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Characterization and in vitro release of methotrexate from gelatin/methotrexate conjugates formed using different preparation variables

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Abstract

The purpose of this study was to evaluate effects of preparation variables on the composition of gelatin-methotrexate conjugates, and to evaluate their in vitro stability. Conjugation variables of pH, amount of conjugating agent 1-ethyl-3-(diaminopropyl)carbodiimide HCl (EDC), and methotrexate (MTX), with unfractionated gelatin were examined. Conjugate composition was determined spectrophotometrically. The molar ratios of MTX to gelatin in the conjugates ranged from 5.9 to 64.9. Molar ratios increased with molecular weight (MW) of gelatin in the conjugate, but the weight ratio was constant. This common conjugating procedure, however, produces by-product crosslinking and produces a mix of covalent MTX binding to carboxyl and amino groups of the gelatin. For release studies, gelatin was fractionated by size exclusion spectra (SEC) into MW of 21, 91, and 195 kDa prior to conjugation. MTX release from conjugates in dialysis cassettes at 25, 37, and 50°C, in isotonic pH 7.4, buffer over 72 h was assayed by high performance liquid chromatography (HPLC). There was no effect of gelatin MW on MTX release. MTX release was approximately linear and attained 2.3, 7.2, and 13% by 72 h at 25, 37, and 50°C, respectively, for the 91 kDa conjugates. First-order release rate constants were 0.23×10^{-3}, 0.95×10^{-3}, and 1.8×10^{-3} h^{-1}, respectively. The calculated activation energy for MTX release was 15.8 kcal/mol. Rate constants and the activation energy for MTX release are consistent with hydrolysis of a peptide bond. Non-degraded MTX species were found in the release medium at amounts similar to free MTX and were attributed to MTX polymers and MTX/gelatin fragments < 10 kDa.

Keywords: Gelatin methotrexate conjugates; Protein conjugation; Protein conjugate stability; Hydrolytic release from protein conjugates; Activation energy of hydrolytic release; Conjugate fragments

1. Introduction

Various approaches have been studied for controlled release and targeting of drugs to selective sites. The use of polymers is increasing in drug

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delivery systems and may enhance drug efficacy, reduce toxicity, and increase patient compliance (Bogdansky, 1990). Gelatin has demonstrated positive results as a carrier for muramyl dipeptide (Tabata and lkada, 1987a, 1995), recombinant interferon alpha (Tabata et al., 1988, 1991), mitomycin *C* (Yoshioka et al., 1981), adriamycin (El-Samaligy and Rohdewald, 1983), bleomycin (Hashida et al., 1979), tumor necrosis factor (Tabata et al., 1993b), recombinant interleukin alpha (Tabata et al., 1993a), and has been conjugated with methotrexate (MTX) to prepare MTX–gelatin microspheres (Narayani and Rao, 1993, 1996).

MTX is a currently employed antineoplastic agent but its limitations include toxic side effects to normal cells, and drug resistance (Calabresi and Chabner, 1996). Efforts to reduce the adverse effects include conjugating the drug to carrier molecules such as antibodies (Ballantyne et al., 1988; Ghosh et al., 1989; Kralovec et al., 1989), albumin (Halbert et al., 1987; Bures et al., 1990; Kim and Hwang, 1993; Stehle et al., 1997), and poly-L-lysine (Ryser and Shen, 1980; Rosowsky, et al., 1985). These carriers have shown promising results but each has its problems. Gelatin may have advantages for such a drug delivery system. These include an intrinsic opsonization (or activation) property for macrophages (Tabata and Ikada, 1987a, 1995), a high tissue distribution after intravenous (IV) administration (Yamaoka et al., 1994), and a susceptibility to bind to mouse tumor cells, rat fibroblasts, natural killer cells, and lymphocytes (Tabata and Ikada, 1987b; Tabata et al., 1993c). In addition, it is plausible that molar ratios in MTX/albumin conjugates $>1{:}1$ which are cleared by the liver in rats (Stehle et al., 1997) may remain viable with gelatin because of its random coil conformation and the absence of similar immune responses in earlier animal work (Hashida et al., 1979; Yoshioka et al., 1981; El-Samaligy and Rohdewald, 1983; Tabata et al., 1993a,b).

Little has been reported on the preparation and characterization details of MTX–gelatin conjugates with particular attention to reaction variables and gelatin molecular weight. Also, there are apparently no reports on the in vitro stability of these conjugates as measured by rate constants and activation energy of hydrolytic MTX release. This paper is an investigation examining these details.

