Characterization and *In Vitro* Methotrexate Release from Methotrexate/Gelatin Conjugates of Opposite Conjugate Bond Polarity

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Received June 23, 2000; accepted July 6, 2000

Purpose. Our laboratory has previously prepared gelatin/methotrexate (MTX) conjugates containing mixed conjugation sites and by-product crosslinking, both of which may alter conjugate effectiveness. In this study, we prepared and evaluated gelatin/MTX conjugates having specific conjugate bond sites and minimal by-product crosslinking.

Methods. Opposite polarity conjugates were produced by coupling gelatin having blocked amino groups with MTX (G-MTX) and by coupling MTX having blocked amino groups with gelatin (M-GEL) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. Amino groups were blocked using citraconic anhydride and deblocked under acidic conditions. Gelatin and MTX contents were determined spectrophotometrically. The stability of each conjugate was determined by evaluating their *in vitro* release of MTX in isotonic buffer at pH 7.4 and 37°C for 7 days.

Results. The G-MTX and M-GEL conjugates contained 21 and 1.2 mole MTX/mole gelatin and released 12 and 17% MTX by 7 days resulting in pseudo-first order release rate constants of 0.76×10^{-3} and 1.0×10^{-3} hr^{-1}, respectively. Alternate MTX species ($\leq$ 10%) were detected during the release study and were attributed to low molecular weight gelatin/MTX fragments and MTX polymers.

Conclusions. Gelatin/MTX conjugates having opposite conjugate bond polarities and minimal by-product crosslinking have been produced and slowly released MTX by hydrolytic cleavage indicating good stability for future cell culture studies.

KEY WORDS: gelatin methotrexate conjugates; protein conjugation; protein conjugate stability; hydrolytic release from protein conjugates; site-specific conjugation; crosslinking; conjugate fragments.

INTRODUCTION

Increasing the specificity and effectiveness of chemotherapeutic agents by using novel drug delivery systems has become a major field of research (1). One such delivery system is the covalent conjugation of low molecular weight drugs to soluble macromolecular carriers. Ideally, these carriers actively or passively accumulate drug at the tumor site and prolong drug residence time in the body thereby limiting systemic toxicities and increasing drug effectiveness. Such carriers may also overcome certain drug resistances by utilizing different cellular uptake mechanisms and altering the drug's intracellular activities.

Gelatin, a partially hydrolyzed form of collagen, has been used as a soluble macromolecular carrier to improve the stability and biological activity of several agents (2–5). The advantages of gelatin include low cost, ease of preparation, biodegradability and biocompatibility (6). Gelatin also possesses several attributes that make it attractive for use as a soluble macromolecular carrier. These include a high intrinsic opsonic property for macrophages (7), phagocytosis and lysosomal degradation by breast tumor cells (8), a high tissue distribution after IV administration (9) and a susceptibility for binding to mouse tumor cells, rat fibroblasts, natural killer cells and lymphocytes (5,10).

Methotrexate (MTX) is a commonly used chemotherapeutic agent but like most of these agents, it has a nonselective toxicity and a high occurrence of drug resistance (11). To overcome these problems, the reactive sites of MTX (Figure 1) have been covalently bound either directly or via a spacer to several soluble macromolecules including monoclonal antibodies (12–14), albumin (15–19) and poly-L-lysine (20). These conjugates, which were formed using either carbodiimide coupling or active ester formation, have shown promising results but each has its problems. Another laboratory has reported the production of gelatin/MTX conjugates; however, these conjugates were used to prepare microspheres and were not extensively evaluated (21,22).

Our lab has prepared soluble gelatin/MTX conjugates using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) as the coupling agent (23). However, this commonly used conjugation procedure provides no control over the polarity of the resulting conjugate bond. A carboxylic acid group on gelatin may couple with an amino group on MTX (G-MTX) or vice versa (M-GEL). Furthermore, gelatin and MTX crosslinking may also occur (Figure 2). After cellular uptake, protein/MTX conjugates are cleaved by lysosomal enzymes and release not only MTX but also MTX oligimers, which significantly contribute to conjugate toxicity (20). Since MTX amino groups are important for enzyme interaction (11), the presence of different conjugate bond polarities as well as MTX crosslinked species in these oligimers may produce varying effects. Therefore, conjugates prepared using this common procedure may have a less than optimal effectiveness and a mechanism of action that is difficult to elucidate.

