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# Preparation of Dextran T70–Methotrexate Conjugate and Dextran T70–Mycophenolic Acid Conjugate, and *in Vitro* Effect of Dextran T70–Methotrexate on Dihydrofolate Reductase

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Alkylenediamine-dextran T70 (T70-$C_n$) might be useful as a polymer support for anti-tumor polymeric drugs, and its preparation was investigated. Decylenediamine-dextran T70 (T70-$C_{10}$) was selected as the most suitable compound. Methotrexate (MTX) or mycophenolic acid was conjugated through an amide bond involving the carboxyl group of the drug and the amino group of T70-$C_{10}$. Stability tests under the conjugation reaction conditions confirmed that T70-$C_{10}$ could be utilized as a polymer support. The conjugation reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as the reagent was satisfactory. The inhibition of dihydrofolate reductase by the conjugate of T70-$C_{10}$ and MTX (T70-$C_{10}$–MTX) was checked *in vitro*.

**Keywords**——decylenediamine-dextran T70; polymer support; conjugate; methotrexate; mycophenolic acid; dihydrofolate reductase

## Introduction

Recently, anti-tumor polymeric drugs have been synthesized and studied in attempts to improve the chemotherapeutic activities of anticancer agents and to achieve selective targeting.[1] Conjugates of polymer and anticancer agents can remain at the site of administration for a long time, accumulate in lymph,[2–4] enter tumor cells through endocytosis and act on drug-resistant cells.[5,6] Therefore, anti-tumor polymeric drugs can be very effective in improving the chemotherapeutic activities of the parent drugs.

It is desirable that a polymer support should be water-soluble for optical measurement, non-toxic, non-immunotoxic and biodegradable for parenteral administration. Thus, dextran, which is commercially available and is used as a plasma expander, was chosen. Further, taking into consideration the mode of degradation or drug release of polymeric drugs, the linking of the parent drug to the polymer support should be carried out at a location far from the active moiety, and the linkage should be hydrolyzable. Studies on the three-dimensional structure of dihydrofolate reductase–methotrexate (MTX) complex show that the carboxyl groups of MTX are located at the surface of the complex.[7,8] Thus, MTX was selected and its carboxyl group was modified. Since dextran accumulates in lysosomes,[9] alkylenediamine-dextran–MTX might be a candidate for an anti-tumor polymeric drug. In this work, such a drug was synthesized, and its activity was checked *in vitro*.

## Experimental

**Materials**——Dextran T70 (T70) was purchased from Tokyo Kasei Industrial Co. MTX and dihydrofolate reductase (DHFR) from chicken liver were obtained from Sigma Chemicals Co. Mycophenolic acid (MPA) was purchased from Hoechst Co., and chymotrypsinogen A (Chymo.A) and bovine serum albumin (BSA) were obtained from Pharmacia Fine Chemicals Co. All other chemicals were commercial reagent-grade products.

**Preparation of the Polymer Support**——The preparation procedure was similar to that of Murachi *et al.*[10,11] T70 was dissolved in water and oxidized with sodium periodate for 1 h at room temperature. The reaction was stopped by the addition of ethylene glycol. The mixture was dialyzed with water for 24 h at 4—5 °C and further against 0.01 M carbonate buffer, pH 9.5, for 12 h. After that, diaminoalkane was added. After 4 h, sodium borohydride was added to reduce the Schiff's base and remaining aldehyde groups. After dialysis of this mixture against water, alkylenediamine-dextran T70 (T70-$C_n$) was obtained. For dialysis, cellulose tubing with a pore diameter of 24 Å was used throughout this work.

1) Degree of Oxidation: Two conditions, 10% oxidation and 20%, were checked. Diaminohexane was used as a spacer. The amount used was appropriate to the degree of oxidization, as was that of sodium borohydride. The polymer supports were stored in a refrigerator at 8 °C for one month, then examined by gel-filtration.

2) Spacer: The degree of oxidization was chosen to be 10% based on the previous experiment. Ethylenediamine, diaminohexane and diaminodecane were used as spacers under equivalent reaction conditions. The final dialyzed samples, T70-$C_2$, T70-$C_6$ and T70-$C_{10}$, were checked with ninhydrin reagent. The reactivity was used as an index of the formation of the effective polymer support, T70-$CH_2NH(CH_2)_nNH_2$.

High-molecular fractions obtained by gel-filtration were used for the conjugation reaction.

