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Growth inhibition, drug load, and degradation studies of gelatin/methotrexate conjugates

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Abstract

Macromolecular gelatin-methotrexate conjugates have potential therapeutic advantages over the free drug. Conjugates with MTX:gelatin molar ratios (MR) ranging from 1:1 to 27:1 were examined for cell growth inhibition, stability, degradation, and methotrexate (MTX) release. Conjugate growth inhibition was less than that of free MTX whose IC_{50} value of 1.3×10^{-8} M was about 10-fold less. Cell uptake of fluorescein labeled gelatin (145 kD) was observed by 24–30 h. Higher MR conjugates produced less growth inhibition, measurably greater stability at pH 7.4 based on MTX release, and had less gelatin degradation in the conjugate by the lysosomal enzyme Cathepsin B (Cat B) compared to low MR conjugates. Cat B conjugate degradation was greater at the in vitro lysosomal pH of 4.8 than the intra-tumor pH of 6.5. The presence of Cat B did not meaningfully affect MTX release, but less MTX was released at pH 4.8 than pH 6.5. The maximum MTX release was a relatively low 7% after 72 h at pH 6.5 for the low MR conjugate. Low molecular weight conjugate fragments were also produced and were also influenced by pH and MR. Reduced growth inhibition by high MR conjugates may be due to a hindered enzymatic degradation in the lysosomes. A strong peptide conjugate bond at lysosomal pH and a 24–30 h delayed gelatin uptake may contribute to reduced growth inhibition of the conjugate compared to free MTX. MTX release under these in vitro conditions occurs by aqueous hydrolysis, not by Cat B cleavage of the conjugate bond.

Keywords: Gelatin; Methotrexate release; Conjugates; HL-60 growth inhibition; Cathepsin B

1. Introduction

Methotrexate (MTX) has a broad range of cytostatic activity, especially when given in high doses with folinic acid rescue (Bertino, 1993). MTX is an antifolate that competively binds to dihydrofolate reductase (DHFR) which inhibits precursors of DNA and RNA and inhibits cell replication (Chabner et al., 2001). It is an important drug in the treatment of acute lymphoblastic leukemia (ALL), choriocarcinoma, related trophoblastic tumors and psoriasis (Chabner et al., 2001). However, it has a short distribution half-life (1.5–3.5 h) (Evans et al., 1986) and its tumor exposure time is considered short. Consequently, its therapeutic efficacy is impaired by its short in vivo half-life, and its low tumor accumulation produces adverse accumulation in healthy tissue. These processes substantially contribute to its severe, and sometimes fatal, life threatening toxicities of bone marrow depression, ulcerative colitis, hepatotoxicity, and nephrotoxicity (Chabner et al., 2001). In addition to these non-selective toxicities, MTX has a high occurrence of drug resistance which also limits its effectiveness. It has been reported that 30% of remission failures are due to MTX resistance in the treatment of ALL (Pui, 1995).

Soluble conjugates of low molecular weight drugs and high molecular weight proteins, peptides, or polymers are being investigated in several laboratories because of their potential therapeutic advantages compared to the free drug. One of the earliest reported potential advantages was overcoming drug resistance in transport deficient, resistant cells (Ryser and Shen, 1980). More recent reports about overcoming drug resistance include avoiding existing ATP-driven efflux pumps for the free drug (Minko et al., 1998) as well as blocking overexpression of these pumps (Minko et al., 1998, 1999). Another potential advantage is the passive accumulation of conjugates in

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solid tumors because of the enhanced permeability and retention (EPR) effect due to leaky tumor vasculature and poorly developed lymphatic drainage (Jang et al., 2003; Maeda et al., 2000). This accumulation reduces systemic toxicity by reducing damage to non-cancerous organs (Minko et al., 2000a). The accumulation may account for a several fold higher maximum tolerated dose of a conjugate compared to the free drug (Thompson et al., 1999). In addition, the EPR effect is amplified by the conjugate cytotoxicity, which increases the average drug concentration in the tumor and enhances the conjugate efficacy (Minko et al., 2000b). It has also been reported that the EPR effect is more effective for macromolecules greater than 40 kDa but negligible for smaller molecules that are cleared more rapidly from tumor interstitium (Maeda et al., 2000; Jang et al., 2003). Finally, ligands such as folic acid (Lu and Low, 2002) and antibodies (Trail et al., 1993) have been used to actively target the drug to enhance efficacy and reduce non-specific toxicities of the free drug.