2. Materials and methods

2.1. Materials

Type B gelatin granules with a bloom strength of 254, a molecular weight (MW) average of 159 kDA, and an approximate moisture content of 11% (w/w) were supplied by Kind and Knox (Sioux City, IW sample No. T7468, lot No. 1). Moisture content was determined by loss on drying at 105°C for 72 h. Solutions of 1% gelita collativ 889904 (MW 100–600 kDa), and cyano bromide (CNBr) fragments of denatured human collagen (MW 13–55 kDa) were also supplied by Kind and Knox. Sepharose 6B, blue dextran (2000 kDa), thyroglobulin (bovine, 669 kDa), apoferritin (horse spleen, 443 kDa), β-amylase (sweet potato, 200 kDa), albumin (bovine, serum 66 kDa), carbonic anhydrase (bovine erythrocytes, 29 kDa), cytochrome *C* (horse heart, 12.4 kDa), aprotinin (bovine lung, 6.5 kDa) tris-(hydroxymethyl)aminomethane base and hydrochloride, sodium azide (ultrapure), MTX ($\pm$ amethopterin, 95% pure, 12% moisture), isotonic phosphate buffered saline (PBS, pH 7.4, 10 mM), 1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride (EDC, ultrapure), were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). BCA protein assay kit and Slide-A-Lyzer dialysis cassettes, 10 kDA MW cut-off with 0.1–0.5 ml sample volume were purchased from Pierce Chemical Co. (Rockford, IL). Water was purified by reverse osmosis. All other chemicals were at least ACS reagent grade.

2.2. Gelatin fractionation

A size exclusion column (SEC) of Sepharose 6B was used to standardize and also to fractionate the gelatin. The column, with an internal diameter of 1.5 cm, was packed in 0.01% sodium azide to a

height of 90 cm, and was held at 40°C (Scholtan et al., 1974). The column was equilibrated with the eluent, pH 5.0 (0.2 M NaH_2PO_4 dihydrate, 0.3 M NaCl, and 0.01% NaN_3) (Ohno et al., 1984). The column was calibrated at 40°C using a standard mixture of CNBr fragments and gelita collativ ranging in molecular weight from 13 to 600 kDa. The eluent was used as the solvent for the standards. Blue dextran was used to determine the void volume. A Fisher peristaltic pump was used to elute the standards and samples at a flow rate of 32 ml/h. Fractions of 3.2 ml were collected and measured at 260 nm (Beckman DU UV-Vis spectrophotometer). Calibration curves converting elution volume (V_e) to log molecular weight were prepared from the chromatograms of absorbance versus V_e of the standards. The globular proteins thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, cytochrome *C*, and aprotinin at pH 7.5 (tris buffer 0.05 M, 0.1 M KCl, 0.01% NaN_3), at pH 5.0 phosphate buffer (same as for gelatin), and in water (unbuffered) were also used as standards. These calibration curves are shown in Fig. 1.

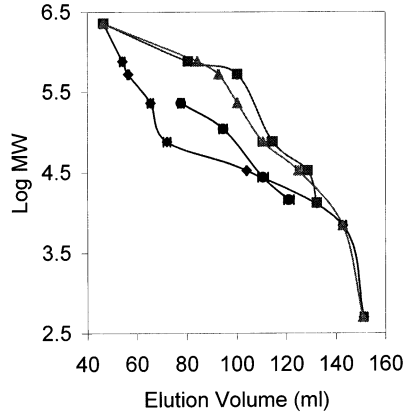

Fig. 1. SEC calibration with gelatin standards and globular protein standards in buffers with swamping electrolytes and in water using a Sepharose 6B column at 40°C. Proteins at pH 5.0 (■), proteins at pH 7.5 (▲), gelatin at pH 5.0 (●), and proteins in water (◆). Each point is the mean of two to three trials. Bars represent S.D.