**Stability of T70-$C_{10}$**——T70-$C_{10}$ (150 mg) was stirred in aqueous solution at pH 6.0 for 24 h in the dark at room temperature, then subjected to gel-chromatography (Sephadex G50). The absorbance at 260 nm, the weight and the ninhydrin reaction of each fraction were determined.

**Preparation of T70-$C_{10}$–MTX Conjugate and T70-$C_{10}$–MPA Conjugate**——The macromolecular T70-$C_{10}$ was separated on a Sephadex G50 column. MTX (or MPA) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were added. The pH was adjusted to 6.0 and the mixture was stirred for 24 h in the dark at room temperature. T70-$C_{10}$–MTX conjugate was estimated at 260 nm, and the spectrum was compared with that of intact MTX. T70-$C_{10}$–MPA conjugate was estimated at 340 nm in alkaline solution and the spectrum was measured.

1) Formulation: The amounts of T70-$C_{10}$ (20 ml) and EDC (300 mg) were fixed, and 30, 3 or 0.3 mg of MTX was used for conjugation. After gel-filtration, the absorbance of each measured at 260 nm.

Several control tests (see Table V) were carried out under the same conditions except for the combination of materials. Spectrophotometry was done with a Hitachi model 200-20 spectrophotometer.

***In Vitro* Activity of T70-$C_{10}$–MTX**——The measurment of activity was based on the fact that the binding of MTX to DHFR causes quenching of the DHFR fluorescence.[12] A Hitachi 204 fluorescence spectrophotometer was used. All experiments were conducted at room temperature in 15 mM Bistris buffer, pH 7.0, containing 500 mM KCl. DHFR, Chymo.A and BSA were each prepared at a concentration of 0.5 μM. The latter two samples were diluted so that the fluorescence could be measured with the same sensitivity as for DHFR. MTX, T70-$C_{10}$–MTX and T70-$C_{10}$ were tested. MTX was used at 0.7 and 1.4 μM. T70-$C_{10}$–MTX samples were prepared to be equivalent to 0.7, 1.4 and 2.8 μM MTX, and T70-$C_{10}$ samples were used at the same concentrations (w/v) as T70-$C_{10}$–MTX. The protein solution (1.5 ml) and the substrate (1.5 ml) were mixed and the fluorescence spectrum was measured after 2 min.

## Results and Discussion

### Polymer Support

Hashida *et al.* reported that T70–mitomycin C or T500–mitomycin C had a good ability to remain at the administration site and to accumulate in lymph, but T10–mitomycin C did not.[2-4] Further, Harding stated that the renal glomerulus retained 1,6-α-glucans whose molecular weight was greater than 65000.[13] Thus, dextran should have a molecular weight of more than 65000 in order to reduce its excretion from the body.

Dextran T70 ($M_r$ 70000) was selected. Aminoethylated dextran–MTX conjugate has been reported by Harding,[13] who prepared the polymer support from 2-chloroethylamine and dextran. We decided to use a polymer support based on oxidized dextran and diaminoalkane, which can be prepared easily in the laboratory.

It was found that T70 (10% oxidized)-$C_6$ maintained a high molecular weight better than T70 (20% oxidized)-$C_6$, as shown in Fig. 1. Thus, T70 was oxidized to the extent of 10% and used for the preparation of the polymer support throughout this work.

In connection with the choice of diaminoalkane as a spacer, the reactivity of T70-$C_n$ with ninhydrin is shown in Table I. T70-$C_{10}$ formed T70-$CH_2NH(CH_2)_nNH_2$ most efficiently. After a comparison between T70-$C_{10}$ and T70-$C_8$, T70-$C_{10}$ was found to give a better polymer support with respect to molecular weight. Thus, diaminodecane was selected as a spacer. T70-$C_{10}$ could be obtained reproducibly by the procedure summarized in Chart 1.