The objectives of this study are 1) to prepare single polarity gelatin/MTX conjugates with minimal crosslinking, 2) to characterize each polarity conjugate and 3) to determine the stability of each conjugate by evaluating their *in-vitro* release of MTX before conducting cell culture studies, which will evaluate the cellular toxicity and cellular uptake of each polarity conjugate.

MATERIALS AND METHODS

Materials

Type B gelatin granules with a bloom strength of 254, an average molecular weight of 159 kDa and an approximate moisture content of 11% (w/w) determined by loss on drying

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ABBREVIATIONS: CA, citraconic anhydride; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; GMPM, gelatin/methotrexate physical mixtures; LOD, loss on drying at 105°C for 72 hr; MTX, methotrexate; PBS, isotonic phosphate buffered saline; SEC, Sephadex G-50 column, 42 cm × 2 cm.

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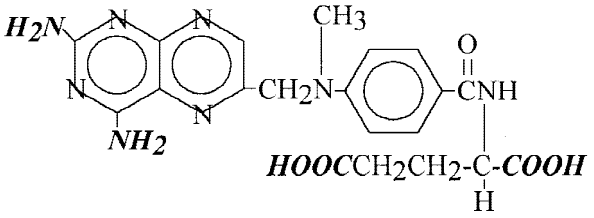

Fig. 1. Chemical structure of methotrexate (MTX) which contains several possible reactive sites for conjugation, molecular weight = 454.

at 105°C for 72 hours (LOD) were supplied by Kind and Knox (Sioux City, IA, sample #T7468, lot #1). Sephadex G-50, MTX (±amethopterin, 95% (w/w) pure, 12% (w/w) moisture), isotonic phosphate buffered saline (PBS, 10 mM, pH 7.4), sodium azide (ultrapure), EDC (ultrapure) and poly-L-lysine HBr (125–250 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). BCA protein assay kits and Slide-A-Lyzer dialysis cassettes (10 kDa molecular weight cut-off and 0.5–3.0 ml sample volume) were purchased from Pierce Chemical Co. (Rockford, IL). Citraconic anhydride (CA, 98% (w/w)) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Water was purified by reverse osmosis. All other chemicals were at least ACS reagent grade.

Conjugate Preparation

Reversible Blocking of Amino Groups

Gelatin and MTX amino groups were reversibly blocked using CA (24). CA (50 μl) was reacted with either 100 mg gelatin dissolved in 6 ml 0.1 M $NaHCO_3$ or 50 mg MTX dissolved in 3 ml 0.1 M $NaHCO_3$ for 1 hr at pH 8.0–9.0 and 25°C with 50 rpm shaking. The blocked gelatin was separated from excess CA on a Sephadex G-50 column, 42×2 cm, eluted with 0.05 M $NaHCO_3$ at 37°C using a Fisher peristaltic pump (SEC). A 12 ml blocked gelatin elution volume containing ~74 mg blocked gelatin was collected. The blocked MTX was separated from excess CA by adding 100 mg poly-L-lysine dissolved in 5 ml 0.1 M $NaHCO_3$ for 1 hr at pH 9.0-10.0 and 25°C with 50 rpm shaking. The blocked MTX was separated from the reacted poly-L-lysine using SEC and lyophilized. Two batches of the blocked MTX were combined and reconstituted in 9 ml water.

