affinity shown by methyl orange. With the polymers investigated, the copolymer P-II exhibits the largest binding constant; the nk values of the copolymers for MTX are of the order of 10^5, whereas those of BSA and PVP are of the order of 10^3. The entropy change accompanying the binding of MTX by the polymers except P-III is small and the largest contribution to the free energy of binding is from the negative enthalpy change. Thus, the binding is mainly enthalpy-controlled. This is presumably due to the presence of two carboxyl groups and less hydrophobic region in MTX. With P-III, which involves the highest ST content, in other word, the most hydrophobic domain and is water-insoluble polymeric matrix, it was found that the complex formation between MTX and P-III is associated with an endothermic enthalpy change and a large and positive entropy change. This binding is mainly entropy-controlled. These observations can be explained as a result of an increase in hydrophobic interactions between the small substrate and the copolymer and a decrease in electrostatic interactions between them, although the contribution of the hydrophobic interaction for MTX is smaller than that for methyl orange.

The nk values are plotted as a function of the content of ST in the copolymer in Fig. 4. For this purpose, the copolymer with ST content of 85% has been synthesized in addition to P-I, P-II, and P-III, and compared. The plots are added in Fig. 4. As is evident in Fig. 4, a maximum appears in

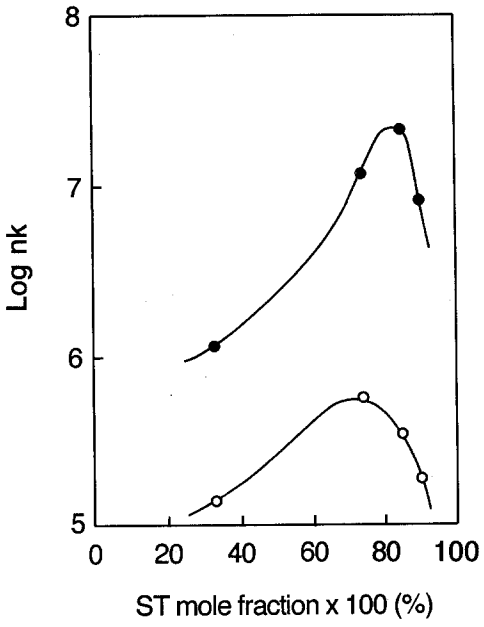


Fig. 4. Relationship between ST content in copolymer and first binding constant accompanying the binding of MTX and methyl orange by copolymer at 25 °C in 0.1M Tris-acetate buffer at pH 7.0: (○) MTX, (●) methyl orange

the binding curve for MTX and methyl orange. The change in the extent of binding of MTX or methyl orange to the copolymers as a function of ST content might be attributed to two effects: the electrostatic interactions between the positively charged groups in the copolymer and carboxylate or sulfonate group in the small substrate and the hydrophobic interaction between the hydrophobic parts of the substrate and the copolymer. Of course, when the amount of ST residues increases, the conformation of the polymer tends to shrink and hence this leads to the increase in the hydrophobic interaction in the binding because of the formation of hydrophobic domains. The balance between two effects, the electrostatic and hydrophobic effects plays a significant role in the enhancement of small substrate binding. The reduction of electrostatic attraction between the quaternized nitrogen atom and the carboxylate or sulfonate anion and the onset of hydrophobic attraction between the hydrophobic moieties of the two binding entities lead to the maximal binding for P-II when the ST content is increased. A similar situation was observed in other binding systems [5].

The relative importance of energetic and hydrophobic contributions in the copolymer binding can be assessed by investigating the interactions of the copolymer with a homologous series of cosolutes, varying in their chemical nature in the systematic fashion. As Fig. 5 clearly indicates, the copolymer (P-I was used representatively) shows a substantially increasing affinity toward small molecules of different hydrophobicity in the following order: methyl orange < ethyl orange < propyl orange < butyl orange. An increase in the hydrophobic nature of the substrate tends to favor interaction with the copolymer. The thermodynamic parameters for the binding of methyl orange, ethyl orange, and propyl orange by P-I in 0.1 M Tris-acetate, pH 7.0, at 25 °C are demonstrated in Table 1 also. With butyl orange the steep rise in binding with increasing concentration of dye is observed. The binding isotherm is not the Langmuir type, suggesting cooperative binding in the binding process. Thus, the value of *nk* and hence the thermodynamic functions cannot be evaluated. As is evident for P-I in Table 1, the longer the alkyl chain of the substrates from methyl orange to propyl orange, the more positive

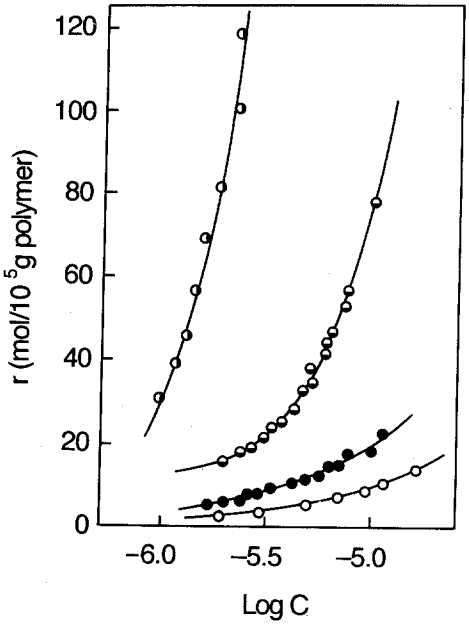


Fig. 5. Extent of binding of methyl orange and its homologs by P-I at 25 °C in 0.1M Tris-acetate buffer at pH 7.0: (○) methyl orange, (●) ethyl orange, (◒) propyl orange, (◑) butyl orange

the enthalpy change (though the value itself is always negative) and, hence, the larger the entropy change. Therefore, the evidence presented here supports the view that the hydrophobic interaction is operating to a greater extent in binding of the apolar substrate by the copolymer.

Now, we are trying to apply these polymers with high binding ability for drugs to a drug-delivery system. The concept is as follows. Polyamide capsules containing the copolymer P-I, P-II, or P-III, which have strong binding affinity for the small substrates such as MTX and methyl orange, were synthesized. The capsule could entrap a large amount of the substrate inside and showed very slow release. The rate of release decreased and the total amount of the released substrate increased with an increase in the strength of interaction between the copolymer and the substrate. The detailed information will be reported in a subsequent paper. The copolymers prepared here show substantially increased affinity for the anticancer drug. However, the other characteristics of the copolymers in vivo are still open. If this concept will be successful, then we must next design biodegradable and biocompatible polymers with increased affinity for drugs.

References

1. Takagishi T, Kim Y-J, Hosokawa T, Morimoto K, Kono K (1993) J Polym Sci Part A Polym Chem 31:365
2. Takagishi T, Kuroki N (1973) J Polym Sci Polym Chem Ed 11:1889
3. Takagishi T, Hayashi A, Kuroki N (1982) J Polym Sci Polym Chem Ed 20:1533
4. Klotz I-M, Walker F-M, Pivan R-B (1946) J Am Chem Soc 68:1486
5. Takagishi T, Hosokawa T, Hatanaka Y (1989) J Polym Sci Part A Polym Chem 27:1
6. Klotz I-M, Shikama K (1968) Arch Biochem Biophys 123:551

Received February 23, 1994;
accepted April 20, 1994

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