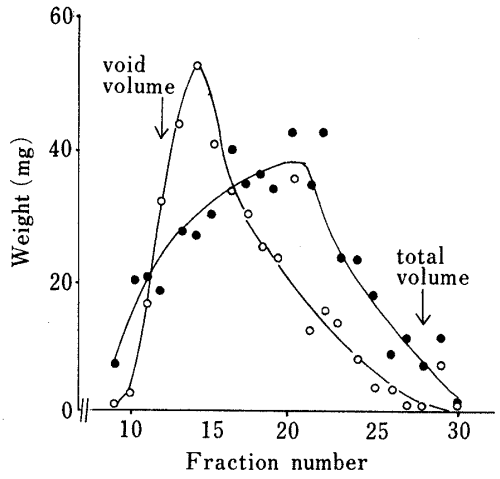


Fig. 1. Gel-Filtration Patterns on a Sephadex G50 Column with Deionized Water

○, T70 (10% oxidized)-$C_6$; ●, T70 (20% oxidized)-$C_6$

T70 (5 g in 100 ml $H_2O$)

$NaIO_4$ 1.139 g
at room temperature, 1 h, in the dark

$(CH_2OH)_2$ 3.828 g
at room temperature, 1 h, in the dark

dialyzed against $H_2O$, 24 h, at 4—5 °C
dialyzed against 0.01 M carbonate buffer, 12 h, 4—5 °C

T70-CHO

$H_2N(CH_2)_{10}NH_2$ 1.063 g
at room temperature, 4 h

$NaBH_4$ 0.467 g
at room temperature, 2 h

dialyzed against $H_2O$, 24 h, 4—5 °C

T70-$CH_2NH(CH_2)_{10}NH_2$ (T70-$C_{10}$)

Chart 1. Preparation of T70-$C_{10}$

Table I. Formation of T70-$CH_2NH(CH_2)_nNH_2$

| Test sample | Reactivity to ninhydrin[a)] |
|---|---|
| T70-$C_2$ | ++ |
| T70-$C_6$ | + |
| T70-$C_{10}$ | ++++ |

*a*) This was carried out in a mixture of each sample (0.5 ml) and 1% aqueous ninhydrin solution (0.5 ml) in 1 : 1 volume ratio. Control (water), −.

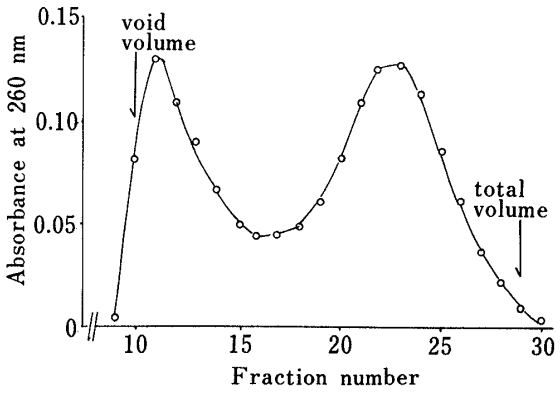


Fig. 2. Gel-Filtration Pattern on a Sephadex G50 Column with Deionized Water after the Stability Test

Table II. Estimation of T70-$C_{10}$ after the Stability Test

| Fraction | Eluted amount (mg) | Reactivity to ninhydrin[a)] |
|---|---|---|
| No. 10—14 | 28.8 | ++++ |
| No. 15—19 | 20.1 | ++ |
| No. 20—24 | 32.6 | + |
| No. 25—32 | 8.4 | + |

*a*) This was tested in a mixture of each sample solution (16 mg/ml) and 1% aqueous ninhydrin solution (5 : 1 in volume ratio). The original T70-$C_{10}$, +++++; control (solvent, water), −.

T70-$C_{10}$ showed a broadened absorption peak at 290—320 nm in neutral or alkaline solution. The reactivity to ninhydrin was high. These characteristics were reproducible.

The stability of T70-$C_{10}$ is important since degradation of the polymer support during the conjugation reaction must be avoided. T70-$C_{10}$ was little degraded in the high-molecular region, as shown in Fig. 2 and Table II, during the conjugation reaction.

Fehling reaction of T70-$C_{10}$ and oxidized dextran T70 (T70-CHO) (formed during T70-$C_{10}$ synthesis) showed that only trace amounts of aldehyde groups were carried over into T70-$C_{10}$ (Table III). Therefore, the formation of T70-$C_{10}$ was considered to have been efficiently executed.

**Conjugation**

Investigation of the conjugation reaction formulation is necessary to ensure efficient

Table III. Reactivity to Fehling's Reagent

| Substance | Concentration (mg/ml) | Reactivity[a] |
|---|---|---|
| T70-CHO | 19.92 | ++++ |
| | 9.96 | +++ |
| | 4.98 | +++ |
| | 2.49 | ++ |
| | 1.29 | + |
| T70-$C_{10}$ | 19.80 | − |

a) Control (water), −.