MTX conjugates are under investigation to address problems associated with the free drug and to explore potential advantages of the conjugate. These conjugates include albumin–MTX (Halbert et al., 1987; Hartung et al., 1999; Scheulen et al., ASCO, Orlando, 2002, Abs. No. 1888), PEG–MTX (Riebessel et al., 2002), fibrinogen–MTX (Boratynski et al., 2000), conjugates from immunoglobulins–MTX (Kralovec et al., 1989; Fitzpatrick and Garnett, 1995), and a gelatin–MTX conjugate (Bowman and Ofner, 2000; Kosasih et al., 2000). Advantages of a gelatin–MTX conjugate include its biodegradability, a low antigenicity with the potential to overcome immune problems reported for high drug load in albumin conjugates (Stehle et al., 1997), the lack of denaturing concerns in native protein carriers, a high tissue distribution after IV administration, and the potential to breakdown into smaller conjugates within a tumor to extend conjugate intra-tumor diffusion and enhance efficacy.

The physical and chemical properties of macromolecular drug conjugates have a substantial influence on conjugate behavior under the various biological conditions it is exposed to during drug delivery. After intravenous administration, the conjugate is exposed to plasma (∼pH 7.4 and enzymes), then tumor interstitium (∼pH 6.5 and enzymes), and after endocytotic uptake into the cell, it is ultimately degraded for drug release under lysosomal conditions (∼pH 4.8 and enzymes). The conjugate bond between drug and macromolecule has been studied to induce rapid release after lysosomal uptake (Putnam and Kopecek, 1995; Minko et al., 2000b). However, lysosomal cleavage of protein–MTX conjugates produces protein–MTX fragments as well as MTX (Fitzpatrick and Garnett, 1995). In addition, there is evidence that some MTX conjugates directly inhibit DHFR (Riebessel et al., 2002). Consequently, conjugate bond polarity, in which different functional groups on the drug and carrier are used to form the same type of conjugate bond, may be important because different functional groups on the non-conjugated "side" of the MTX molecule would be available for interaction with DHFR. The effect of drug load on the conjugate is not entirely clear. The molar ratio of drug to carrier was varied in early in vitro studies (Matsumoto et al., 1986; Halbert et al., 1987), but in later in vivo studies it was reported that a low immunological response from an albumin conjugate required a low molar ratio (Stehle et al., 1997). While a high molecular weight of the conjugate is important for its passive accumulation in solid tumors, intra-tumor diffusion, however, is impeded by this high molecular weight and by the high interstitial pressure of solid tumors. Intra-tumor conjugate degradation into smaller fragments may offer an approach to extend conjugate tumor penetration and enhance efficacy.

The objectives of this study were (1) to evaluate drug molar ratio effects of the conjugate on growth inhibition, conjugate degradation, and MTX release, (2) to evaluate the potential for cellular uptake of an anionic high molecular weight conjugate, and (3) to evaluate gelatin–MTX conjugate stability and conjugate bond polarity under different in vitro biological conditions during drug delivery.