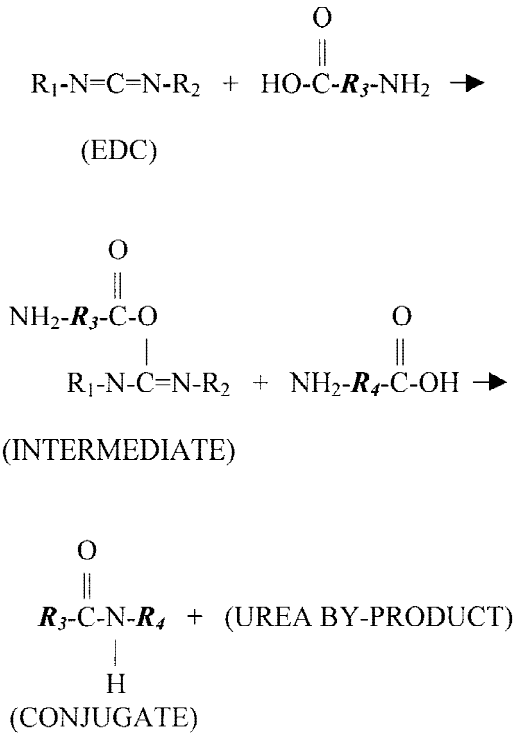

Fig. 2. Conjugation of gelatin and MTX. G-MTX: R_3 = gelatin and R_4 = MTX; M-GEL: R_3 = MTX and R_4 = gelatin; gelatin crosslinking: both R_3 and R_4 = gelatin; MTX crosslinking: both R_3 and R_4 = MTX.

Gelatin/MTX Conjugation

G-MTX was prepared by mixing the 12 ml blocked gelatin elution volume containing ~74 mg blocked gelatin with 37.5 mg MTX dissolved in 3 ml 0.1 M $NaHCO_3$ followed by coupling with 75 mg EDC. M-GEL was prepared by mixing the 9 ml blocked MTX solution with 100 mg gelatin dissolved in 6 ml 0.1 M $NaHCO_3$ followed by coupling with 112.5 mg EDC. Mixing and coupling were conducted for 2 and 24 hr, respectively, at pH 7.0 and 25°C with 50 rpm shaking. The conjugates were purified using SEC and lyophilized. Elution profiles were prepared by collecting the eluent in 6 min fractions (2.8–3.0 ml) and measuring their absorbance at 372 nm using a Shimadzu UV-1601 UV/Visible spectrophotometer.

Amino Group Deblocking

The conjugates were reconstituted in 5 ml water and their respective amino groups were deblocked for 5 hr at pH 4.0–5.0 and 25°C with 50 rpm shaking (24). The conjugates were purified using SEC eluted with water, lyophilized and stored in a desiccater at room temperature.

Conjugate Characterization

G-MTX and M-GEL samples were dissolved in release medium (PBS at pH 7.4 containing 0.05% NaN_3 as preservative). Absorption spectra were determined using a Molecular Devices SpectraMax plus spectrophotometer. Gelatin content was determined using a BCA protein assay and reproducible calibration curves, 0.08–1.2 mg/ml, were fit to a quadratic equation. The calibrations showed no MTX interference at MTX concentrations of 1.6×10^{-3} and 3.3×10^{-2} mg/ml. MTX content was determined spectrophotometrically at 372 nm using linear calibration plots from 5.0×10^{-4}–4.0×10^{-2} mg/ml. It was assumed that the molar absorbtivity of MTX did not change significantly after conjugation. Absorbance due to gelatin at 372 nm was accounted for in the blanks. Moisture content was determined by LOD.

Conjugate Release

The *in vitro* release of MTX from each conjugate was evaluated over 7 days. G-MTX amounts equivalent to 0.12 and 1.7 mg MTX, designated G-MTX-L and G-MTX-H, respectively, and M-GEL amounts equivalent to 0.10 mg MTX in 3 ml release medium were injected into dialysis cassettes. The cassettes were placed into capped, wide-mouth jars containing 70 ml release medium maintained at 37°C in a shaker

water bath at 100 rpm. Each sample had a corresponding MTX solution and gelatin/MTX physical mixture (GMPM) as controls. Experiments were conducted with 3 replicates. One milliliter of release medium was sampled at desired times and replaced with fresh medium to maintain a constant volume. Samples were assayed by reverse phase HPLC on a Nova-Pak C18, 3.9×150 mm column at 303 nm with a 88:12 phosphate buffer, pH 2.7, and acetonitrile mobile phase (25). Low and high MTX concentration calibration plots, 4.0×10^{-6}–8.0×10^{-5} and 8.0×10^{-5}–1.6×10^{-3} mg/ml, respectively, were linear. The MTX chromatographic peak retention time ranged from 4.06 to 4.19 minutes.