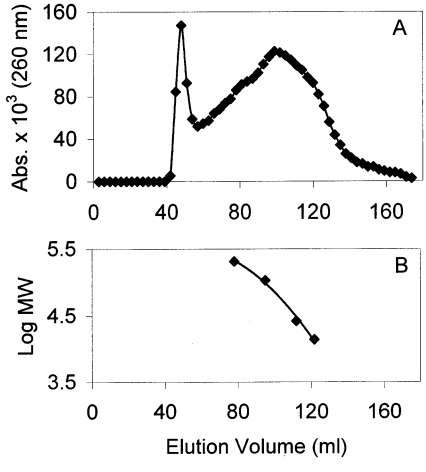

Fig. 2. SEC elution profile of unfractionated gelatin using swamping electrolyte concentration at pH 5.0 on a Sepharose 6B column at 40°C and (B) calibration plot of gelatin for molecular weight determination under the same conditions.

The molecular weight distribution of a 2.5-ml solution containing 100 mg unfractionated gelatin was determined at pH 5.0 using the above procedure and is shown in Fig. 2. From several of these distribution profiles, the V_e ranges of 75–86, 95–108, and 118–128 ml were collected and pooled to obtain fractions of three different molecular weights. These fractions were purified by dialyzing with 12.4 kDa molecular weight cut-off tubings at 4°C for 48 h using seven changes of one liter of water then lyophilized and stored in a desiccator. The molecular weight values of these fractions were determined using the V_e values of 79.9 ± 0.9, 94.6 ± 1.0, and 115.8 ± 2.1 ml from chromatograms of each pooled fraction. The calculated molecular weights were 195, 91, and 21 kDa, respectively, using the calibration equation of log $MW = 5.18 + 0.0207\ V_e - 2.43 \times 10^{-4}\ V_e^2$ ($r^2 = 0.992$).

2.3. Conjugate preparation

The effects of pH, the amount of coupling agent EDC, the amount of MTX, and the molecular weight of gelatin were examined on the conjugate. The specific conditions are listed in Table 1. Three milliliters of a solution of MTX dissolved in 0.05 M sodium bicarbonate were added to a 6-ml solution containing 100 mg of gelatin at 40°C dissolved in the same buffer. The solutions were mixed and the pH was adjusted to pH 6.0 with HCl to a final volume of 10 ml. After

Table 1
Variables examined in preparation of MTX–gelatin conjugates and the resulting composition[a]

Preparation variable	Water content (% w.w)	MTX:gelatin ratio (mg/mg)	MTX:gelatin molar ratio[b]
pH[c]			
4.7	11.7 ± 2.0	0.029 ± 0.009	10.0 ± 2.7
6.0	11.2 ± 0.6	0.100 ± 0.002	35.0 ± 0.6
7.0	12.0 ± 0.7	0.076 ± 0.002	26.1 ± 0.7
8.0	12.0 ± 1.9	0.041 ± 0.001	14.2 ± 0.3
EDC[d]			
25 mg	11.2 ± 0.6	0.100 ± 0.002	35.0 ± 0.6
75 mg	10.1 ± 0.3	0.145 ± 0.004	50.6 ± 1.0
150 mg	9.2 ± 0.7	0.160 ± 0.011	56.0 ± 3.2
MTX[e]			
25 mg	13.5 ± 1.8	0.131 ± 0.003	45.9 ± 0.8
36.7 mg	9.2 ± 0.7	0.160 ± 0.011	56.0 ± 3.2
50 mg	11.4 ± 2.8	0.186 ± 0.010	64.9 ± 2.9
Gelatin MW[f]			
195 kDa	8.1 ± 2.6	0.129 ± 0.001	55.3 ± 0.2
91 kDa	10.4 ± 0.7	0.124 ± 0.001	24.8 ± 0.2
21 kDa	10.5 ± 0.7	0.128 ± 0.000	5.9 ± 0.0

[a] $n = 2$–4, ± S.D.
[b] Using average gelatin MW of 159 kDa from manufacturer.
[c] 100 mg unfractionated gelatin, 36.7 mg MTX and 25 mg EDC.
[d] pH 6.0, 100 mg unfractionated gelatin and 36.7 mg MTX.
[e] pH 6.0, 100 mg unfractionated gelatin and 150 mg EDC.
[f] pH 6.0, 100 mg fractionated gelatin, 150 mg EDC and 25 mg MTX.

shaking at 50 rpm in a water bath at 25°C for 2 h the EDC was added and the mixture was returned to the water bath for another 4 h. The resulting conjugate was purified on a Sephadex G50 SEC column (1.5 × 40 cm) using water eluent at 37°C and a peristaltic pump. Chromatograms of the separations were determined by measuring UV absorbance of these fractions at 372 nm and plotted as a function of V_e.