Table IV. Optimization of Reaction conditions

| Formulation | T70-$C_{10}$ (ml) | EDC (mg) | MTX (mg) | $A_{260}$[a] |
|---|---|---|---|---|
| A | 20 | 300 | 30 | 1.54 |
| B | 20 | 300 | 3 | 1.52 |
| C | 20 | 300 | 0.3 | 0.41 |

a) Sum of absorbances at 260 nm shown by fractions No. 10, No. 11 and No. 12, which are high-molecular fractions around the void volume.

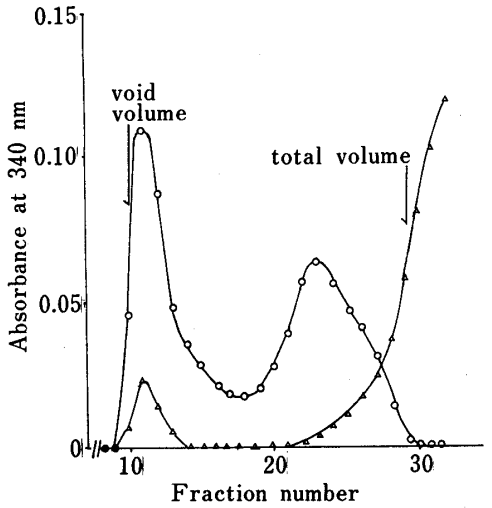


Fig. 3. Gel-Filtration Patterns on a Sephadex G50 Column with Deionized Water

○, T70-$C_{10}$+MPA+EDC; △, T70-$C_{10}$+MPA.

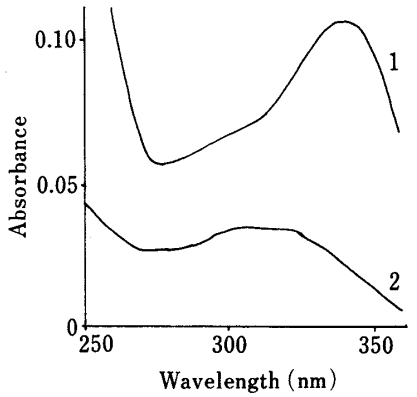


Fig. 4. Spectra of the High-Molecular Fractions under Alkaline Conditions

1, T70-$C_{10}$+MPA+EDC; 2, T70-$C_{10}$+MPA.

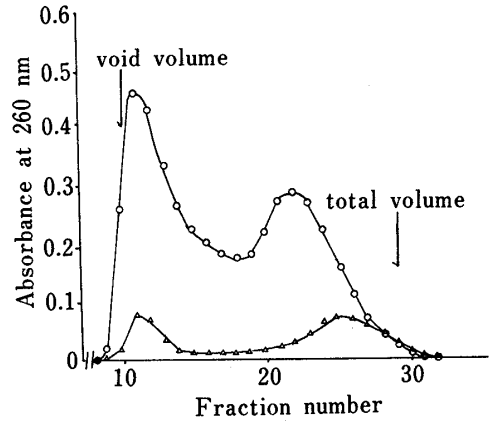


Fig. 5. Gel-Filtration Patterns on a Sephadex G50 Column with Deionized Water

○, T70-$C_{10}$+MTX+EDC; △, T70-$C_{10}$+MTX.

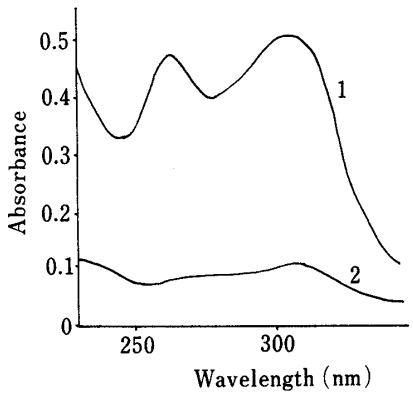


Fig. 6. Spectra of the High-Molecular Fractions

1, T70-$C_{10}$+MTX+EDC; 2, T70-$C_{10}$+MTX.

coupling of the anticancer agent. Formulation B in Table IV was found to be effective. MPA was set at 5 mg for convenience in detecting the drug binding spectrophotometrically.