RESULTS

Conjugate Preparation

The SEC elution profiles after conjugation showed peaks at fractions 6-7 and 23-25 while the elution profiles for GMPM showed a single peak at fractions 23-25 (Figure 3). The absence of MTX absorbance in the macromolecular fractions (3–12) of the GMPM elution profiles indicates that there are no significant physical attractions between gelatin and MTX and that the MTX present in the macromolecular fractions after conjugation is covalently conjugated to the gelatin.

Conjugate Characterization

The absorption spectrum of a GMPM showed peaks at 303 and 372 nm which are characteristic of MTX; however, the absorption spectra of the G-MTX and M-GEL show peak shifts from 303 nm to 310, and to 305 nm, respectively, and a flattening of the 372 nm peak (Figure 4). These results support the covalent conjugation of MTX to gelatin. Table I summarizes each conjugate's composition, molar ratio, conjugation efficiency and yield.

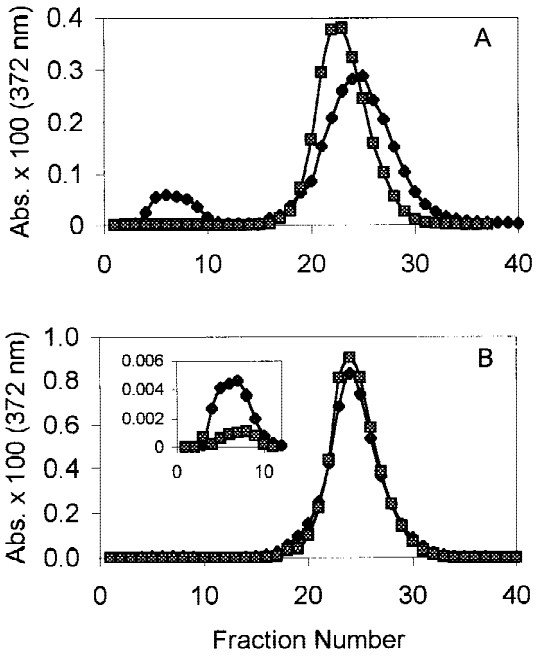

Fig. 3. SEC elution profiles for (A) G-MTX (♦) and a blocked gelatin/MTX physical mixture (■) and (B) M-GEL (♦) and a gelatin/blocked MTX physical mixture (■).

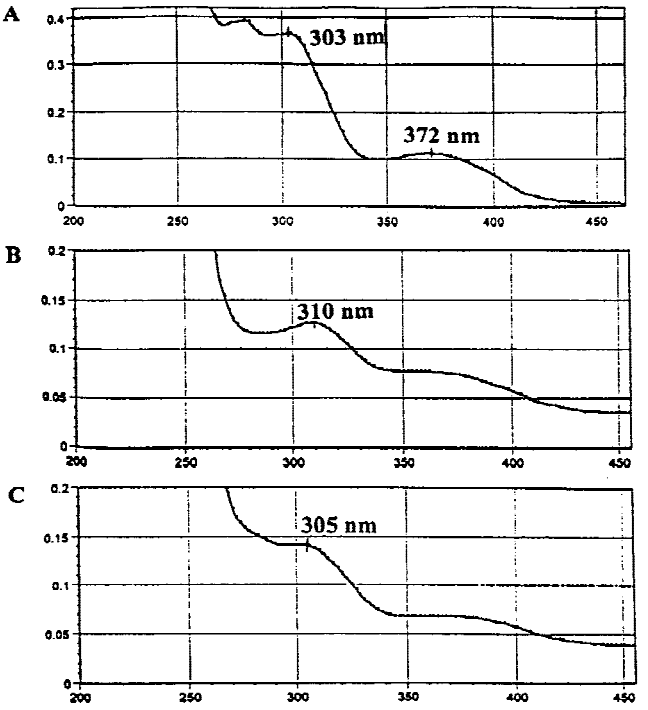

Fig. 4. UV absorbance profiles for (A) gelatin/MTX physical mixture, (B) G-MTX and (C) M-GEL.