2.4. Conjugate characterization

MTX content was determined from accurately weighed amounts of the conjugate dissolved in 0.1 N NaOH and UV absorbance at 372 nm using a calibration plot of dissolved MTX that was linear from 6.4×10^{-4} to 4.0×10^{-2} mg/ml. It was assumed that the molar absorbtivity of MTX was unchanged by conjugation. Gelatin absorbance was negligible at this wavelength. Gelatin content of the conjugate was determined using a BCA protein assay kit by absorbance at 562 nm after hydrolysis of the conjugate in 5 N NaOH for 1 h at 64°C then neutralization with 5 N HCl. Gelatin prepared in the same manner was used for the calibration plot in the concentration range of 0.38–2.21 mg/ml. MTX did not interfere with these measurements. Residual moisture content of the conjugate was determined by loss on drying at 105°C for 72 h.

2.5. Conjugate release

The conjugate stability was evaluated by measuring in vitro release of MTX from the conjugate over 72 h. A 0.5 ml conjugate solution in PBS at pH 7.4 containing 0.5 mg MTX was injected into a dialysis cassette and placed into a capped, wide-mouth jar containing 20 or 40 ml of PBS as the release medium at 37°C. A shaker water bath maintained temperature (25, 37, 50°C) and shaking at 100 rpm. The same procedure was used for the controls containing a physical mixture of MTX and gelatin. A 1-ml sample was periodically

removed and replaced to maintain constant release medium volume. Experiments were conducted with three replicates. Samples were analyzed for MTX content by high-performance liquid chromatography (HPLC; Nernberg et al., 1989) on a NovaPak C18, 3.9×150 mm column at 303 nm with a 88:12 phosphate buffer pH 2.7 and acetonitrile mobile phase. MTX calibration plots were linear for the concentration range of 1.28×10^{-4}–6.5×10^{-3} mg/ml. The statistical analysis of MTX release rate constants from different molecular weight fraction conjugates was conducted by comparing 95% confidence intervals of the release profile slopes.

3. Results and discussion

3.1. Gelatin fractionation

The SEC calibration plots with globular proteins show a dramatic difference between elution in pH 5.0 and in water, and a small but noticeable difference between elution in pH 5.0 and 7.5. These differences can be due to changes in ionic strength, pH and occasionally the specific ions in the buffers. Only four collagen standards for gelatin could be resolved on this column. These V_e values were 121, 111, 94.4, and 77.8 ml representing the molecular weight values of 13.5, 25, 100, and 200 kDa, respectively. The random coil conformation of collagen/gelatin standards had smaller V_e values than V_e values from globular proteins of the same molecular weight indicating a larger molecular volume.

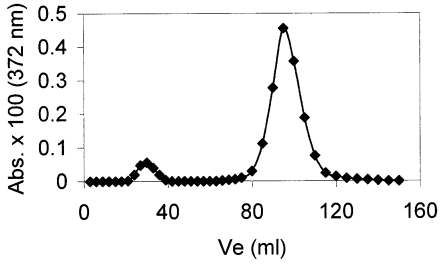

Fig. 3. SEC elution profile of MTX–gelatin conjugates and unreacted MTX from a Sephadex G-50 column eluted with water at 37°C.

Fig. 1 shows the importance of proper standards and conditions for SEC calibration plots. For example, similar V_e values of 78 ml for gelatin and 81 ml for thyroglobulin values at the same pH actually represent more than a three-fold difference in molecular weight. In addition, the gelatin eluent is at a high ionic strength from a combined buffer and salt concentration of 0.5 M to minimize or swamp out electrostatic effects of attraction or repulsion within the gelatin molecule. Both molecules occupy about the same overall volume under these conditions but the expanded random coil of gelatin is less dense because of the solvent within its domain.

The molecular weight distribution of unfractionated gelatin shown in Fig. 2 is broad with a peak at about 100 ml. It has some molecular species that are too large to be resolved which elute in the early, sharp excluded volume peak. The gelatin calibration plot from collagen standards is also shown in this figure. The elution profiles of the three pooled gelatin fractions each had clearly identified and more narrow peaks indicating a relatively narrow molecular weight distribution (data not shown). Different molecular weight gelatin fractions produce different sized conjugates at the molecular level and may produce different effects of the drug on cells in future studies.