2. Materials and methods

2.1. Materials

Type B gelatin granules having a Bloom strength of 254 g, average molecular weight of 159 kDa, and an approximate moisture content of 11% (w/w) determined by loss on drying at 105 °C for 72 h were supplied by Kind and Knox (Sioux City, IA, sample #T7468). Methotrexate (MTX, ±amethopterin, 95% (w/w) pure, 12% (w/w) moisture), 1-ethyl-3-(dimethylaminopropyl) carbodiimide HCl (EDC, ultrapure), poly-L-lysine HBr (125–250 kDa), Sephadex G-50, Dulbecco's phosphate buffered saline (DPBS, endotoxin tested, sterile filtered), RPMI-1640 medium with L-glutamine and $NaHCO_3$ (endotoxin tested, sterile filtered), fetal bovine serum (FBS, hybridoma tested, sterile filtered), propidium iodide (PI), gentamicin (10 mg/ml in deionized water, endotoxin tested, sterile filtered), sodium azide (ultra pure), isotonic phosphate buffered saline (PBS, 0.01 M, pH 7.4) and Cathepsin B (Cat B, Bovine Spleen) were purchased from Sigma Chemical Co. (St. Louis, MO). Falcon 25 cm^2 polystyrene culture flasks (tissue culture treated, 0.2 μm vented cap, canted neck) and Falcon 96-well polystyrene plates (tissue culture treated, flat bottom, low evaporation lids) were purchased from Fisher Scientific (Fair Lawn, NJ). Citraconic anhydride (98%, w/w) was purchased from Aldrich Chemical Co. (Milwaukee, WI). BCA protein assay kits and Slide-A-Lyzer Dialysis cassettes (10,000 Da MWCO) were purchased from Pierce Chemical Co. (Rockford, IL). Trypan blue dye (0.4% in phosphate buffered saline) was purchased from ICN Biomedicals, Inc. Gelatin-fluorescein (GF, 7.6 mol dye/mol, 145 kD) and fluorescein were purchased from Molecular Probes (Eugene, OR). Water was purified by reverse osmosis. All other chemicals were at least ACS reagent grade.

2.2. Cells and cell conditioned media

The HL-60 promyelocytic leukemia cell line was kindly donated by Dr. Ruy Tchao (Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, Philadelphia, PA). Suspensions of HL-60 cells were grown in 25 cm^2 polystyrene culture

flasks containing 7 ml of RPMI-1640 with 20% (v/v) FBS and 50 μg/ml gentamicin at 37 °C, 5% CO_2, and 100% relative humidity. The cells were subcultured weekly at a seeding density of 1.0×10^5 cell/ml, grown to a peak cell concentration of 2.0×10^6 cell/ml, and determined to have a 25 h doubling time. A cell conditioned medium was prepared by incubating HL-60 cells for 72 h in the above growth medium with FBS and gelatin at a concentration equivalent to gelatin in the G-MTX-L experiment followed by separation from the cells by centrifuging.

2.3. Conjugate preparation

Conjugates were prepared using previously published methodology (Bowman and Ofner, 2000). Briefly, 100 mg of gelatin or 50 mg of MTX were reacted with 50 μl of citraconic anhydride for 1 h at pH 8.0–9.0 to temporarily modify either the gelatin or MTX amino groups, respectively. The modified gelatin was separated from excess citraconic anhydride on a Sephadex G-50 column with a 0.05 M $NaHCO_3$ eluent. The modified MTX reaction solution was mixed with 100 mg of poly-L-lysine HBr for 1 h at pH 9.0–10.0 to react any excess citraconic anhydride and then eluted on a SEC column with 0.05 M $NaHCO_3$ to separate the modified MTX from the modified poly-L-lysine.

G-MTX conjugates (MTX amino groups bound to gelatin carboxylic acid groups) were prepared by mixing the modified gelatin with 37.5 mg of MTX followed by coupling with 75 mg of EDC. These conjugates were designated as G-MTX-H because of their high drug load. G-MTX conjugates having a low drug load (G-MTX-L) were prepared by mixing the modified gelatin with 15 mg of MTX followed by coupling with 12.5 mg of EDC. M-GEL conjugates (MTX carboxylic acid groups bound to gelatin amino groups) were prepared by mixing the modified MTX with 100 mg of gelatin followed by coupling with 112.5 mg of EDC. Mixing and coupling were conducted for 2 and 24 h, respectively, at pH 7.0. All reaction solutions were eluted on a SEC column with 0.05 M $NaHCO_3$ to separate the conjugates from excess reagents. The conjugates were then incubated for 5 h at pH 4.0–5.0 to deacylate the modified amino groups. The reaction solutions were eluted on a SEC column with water to separate the conjugates from the citraconate. The purified conjugate fractions were pooled, frozen, and lyophilized.