Conjugate Release

MTX release from controls reached 100% by 4 hr and remained there throughout the 7 days. MTX release from the G-MTX and M-GEL reached 6.9±0.4 and 11±0% (±SD) after 72 hr and 12±0 and 17±2% (±SD) after 7 days, respectively (Figure 5). Each G-MTX sample showed a similar release profile even though a 14-fold difference in starting amounts was used. The amount of MTX released from the G-MTX-L, G-MTX-H and M-GEL corresponds to 0.10, 1.6 and 0.14 μM after 72 hr and 0.19, 2.7 and 0.23 μM after 7 days, respectively. In comparison, therapeutic plasma concentrations of MTX generally range from 1.0–10.0 μM (26). MTX release was fit

Table 1. Summary of Conjugate Characterization

	G-MTX[a]	M-GEL[b]
Sample weight	100.4 mg	102.0 mg
Gelatin content[c]	92 ± 3 mg	97 ± 1 mg
MTX content[c]	5.6 ± 0.0 mg	0.34 ± 0.00 mg
Moisture content[c]	4.5 ± 1.2 mg	3.8 ± 0.7 mg
Molar ratio[d]	21	1.2
Conjugation efficiency[e]	18%	3.7%
Conjugation yield[f]	49%	74%

[a] Conjugate bond between gelatin carboxyl and MTX amino groups.
[b] Conjugate bond between MTX carboxyl and gelatin amino groups.
[c] Mean value of three determinations ± SD.
[d] MTX = 454 Da, gelatin = 159,000 Da (21).
[e] (calculated molar ratio/theoretical ratio) × 100.
[f] (recovered conjugate mass/theoretical mass) × 100.

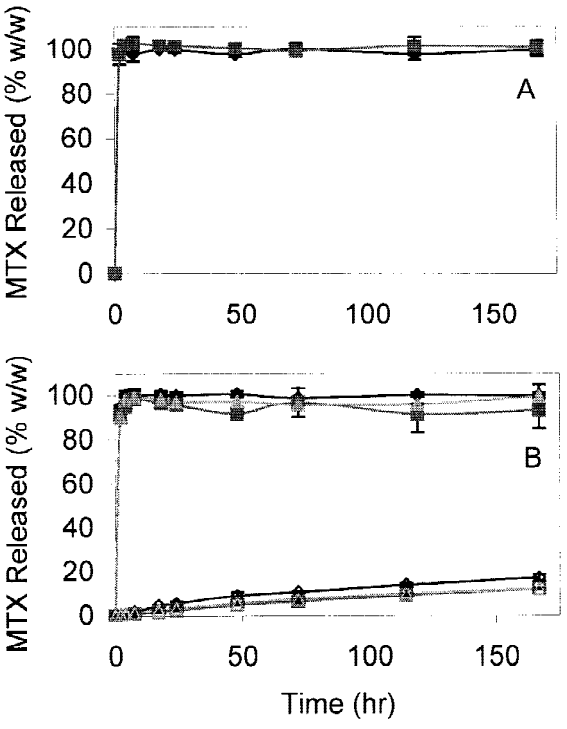

Fig. 5. MTX release profiles from (A) MTX solutions (♦: 7.31×10^{-5} M, ■: 1.02×10^{-3} M) and (B) gelatin/MTX physical mixtures of concentrations corresponding to ♦: M-GEL, ■: G-MTX-L, ▲: G-MTX-H, and conjugates (◇: M-GEL, □: G-MTX-L, △: G-MTX-H) in PBS at pH 7.4 and 37°C containing 0.05% NaN_3. Error bars = SD, n = 3.