3.2. Conjugate preparation and characterization

The SEC elution profile after the conjugation reaction shows a conjugate peak at about 30 ml and an unreacted MTX peak at about 90 ml in Fig. 3. This separation indicates that MTX is either carried along with the gelatin by physical attractions or by covalent bonds. A UV scan of a typical conjugate dissolved in 0.1 N NaOH shows a peak at 306 nm that is shifted from one at 302 nm for a physical mixture of MTX and gelatin (data not shown). This shift supports the covalent conjugation of MTX to gelatin. This shift, however, differs from the shift reported for the earlier MTX–gelatin conjugate. For the earlier conjugate, the shift is to shorter wavelengths (Narayani and Rao, 1993), but in the current study, there is a 4 nm shift to longer wavelengths.

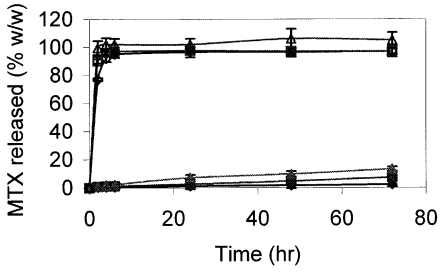

Fig. 4. Release of MTX from 91 kDa MTX–gelatin conjugates and from controls of MTX gelatin physical mixtures (upper curves) in pH 7.4 PBS at 25°C (◆), 37°C (■), and 50°C (▲). Bars represent S.D., $n = 3$.

Table 1 shows the effects of reaction pH, amount of conjugating agent, amount of MTX and gelatin molecular weight on conjugate composition. The conditions producing the highest molar ratio of MTX to gelatin of 64.9 are pH 6.0, 150 mg EDC, and 50 mg MTX using unfractionated gelatin, which represents 0.19 mg MTX/mg gelatin. These results are similar to the 0.2 mg MTX/mg gelatin reported for the earlier conjugate (Narayani and Rao, 1993). A low molar ratio of 10.0 occurred at pH 4.7, which is the optimum pH for the EDC conjugation reaction (Hoare and Koshland, 1967). This pH is also the optimum conjugation pH reported earlier for an MTX–gelatin conjugate using the same conjugating agent and similar procedure (Narayani and Rao, 1993). In the current study, the low solubility of the MTX at pH 4.7 induced precipitation, which probably caused the low molar ratio. Conjugates with fractionated gelatin were prepared with 25 mg of MTX for a maximum molar ratio of 55.3 from the 195 kDa gelatin fraction. The 21 kDa gelatin fraction produced the smallest molar ratio of 5.9, but all three gelatin molecular weight fractions produced the same 0.12 mg MTX/mg gelatin. This suggests that the conjugates are homogenous in their MTX composition. The increase in the molar ratio as the gelatin fraction molecular weight increased (Table 1) is ascribed to more conjugation sites on the larger molecules.

There are two possible sites for this conjugation reaction. The EDC conjugating agent first reacts with a carboxylic acid group that is present on both gelatin and MTX molecules to form an *O*-acylisourea intermediate (Hoare and Koshland, 1967). The intermediate then reacts with a primary amino group that also is present on both molecules. Consequently, activated carboxyl groups on the MTX may conjugate to the 33 primary amino groups per gelatin molecule (Bubnis and Ofner, 1992), or activated carboxylic acid groups on gelatin [120 initial groups per gelatin molecule (Veis, 1964)], may conjugate to the MTX amino groups. These numbers correspond to a theoretical maximum of 0.44 mg MTX/mg gelatin. This procedure most likely produces MTX conjugation at both the carboxyl and amino groups of the protein carrier molecule. In addition, both gelatin crosslinking and MTX polymerization probably occur since each specie contains both amino and carboxylic acid groups. The extent of conjugation or by-product crosslinking at each of these two sites will depend upon reaction conditions. In spite of these potential complications, EDC is an attractive conjugating agent because the resulting conjugate peptide bond retains none of the conjugating agent and is susceptible to aqueous and enzymatic hydrolysis for drug release.