MPA is an antineoplastic agent that inhibits nucleic acid synthesis. It has a carboxyl group but no amino group, and shows characteristic absorption at 340 nm in alkaline

solution, pH > 10.0. The elution pattern on gel-filtration is shown in Fig. 3. The absorption spectra of the high-molecular fractions are given in Fig. 4. Binding between T70-$C_{10}$ and MPA in the presence of EDC was apparent but only T70-$C_{10}$ was detected without EDC. The substance eluted in front of the total volume might be degraded T70-$C_{10}$–MPA conjugate or a compound derived from MPA and EDC.

MTX, in each fraction was estimated spectrophotometrically at 260 nm and the elution pattern is shown in Fig. 5. The spectra of the high-molecular fractions are shown in Fig. 6. When the mixture of T70-$C_{10}$ and MTX was stirred in the presence of EDC, the same spectral profile as that of free MTX was obtained in the high-molecular fractions. T70-$C_{10}$ and MTX without EDC showed a small shoulder near 260 nm, which may be due to adsorption of a small amount of MTX on T70-$C_{10}$. Chemical interaction between the remaining aldehyde groups of the polymer and the amino groups of MTX pteridine ring is possible, but T70-$C_{10}$ was negative in the Fehling reaction test, so that the content of aldehyde groups, if any, must be very small. From a further study in which the reaction times for Schiff's base formation and $NaBH_4$ reduction were extended, a more stable T70-$C_{10}$ was obtained. In this case, a similar result was obtained. In any case, the adsorption was slight and a T70-$C_{10}$–MTX conjugate was obtained efficiently by the use of EDC.

The conjugation reaction procedure is described in Chart 2, and the effectiveness of high-molecular T70-$C_{10}$–drug conjugate formation is summarized in Table V. The structures of

T70-$C_{10}$ (20 ml)
↓ Sephadex G50
high-molecular T70-$C_{10}$
↓ MTX 3 mg (or MPA 5 mg)
EDC 300 mg
at room temperature, pH 6.0, 24 h
in the dark
↓ Sephadex G50
T70-$C_{10}$–MTX (or T70-$C_{10}$–MPA)

Chart 2. Preparation of T70-$C_{10}$ – MTX and T70-$C_{10}$ – MPA

Table V. High-Molecular T70-$C_{10}$–Drug Conjugate Formation

| Conditions | X = MTX | X = MPA |
|---|---|---|
| T70-$C_{10}$ + X + EDC | + + + | + + + |
| T70-$C_{10}$ + X | ± | – |
| T70-$C_{10}$ + EDC | – | – |
| T70-$C_{10}$ | – | – |
| X + EDC | – | – |
| $C_{10}$ + X + EDC | – | – |

+ + +, large amount; ±, small amount; –, not recognized.

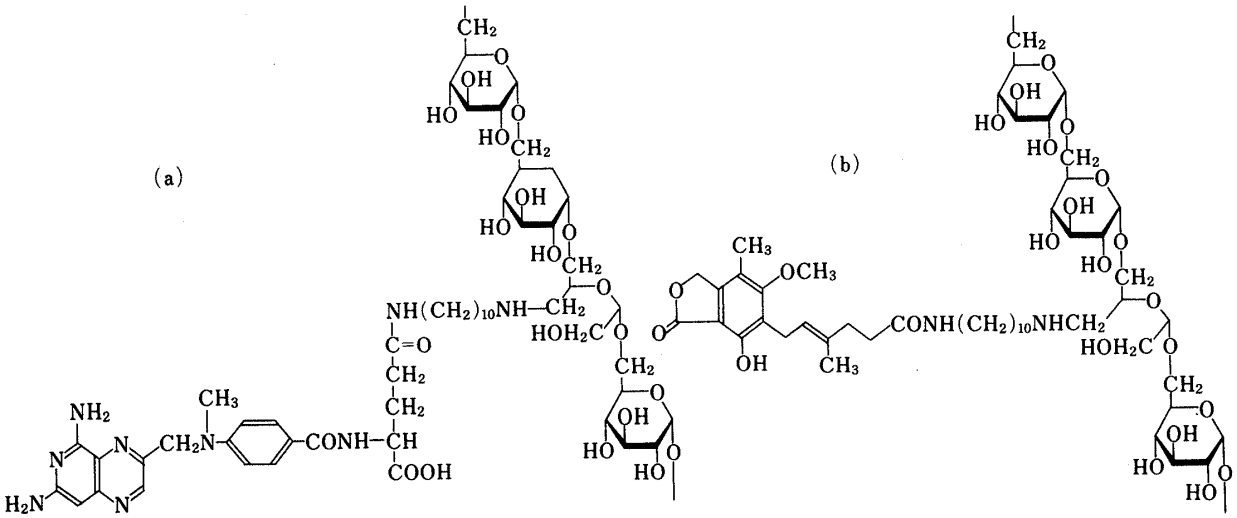


Fig. 7. Proposed Structures of T70-$C_{10}$–MTX Conjugate (a) and T70-$C_{10}$–MPA Conjugate (b)