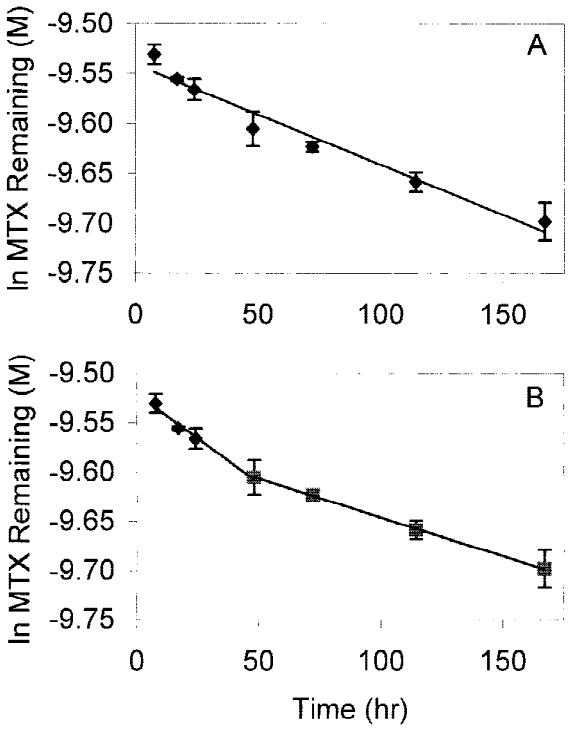

Fig. 6. M-GEL release shows the apparent biphasic nature of MTX release from the conjugates, (A) overall and (B) biphasic fits. Error bars = SD, n = 3.

to pseudo first-order kinetics; however, the release suggests an apparent biphasic nature with faster release up to 48 hr and slower thereafter (Figure 6). The 2 and 4 hr data were not used for the kinetic analysis due to a lack of sensitivity at these time points. Table II summarizes the conjugate kinetic release data.

HPLC chromatograms of the G-MTX and M-GEL release samples showed several peaks in addition to MTX as early as 2 and 16 hr, respectively (data not shown). These peaks were not detected in controls and do not represent gelatin or MTX degradation products. The retention times of these peaks ranged from 2.85 to 7.68 minutes with a majority detected in the G-MTX samples. Estimates using these peak areas indicated that G-MTX and M-GEL released 7.0 and 3.0% of the initial amount of MTX as alternate MTX species after 72 hr and 10 and 5.0% after 7 days, respectively.

DISCUSSION

Conjugate Preparation

Our lab has prepared gelatin/MTX conjugates using a common carbodiimide conjugation procedure; however, this procedure produced mixed polarity conjugates along with gelatin and MTX crosslinking (23). The side reaction of protein crosslinking during carbodiimide conjugation has been reported in some studies (12,13,16,18) but is not always taken into account. The side reaction of MTX crosslinking may also occur, but was only recently reported (23,31). The amino groups of the MTX pteridine ring (Figure 1) have been generally believed to be insufficiently nucleophilic to participate in carbodiimide conjugation reactions (27) and consequently, only the M-GEL polarity was believed to be produced. However, in preliminary experiments, MTX was polymerized using EDC indicating that the amino groups on MTX are able

Table 2. Summary of Kinetic Release Data

	Rate constant[a] ($hr^{-1} \times 10^3$)	Upper 95% ($hr^{-1} \times 10^3$)	Lower 95% ($hr^{-1} \times 10^3$)
G-MTX-L[b]			
Overall[c]	0.78 ± 0.03	0.86	0.69
Early[d]	1.0 ± 0.0	1.1	0.95
Late[e]	0.69 ± 0.01	0.75	0.63
G-MTX-H[f]			
Overall	0.74 ± 0.05	0.87	0.61
Early	1.0 ± 0.1	1.3	0.83
Late	0.62 ± 0.06	0.85	0.38
M-GEL[g]			
Overall	1.0 ± 0.1	1.2	0.77
Early	1.8 ± 0.2	2.4	1.2
Late	0.78 ± 0.01	0.84	0.73

[a] ± SE, n = 3, in PBS at pH 7.4 and 37°C containing 0.05% NaN_3.
[b] Conjugate bond between gelatin carboxyl and MTX amino groups; MTX amount of 0.12 mg.
[c] Full 7 days of the study.
[d] First 2 days of the study.
[e] Days 2–7 of the study.
[f] Conjugate bond between gelatin carboxyl and MTX amino groups; MTX amount of 1.7 mg.
[g] Conjugate bond between MTX carboxyl and gelatin amino groups; MTX amount of 0.10 mg.

to participate in such reactions. Spectophotometric evaluation indicated that dissolved, polymerized MTX has its greatest stability at pH 7.0 compared to pH 2.0 and 12.0 (data not shown). The reactivity of the MTX amino groups is also confirmed by the successful production of G-MTX in the current study. Therefore, mixed polarities as well as by-product crosslinking are probably present in many protein/MTX carriers including the first gelatin/MTX conjugates reported (21).