3.3. Conjugate release

Release profiles of conjugates prepared with the 91 kDa gelatin fraction along with their controls are shown in Fig. 4. By 2 h most, and by 4 h all, MTX in the physical mixture was released into the medium. The rapid diffusion of MTX out of the cassettes in the controls (Fig. 4) indicated little if any physical attraction between MTX and gelatin. There was no evidence of MTX degradation during the study. The release profiles from conjugates of different molecular weights at the same temperature showed essentially no statistically significant differences. The percent MTX released after 72 h for the three fractions was 2.1–2.8, 7.1–8.3, and 12.5–13.8% for 25, 37, and 50°C, respectively. These results were similar to MTX release from unfractionated gelatin conjugates: 4.2, 8.1, and 12.1%, respectively (not shown). The conjugates demonstrated a slow and small amount released, which is ascribed to aqueous hydrolysis of the conjugate bond and is

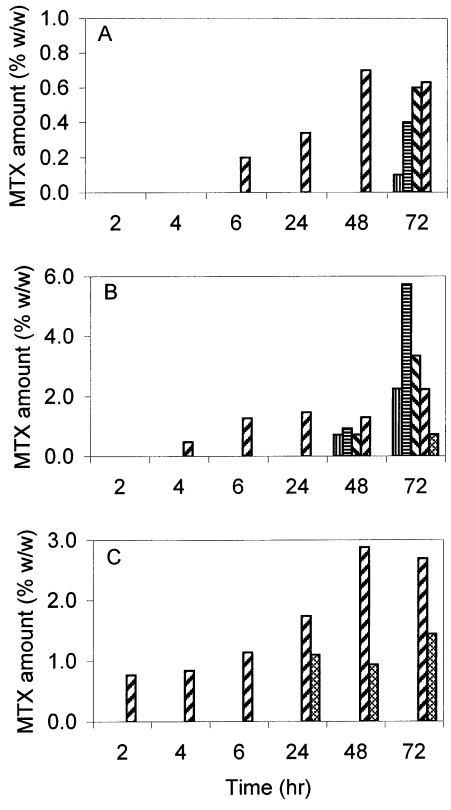

Fig. 5. Tentatively identified conjugate fragments released from the 91 kDa MTX–gelatin conjugate in pH 7.4 PBS at 25°C (A), 37°C (B), and 50°C (C). HPLC peak retention times: 1.7 min, 1.9 min, 2.2 min, 2.7 min, 4.8 min.

similar to that reported for an albumin-MTX conjugate (Halbert et al., 1987)

The controls produced one MTX peak in the HPLC chromatograms but the release samples produced two to five additional, but smaller peaks with reproducible retention times that occurred before and after the MTX 4-min retention time. While there was not a large difference between production of these extra peaks by the different molecular weight conjugates, there was a noticeable difference with temperature. Fig. 5 shows the extra peaks from the 91 kDa fraction conjugate at 25, 37, and 50°C. By the end of the study, about 2% of MTX is represented in these extra peaks at 25°C, about 14% at 37°C, and about 5% at 50°C.

The extra chromatographic peaks in the samples (Fig. 5) represent a substantial amount of MTX in a form that differs from the free MTX. They do not represent degradation species or gelatin. These alternate forms represent an equal amount of the free MTX released at 25°C, double the free MTX at 37°C, and almost one half the free MTX at 50°C. These species are tentatively identified as either conjugate fragments composed of small peptide chains of varying length and one or more MTX molecules, or MTX polymers formed during conjugation, or a mixture of each. Such species would be smaller than the 10 kDa cut-off of the dialysis membrane in the cassettes. Regardless of the unclear composition of these conjugates, a kinetic analysis of MTX release produces useful parameter estimates; hydrolysis of the peptide conjugate bond is still required to release an MTX molecule.

Due to the small MTX release, there was little difference between fitting the results to a zero-order model or a first-order model. MTX release from the 91 kDa conjugate was fit to a pseudo first-order model based on theory for a hydrolytic reaction. The data at 50°C has the greatest varia-

Table 2
Kinetic and arrhenius analysis of MTX release from 91 kDa conjugates

Temperature (°C)	% Release[a] (w/w) ± S.D.	Rate constant ($h^{-1} \times 10^3$) ± S.E.	Rate constant combined fractions[b] ($h^{-1} \times 10^3$) ± S.E.
25	2.28 ± 0.02	0.23 ± 0.01	0.26 ± 0.03
37	7.24 ± 0.08	0.95 ± 0.04	1.02 ± 0.06
50	13.2+0.2	1.81 ± 0.14	1.85 ± 0.15
Activation energy (kcal/mol) ± S.E.		15.8 ± 3.4	15.0 ± 3.4[b]

[a] After 72 h. at pH 7.4 in PBS with 100 rpm shaking; $n = 3$.
[b] Combining data from 21, 91, and 194 kDa gelatin fractions.

tion and deviation from linearity. Table 2 lists the rate constants of release at each temperature obtained from a first-order model. These values were used for an Arrhenius plot to calculate an activation energy, E_a, for MTX release. The release rate constants for the 91 kDa conjugate are no different from values calculated after combining data from the three molecular weight fraction conjugates (see Table 2). The relatively broad variation of the calculated activation energy (15.8 kcal/mol $\pm$ 3.4 S.E.) probably reflects the mixed composition of the conjugate. This value for the activation energy is within the reported range of 13.4–26.3 kcal/mol for several peptide bonds in gelatin (Veis, 1964).