2.4. Conjugate composition analysis

Conjugates were characterized using previously published methodology (Bowman and Ofner, 2000). Briefly, the gelatin content of each conjugate was determined using a BCA protein assay. The MTX content of each conjugate was determined spectrophotometrically at 372 nm. Absorption values were determined using a Molecular Devices SpectraMax plus spectrophotometer. G-MTX-L had virtually the same drug load, or molar ratio, as M-GEL, which for these studies ranged from 1.1:1 to 2:1. A typical G-MTX-L batch weighed 50.6 mg and contained 42 mg of gelatin, 0.14 mg of MTX, and had a moisture content of 8.5 mg. The molar ratio for G-MTX-H ranged from 23:1 to 27:1. Conjugates with an intermediate molar ratio ranging from 14:1 to 18:1 (G-MTX-M) were used in conjugate degradation studies.

$$\text{Effect} = (E_{max} \times \text{Conc.})/(EC_{50} + \text{Conc.})$$

Effect = % Growth Inhibition = 100 - % Growth Relative to Control Cells

E_{max} = Maximum Effect = Maximum Growth Inhibition

Conc. = MTX or MTX Equivalent Concentration

$EC_{50} = IC_{50}$ = Conc. Causing 50% Growth Relative to Control Cells

Fig. 1. Pharmacodynamic maximum effect model.

2.5. HL-60 cell growth inhibition

HL-60 cell suspensions (200 μl) were seeded in a 96-well plate at 1.0×10^5 cell/ml and incubated at 37 °C, 5% CO_2, and 100% relative humidity. After 24 h, MTX, M-GEL conjugate and G-MTX conjugate samples were added to the cells in 50 μl of DPBS resulting in MTX or MTX equivalent concentrations ranging from 1×10^{-9} to 5×10^{-6} M. Control cells were exposed to 50 μl of DPBS and grown in drug-free media. Experimental control samples included gelatin solutions and gelatin/MTX physical mixture solutions of concentrations corresponding to each conjugate sample. Cell concentrations were determined using a hemacytometer with trypan blue dye exclusion. Experiments were conducted with three replicates. Cell growth was calculated by subtracting the cell concentration immediately prior to drug exposure from the cell concentrations determined at each time point and expressed as a % growth relative to control cells. IC_{50} values (concentration causing 50% growth relative to control cells) were determined by non-linear regression using the pharmacodynamic maximum effect model (Fig. 1).

2.6. Incubation of fluorescein labeled gelatin with HL-60 cells

HL-60 cell suspensions (200 μl) were seeded in a 96-well plate at 6.25×10^5 cell/ml and incubated at 37 °C, 5% CO_2, and 100% relative humidity. After 24 h of cell growth, 50 μl of an aqueous GF solution (0.75 mg/ml fluorescent labeled gelatin) was added to the cell suspension. Fluorescein (0.02 mg/ml) was used as a control. Cells were transferred to a microcentrifuge tube at 0.5, 4, 8, 12, 24, 30, 48, 72, and 96 h, and were incubated with PI (0.075 μg/ml) for 15 min, then washed three times with DBPS. Slides of cells were examined for GF, PI, and fluorescein uptake using an Olympus fluorescence microscope. Images were obtained with a digital camera and stored on computer. Cell viability was evaluated by PI staining.

2.7. MTX release in cellular conditioned growth medium

Conjugates were dissolved in 0.5 ml of cell conditioned medium at MTX concentrations equivalent to 0.04 and 0.57 mg/ml for the low and high molar ratio, respectively. The solutions were injected into the donor compartment of a 0.5 ml

10,000 Da MWCO dialysis cassette. The cassettes were placed in screw cap, wide-mouth jars containing 50 ml of isotonic phosphate buffered saline (PBS), maintained at 37 °C in a shaker water bath at 100 rpm. Each conjugate had a corresponding MTX solution and gelatin/MTX physical mixture as controls. Three milliliter samples for MTX analysis were removed at appropriate times and replaced with buffer.