The second reported gelatin/MTX conjugates were prepared using an azide coupling-grafting method that produced opposite polarity conjugates but there was no report of by-product crosslinking (22). We attempted to overcome these problems using a less intensive procedure than azide coupling. Gelatin and MTX amino groups were blocked using CA to prevent their participation in the conjugation reaction. This produced single polarity conjugates and prevented crosslinking of the specie whose amino groups had been blocked. Unfortunately, the specie whose amino groups remain unblocked may still crosslink. The efficiency and stability of the amino group blocking was evaluated to ensure production of single polarity conjugates (28). Results showed that 93% of gelatin amino groups were initially blocked and 84% of gelatin amino groups remained blocked at the end of the conjugation reaction. It was also determined that 93% of gelatin amino groups were recovered after deblocking.

Conjugate Characterization

Our previously prepared mixed polarity conjugate showed a peak shift from 302 to 306 nm (23) compared to the peak shifts from 303 to 310, and to 305 nm for the G-MTX and M-GEL, respectively. By comparison, the first reported gelatin/MTX conjugates prepared using EDC, which were described as having an M-GEL polarity, showed a peak shift from 302 to 309 nm (21) suggesting more of a G-MTX polarity. When gelatin/MTX conjugates were prepared using azide coupling, the M-GEL polarity conjugates showed a peak shift from 302 to 300 nm and the G-MTX polarity showed no significant shift (22). These results differ from both the first reported gelatin/MTX conjugates (21) and the current study.

We modified our conjugation conditions in the current study to minimize by-product crosslinking of the specie whose amino groups were not blocked. However, this lowered the resulting drug load (Table I) to less than that of the mixed polarity conjugates (23). Experiments were conducted to improve the G-MTX molar ratio by including N-hydoxysulfosuccinimde in the conjugation reaction but this actually inhibited drug conjugation (29), which is opposite to that reported for conventional globular proteins (30). Generally, it has been believed that conjugating a greater amount of drug to the carrier protein should result in greater effectiveness. However, it has recently been reported that in rats the liver cleared high molar ratio albumin/MTX conjugates, while 1:1 molar ratio conjugates provided optimal tumor targeting (19). Molar ratios of the gelatin/MTX conjugates reported earlier (21,22) are not available for comparison.

The difference between the G-MTX and M-GEL molar ratios (Table I) is attributed to the different number of gelatin reactive groups. The G-MTX uses gelatin carboxylic acid groups (~120/mole of gelatin) whereas the M-GEL uses gelatin amino groups (~33/mole of gelatin). The difference between the G-MTX and M-GEL conjugation yields (Table I) is attributed to differences between each conjugate's preparation. During G-MTX preparation, only ~74 mg of the original 100 mg gelatin is recovered after amino group blocking. This results in an additional 26% loss of materials, which does not occur during M-GEL preparation, and accounts for a major portion of the low G-MTX conjugation yield.

Conjugate Release

An *in vitro* release study was conducted to evaluate the stability of each polarity conjugate. If the conjugates have a low stability and release a significant amount of MTX before entering cells, then the determination of their cellular toxicity will be influenced by the presence of free drug in the extracellular media. MTX solution controls showed no MTX degradation in the release medium during the 7 day study and GMPM controls showed no physical attraction between gelatin and MTX and no influence from gelatin concentration upon the diffusion of MTX from the dialysis cassettes (Figure 5). Each polarity conjugate showed a slow release of MTX (Figure 5), which indicates that they have good stability for future short-term cell culture studies. Since the G-MTX presumably contains MTX polymers and therefore a smaller amount of monomeric MTX, the calculated percent of MTX released from the G-MTX may be an underestimate. This may be contributing to the small difference between each conjugate's release profile. Our earlier mixed polarity conjugate showed a MTX release of 8.1% by 72 hr (23) compared to 6.9 and 11% for G-MTX and M-GEL, respectively. MTX release from our conjugates is also comparable to the release from albumin/MTX conjugates (15,17).