4. Conclusions

Accurate SEC measurements of random coil molecules, such as gelatin, require appropriate standards and conditions that take into account molecular conformation, pH, and ionic strength. MTX–gelatin conjugates were prepared with a carbodiimide conjugating agent under different reaction conditions using different molecular weights of gelatin. The molar ratios of MTX to gelatin for these conjugates ranged from 5.9 to 64.9. This common procedure probably produces MTX conjugation at both the carboxyl and amino groups of the protein carrier molecule as well as by-product crosslinking. The extent of conjugation and crosslinking at each of these two sites will depend on reaction conditions. There is no effect of varying gelatin molecular weight on the MTX content of the conjugate measured on a weight basis but the molar ratio increases with higher gelatin molecular weight. There is no effect of gelatin molecular weight on MTX release. MTX release over 72 h is approximately linear at 25, 37, and 50°C, and is $< 14\%$. Apparent conjugate fragments and MTX polymers (< 10 kDa) up to double the small amount of free MTX released were observed. The rate constants and energy of activation for MTX release are consistent with hydrolysis of a peptide bond. The site-specific conjugation of MTX to gelatin and minimization of by-product crosslinking is currently being investigated. These conjugates are also being investigated in cell culture studies for toxicity and uptake.

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References

Ballantyne, K.C., Perkins, A.C., Pimm, M.V., Garnett, M.C., Clegg, J.A., Armitage, N.C., et al., 1988. Biodistribution of a monoclonal antibodymethotrexate conjugate (791T/36-MTX) in patients with colorectal cancer. Int. J. Cancer (Suppl.) 2, 103–108.

Bogdansky, S., 1990. Natural polymers as drug delivery systems. In: Chasin, M., Langer, R. (Eds.), Biodegradable Polymer as Drug Delivery Systems. Marcel Dekker, New York Chapter 7.

Bubnis, W.A., Ofner III, C.M., 1992. The determination of e-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. Anal. Biochem. 207, 129–133.

Bures, L., Lichy, A., Bostik, I., Spundova, M., 1990. The use of protein as a carrier of methotrexate for experimental cancer chemotherapy. V. Alternative method for preparation of serum albumin-methotrexate derivative. Neoplasma 37, 225–231.

Calabresi, P., Chabner, B.A., 1996. In: Hurdman, L., Limbird, E. (Eds.), Goodman and Gilman's Pharmacological Basis of Therapeutics. McGraw-Hill, New York, pp. 1243–1247.

El-Samaligy, M.S., Rohdewald, P., 1983. Reconstituted collagen nanoparticles, a novel drug carrier delivery system. J. Pharm. Pharmacol. 35, 537–539.

Ghosh, M.K., Kildsig, D.O., Mitra, A.X., 1989. Preparation and characterization of methotrexate-immunoglobulin conjugate. Drug Design Del. 4, 13–25.

Halbert, G.W., Florence, A.T., Stuart, J.F.B., 1987. Characterization of in vitro drug release and biological activity of methotrexate-bovine serum albumin conjugates. J. Pharm. Pharmacol. 39, 871–876.

Hashida, W., Muranishi, S., Sezaki, H., Tanigawa, N., Satomura, K., Hikasa, Y., 1979. Increased lymphatic delivery of bleomycin by microsphere emulsion and its effect on lymph node metastasis. Int. J. Pharm. 2, 245–256.

Hoare, D.G., Koshland Jr, D.E., 1967. A method for quantitative modification and estimation of carboxylic acid groups in proteins. J. Biol. Chem. 242, 2447–2453.

Kim, C.K., Hwang, S.A., 1993. Pharmacokinetics and organdistribution of ^{3}H methotrexate and ^{3}H-methotrexate-human serum albumin conjugates in mice. Drug Dev. Indust. Pharm. 19, 961–970.