2.8. MTX analysis

Samples were assayed by reverse phase HPLC on a Nova-Pak C18, 3.9 mm × 150 mm column with Nova-Pak Guard-Pak Inserts (4 μm, 60A, C18) at 303 nm with a 88:12 phosphate buffer, pH 2.7, and acetonitrile mobile phase (Nuernberg et al., 1989). Low and high MTX concentration calibration plots, 4.0×10^{-6} to 1.0×10^{-4} and 8.0×10^{-5} to 2.0×10^{-3} mg/ml, respectively, were linear. The MTX retention time ranged from 3.8 to 4.6 min. A small amount of MTX was detected at 2 and 4 h of release but was below the quantifiable limit of 0.5% MTX release. At these early time points, this small amount represents residual free MTX. Conjugate fragments containing MTX, or MTX polymers (Bowman and Ofner, 2000) were also detected which had retention times ranging from 3.0 to 10.1 min. The assayed amounts of released free MTX were expressed as a percent of the determined MTX content of the conjugate. Fragment release was calculated by adding their chromatogram peak areas to obtain the MTX amount in these conjugates, and was expressed as a percent of the MTX content of the conjugate.

2.9. Conjugate degradation by Cathepsin B

A gelatin control and G-MTX conjugates of three molar ratios were dissolved at equivalent gelatin concentrations (7 mg/ml) in 0.1 M potassium phosphate buffer and 0.025% sodium azide at pH 4.8 (lysosomal environment) and 6.5 (interstitial tumor environment). One-half milliliter of each solution was pipetted into six different microcentrifuge tubes for every time point to be analyzed. After 30 min of pre-incubation at 37 °C, 100 μl of the appropriate buffer (without enzyme) was added to three of the samples, and 100 μl of Cat B enzyme solution (1/2 unit) reconstituted with the same buffer was added to the other three samples. At selected times the samples were removed from the incubator and analyzed for degraded gelatin as described below. This method was also used to measure gelatin degradation by Cat B as a control assay during MTX release experiments to verify enzyme activity.

2.10. Assay of degraded gelatin

Conjugate degradation by Cat B was measured using a non-radioactive modification of a previously published assay for gelatin degradation by gelatinase A (Murphy and Crabbe, 1985). After the selected incubation times, 84 μl of 90% (w/v) trichloroacetic acid (TCA) was added to each of the samples to precipitate the large undigested gelatin molecules leaving the smaller digested molecular fragments in solution. The samples were centrifuged at 7800 × *g* for 15 min. The supernatant was removed, vortexed, and 300 μl was removed to which was added 20 μl of 12.5 M NaOH to bring the pH back to values ≥1 suitable for BCA assay. The solution was then analyzed for gelatin content using the BCA assay. A set of gelatin standards with and without enzyme was prepared for each time point. Calibration plots were prepared at each time point and treated in the same manner as samples. Gelatin calibration plots were reproducible quadratic equations; concentrations ranged from 0.24 to 4.8 mg/ml. Percent degradation was calculated by expressing the amount of assayed gelatin in the sample supernatant as a percent of the initial gelatin. Gelatin remaining in the supernatant after TCA precipitation in the controls, which represented undegraded gelatin in the samples (∼10%), was subtracted from the original gelatin.

2.11. MTX release from conjugates under in vitro lysosomal and intra-tumor conditions

Conjugates containing MTX amounts of 0.02 mg in 0.5 ml of 0.1 M potassium phosphate buffer with 0.025% sodium azide at pH 4.8 and 6.5 were injected into dialysis cassettes. Each dialysis cassette was placed in 50 ml of the appropriate medium. Physical mixture controls contained MTX, MTX/gelatin, and MTX/gelatin/enzyme for both in vitro experimental conditions. Controls and samples were conducted in triplicate. Jars were maintained at 37 °C with 100 rpm shaking, while samples were removed at appropriate times for MTX analysis and replaced with buffer.

2.12. Statistics

Statistical analysis of IC_{50} values was conducted using one-way ANOVA and Newman–Keuls tests. Statistical analysis of MTX release in cell conditioned media was conducted using one-way ANOVA and *t*-tests. Statistical analysis of in vitro lysosomal and intra-tumor MTX release was conducted using *t*-tests. Statistical significance was determined at a P value of 0.05.