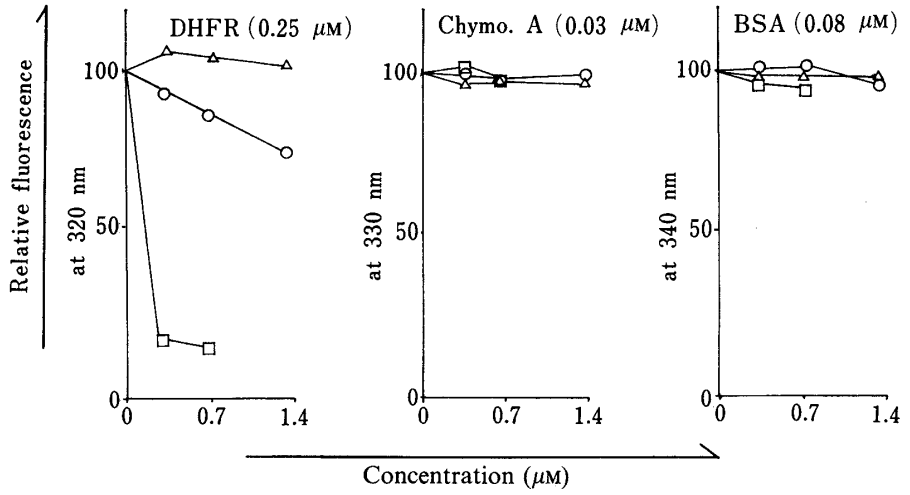


Fig. 8. Quenching of Fluorescence

○, T70-$C_{10}$–MTX; □, MTX; △, T70-$C_{10}$.
Concentration is given as that of MTX in T70-$C_{10}$–MTX. T70-$C_{10}$ was used at the same concentration (w/v) as T70-$C_{10}$–MTX.

T70-$C_{10}$–MTX and T70-$C_{10}$–MPA are proposed to be as shown in Fig. 7.

### *In Vitro* Activity of T70-$C_{10}$–MTX

T70-$C_{10}$–MTX (2 mg/ml) showed an absorbance of 2.1 at 260 nm in 0.1 N NaOH, while T70-$C_{10}$–MPA (2 mg/ml) showed an absorbance of 0.74 in 0.1 N NaOH. The content of MTX in T70-$C_{10}$–MTX was estimated to be 40 μM/g, while that of MPA in T70-$C_{10}$–MPA was 27 μM/g. DHFR suffers rapid fluorescence quenching due to the specific binding of MTX.[14,15] As Chymo.A and BSA gave stronger fluorescence than DHFR at the same concentration, they were diluted to enable measurement at the same sensitivity as for DHFR. Chymo.A and BSA suffered no fluorescence quenching with any sample. DHFR showed almost complete fluorescence quenching by MTX which was 1.4-fold molar (0.35 μM) (Fig. 8). T70-$C_{10}$–MTX corresponding to about 5.6-fold molar excess of MTX gave about 25% fluorescence quenching of DHFR and none with other proteins. This means that T70-$C_{10}$–MTX has only 8% of the activity of free MTX. This result is very similar to that of Whitely[16] or Shen and Ryser.[6] Carboxyl groups of the glutamate residues of MTX exist on the surface side of DHFR but interact with DHFR by hydrogen-bonding. Since the polymer backbone is very bulky, the modification might sterically hinder specific binding. Our T70-$C_{10}$–MTX may contain adsorbed MTX, as stated previously. Thus, the activity of T70-$C_{10}$–MTX, 8%, might be due to T70-$C_{10}$–MTX polymer conjugate or T70-$C_{10}$–MTX polymer conjugate and adsorbed MTX. The activity of T70-$C_{10}$–MTX polymer conjugate will be discussed in detail in a subsequent paper.

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