The alternate MTX species detected in the release samples may have originated from two sources. The conjugates may hydrolyze into gelatin/MTX fragments smaller than 10 kDa, which are detected in the release medium. In addition, MTX may crosslink during conjugation to form MTX polymers, which may be bound to the gelatin and subsequently released. Evidence for this is the different amount of alternate MTX species released from each polarity conjugate. The amount of alternate species released from M-GEL, in which MTX crosslinking is blocked, is one-half the amount from G-MTX. A polymer of about 22 MTX molecules represents the maximum molecular weight that can pass through the dialysis membrane.

In our earlier report of a mixed polarity conjugate, these extra peaks accounted for 14% of the initial amount of MTX and represented up to double the amount of MTX released after 72 hr (23). In the current study, these extra peaks from the G-MTX and M-GEL accounted for 7.0 and 3.0% of the initial amount of MTX after 72 hr, and 10 and 5.0% after 7 days, respectively. These alternate species represented equal and 25% of the amount of MTX released after 72 hr, and 83% and 30% after 7 days, respectively. The lower percentages in the current study indicate that the procedural modifications successfully reduced by-product crosslinking. No such alternative MTX species have been reported in other protein/MTX conjugate investigations. However, it should be noted that one of these species, MTX/gelatin fragments, may be more likely to result from random coil conformations like gelatin than from fixed globular proteins like albumin. Hydrolysis of a single peptide bond could release a fragment from a random coil, but the hydrogen and hydrophobic bonds

that maintain the tertiary structure of globular proteins may bind a potential fragment and prevent its release.

It is difficult to thoroughly evaluate the kinetic release of MTX from the conjugates due to low drug release. Both G-MTX samples showed similar release profiles even though different starting amounts were used which indicates a first order process. In addition, the data fit slightly better to a first-order model than a zero-order model. Consequently, the release of MTX is ascribed to hydrolytic cleavage of the conjugate bond, which is the theoretical mechanism of release. However, additional influences may exist because of its apparent biphasic nature (Table II). The release rate constant for our earlier mixed polarity conjugate over 72 hr was 1.0×10^{-3} hr^{-1} (23) compared to 1.0×10^{-3} and 1.8×10^{-3} hr^{-1} for the early 48 hr release from G-MTX and M-GEL, respectively. The G-MTX appears to exhibit a slower MTX release than the M-GEL in this early time period. The G-MTX utilizes amino groups of the MTX pteridine ring system, which introduces a large bulky structure around the conjugate bond that may hinder hydrolysis and slow release. In comparison, some albumin/MTX conjugates also displayed a biphasic MTX release, but this was attributed to fast release from a physical attraction between the MTX and albumin for the first 6 hr, and slow release from hydrolytic cleavage of the conjugate bond for 24–72 hr (15,17). A physical attraction between gelatin and MTX was not observed in the current study. However, the early hydrolytic release of MTX from our conjugates is comparable to the hydrolytic release from the albumin/MTX conjugates.

In summary, the problems of mixed polarity conjugates and MTX crosslinking resulting during carbodiimide coupling have rarely been sited in the literature; however, these problems may dramatically affect the resulting *in vivo* performance of the conjugates. In this paper, we have reported the production of two gelatin/MTX conjugates having opposite conjugate bond polarities using an amino group blocking agent and a carbodiimide conjugating agent. The blocking step also minimizes, but does not eliminate, by-product crosslinking. Gelatin/MTX conjugates for microsphere preparation were reported (21,22) but little data are available for comparison to our conjugates. In addition to MTX, the single polarity conjugates released alternate MTX species, which are attributed to low molecular weight gelatin/MTX fragments and MTX polymers. The G-MTX and M-GEL released 12 and 17% MTX, respectively, by 7 days indicating good stability for future short-term cell culture studies. These studies will evaluate the cellular toxicity and cellular uptake of each polarity conjugate. The slow release also indicates the possible use of these conjugates as long-term delivery systems after intratumor injection.

ACKNOWLEDGMENTS

We would like to thank Dr. J. Michael Dunn (Kind & Knox, Sioux City, IA) for donation of the gelatin granules and SmithKline Beecham for donation of HPLC equipment. We would also like to thank Chao-Sheng Chen, Joseph Crisafulli and Ingrid Freeny for their excellent technical assistance.

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