3. Results

3.1. HL-60 cell growth inhibition

The HL-60 cell growth within the samples containing gelatin alone remained at 100% relative to the growth of control cells throughout the 72 h study. Cell growth inhibition due to the gelatin/MTX physical mixtures showed no statistical difference compared to MTX alone. At 24 h, the extent of growth inhibition was insufficient to determine IC_{50} values (Fig. 2A). At 48 and 72 h, both MTX and the conjugates showed sigmoidal concentration–response curves with maximum growth inhibitions of 80 and 95%, respectively (Fig. 2B and C). However, the conjugate effects were shifted to a 10–25-fold higher concentration level. The IC_{50} values determined for MTX and the conjugates after 48 and 72 h are summarized in Table 1. At 48 h, the IC_{50} values showed no statistical difference. However, at 72 h, statistical analysis indicated a lower IC_{50} value

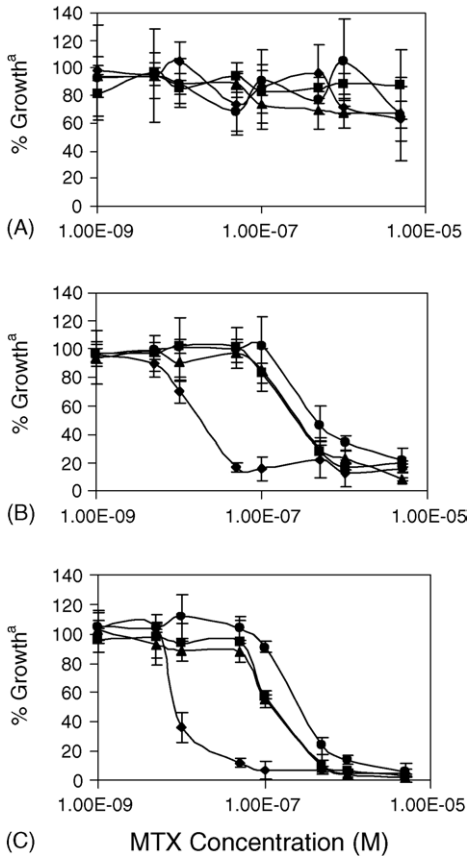

Fig. 2. Growth inhibition curves for (♦) MTX and the (■) M-GEL, (▲) G-MTX-L, and (●) G-MTX-H conjugates at (A) 24, (B) 48, and (C) 72 h. Superscript a: Percentage growth relative to control cells; after 24, 48, and 72 h, control cell growth reached $(1.6 \pm 0.4) \times 10^5$, $(4.4 \pm 0.8) \times 10^5$, and $(8.6 \pm 1.3) \times 10^5$ cell/ml, respectively.

Table 1
Summary of the IC_{50} values determined for MTX and the gelatin–MTX conjugates[a]

	48 h	72 h
MTX	$(1.5 \pm 0.3) \times 10^{-8}$	$(1.3 \pm 0.2) \times 10^{-8}$[b]
M-GEL	$(2.5 \pm 0.5) \times 10^{-7}$	$(1.9 \pm 0.4) \times 10^{-7}$
G-MTX-L[c]	$(2.8 \pm 0.6) \times 10^{-7}$	$(1.4 \pm 0.0) \times 10^{-7}$
G-MTX-H[d]	$(5.5 \pm 3.2) \times 10^{-7}$	$(3.4 \pm 0.5) \times 10^{-7}$[e]

[a] Mean of three replicates ± S.D.; values are molar (M) concentrations.
[b] Statistical difference ($P < 0.05$) compared to all three gelatin–MTX conjugates.
[c] MTX to gelatin molar ratio of 1.2:1.
[d] MTX to gelatin molar ratio of 23:1.
[e] Statistical difference ($P < 0.05$) compared to the M-GEL and G-MTX-L.

for MTX compared to the conjugates, a higher IC_{50} value for the G-MTX-H conjugates compared to the G-MTX-L and M-GEL conjugates, and no difference between the IC_{50} values for the M-GEL and G-MTX-L conjugates.