Kralovec, I., Singh, M., Mammen, M., Blair, A.H., Ghose, I., 1989. Synthesis of sitespecific methotrexate-lgG conjugates. Cancer Immun. Immunother. 29, 293–302.

Narayani, R., Rao, K.P., 1993. Preparation, characterization and in vitro stability of hydrophilic gelatin microspheres using a gelatin-methotrexate conjugate. Int. J. Pharm. 95, 85–91.

Narayani, R., Rao, K.P., 1996. Biodegradable microspheres using two different gelatin drug conjugates for the controlled delivery of methotrexate. Int. J. Pharm. 128, 261–268.

Nernberg, B., Kohibrenner, M., Faulkner, R., 1989. Rapid quantitation of methotrexate and its metabolites in human serum, urine, and bile, using solid-phase extraction and high performance liquid chromatography. J. Chromatogr. 487, 476–482.

Ohno, T., Kobayashi, H., Mizusawa, S., 1984. Determination of molecular weight distribution of gelatin by gel permeation chromatography (III). Chiba Daigaku Kogakubu Kenkyo Hokoku 59, 237–247.

Rosowsky, A., Forsch, R.A., Galivan, I., Susten, S.S., Freisheim, J.H., 1985. Regiospecific gamma-conjugation of methotrexate to poly(L-lysine), chemical and biological studies. Mol. Pharmacol. 27, 141–147.

Ryser, H.J., Shen, W.C., 1980. Conjugation of methotrexate to poly(L-lysine) as a potential way to overcome drug resistance. Cancer 45, 1207–1211.

Scholtan, V.W., Lange, H., Rosenkranz, H., Moll, F., 1974. Bestimmung des molekulargewichts und der molekulargewitchsverteilung von gelatine mit hilfe der gelchromatographie und der ultrazentrifuge. Colloid Polym. Sci. 252, 949–970.

Stehle, G., Sinn, H., Wunder, A., Schrenk, H.H., Schutt, S., Maier-Borst, W., et al., 1997. The loading rate determines tumor targeting properties of methotrexatealbumin conjugates in rats. Anti-Cancer Drugs 8, 677–685.

Tabata, Y., Ikada, Y., 1987a. Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. Jpn. J. Cancer Res. 39, 698–704.

Tabata, Y., Ikada, Y., 1987b. Pinocytosis of proteins by tumor cells. Sixteenth Iyoukoubunshi Symposium 16, 89–90.

Tabata, Y., Ikada, Y., 1995. Enhanced macrophage activation for antitumor function by muramyl dipeptide chemically modified with gelatin of different molecular weights. Proc. Int. Symp. Control. Rel. Bioact. Mater. 22, 552–553.

Tabata, Y., lkada, Y., Uno, K., Muramatsu, S., 1988. Potentiation of antitumor activity of macrophages by recombinant interferon alpha A/D contained in gelatin microspheres. Jpn. J. Cancer Res. 79, 636–646.

Tabata, Y., Ikada, Y., Uno, K., Yamaoka, I., Muramatsu, S., 1991. Effects of recombinant alpha-interferon-gelatin conjugate on in vivo murine tumor cell growth. Cancer Res. 51, 5532–5538.

Tabata, Y., Ikada, Y., Uno, K., Kishida, I., Muramatsu, S., 1993a. Potentiation of in vivo antitumor effects of recombinant interleukin 1-alpha by gelatin conjugation. Jpn. J. Cancer Res. 84, 681–688.

Tabata, Y., lkada, Y., Uno, K., Muramatsu, S., 1993b. Suppressive effect of recombinant TNF-gelatin conjugate on murine tumor growth in vivo. J. Pharm. Pharmacol. 45, 303–308.

Tabata, Y., Uno, K., Ikada, Y., Kishida, I., Muramastsu, S., 1993c. Potentiation of in vivo antitumor effects of recombinant interleukin-1α by gelatin conjugation. Jpn. Cancer Res. 84, 681–688.

Veis, A., 1964. The Macromolecular Chemistry of Gelatin. Academic Press, New York.

Yamaoka, T., Tabata, Y., lkada, Y., 1994. Body distribution of intravenously administered gelatin with different molecular weights. J. Contr. Rel. 31, 1–8.

Yoshioka, I., Hashida, W., Muranishi, S., Sezaki, H., 1981. Specific delivery of mitomycin C to the liver, spleen and lung: nano- and microspherical carriers of gelatin. Int. J. Pharmacol. 8, 131–141.