3.2. Gelatin uptake into HL-60 cells

HL-60 cells were incubated up to 96 h with fluorescein labeled gelatin, GF. The first GF association with the cells appears at 24 h (Fig. 3B). Punctate images of GF are also seen at 30 and 72 h in Fig. 3C and D, respectively. A central shadow can be seen in these images surrounded by the GF which appears to be the nucleus and compartmentalized gelatin in the cytoplasm. These results indicate cellular uptake of the anionically charged gelatin carrier. This gelatin uptake was observed to the end of the 96 h study. Estimates of the percentage of cells with GF uptake were ~35% of cells at 30 h to ~85% of cells at 72 and 96 h. Cell viability was high throughout the study as indicated by the few

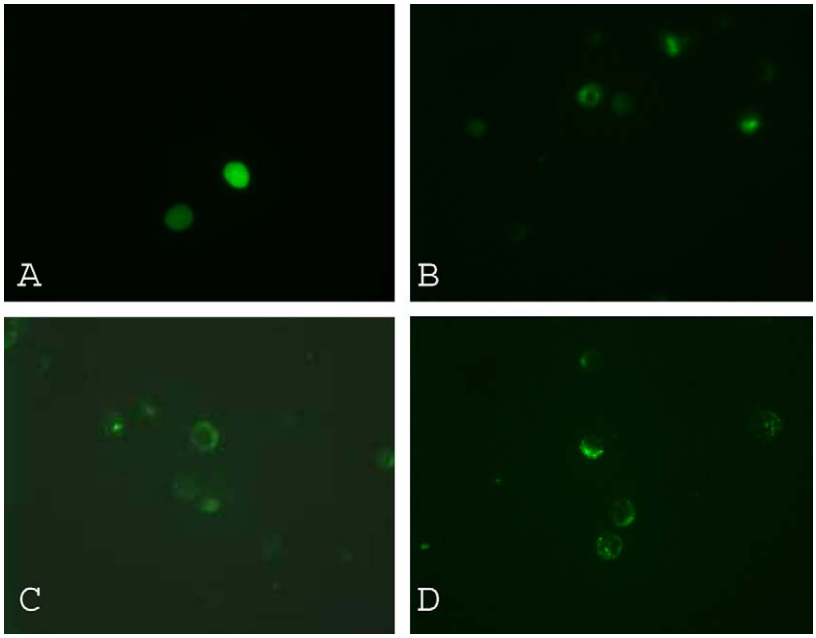

Fig. 3. Intracellular accumulation of gelatin under 400× magnification by fluorescence microscopy. HL-60 cells were incubated at 37 °C in the presence of fluorescein for 4 h (A) or fluorescein labeled anionic 145 kDa gelatin for 24 (B), 30 (C), and 72 h (D).

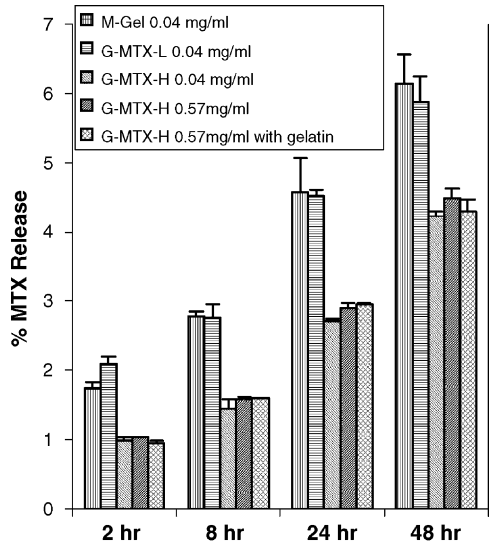

Fig. 4. In vitro stability of gelatin–MTX conjugates in cellular conditioned media for 2, 8, 24, and 48 h. M-GEL and G-MTX-L are opposite conjugate bond polarity and have drug to conjugate molar ratios of 1.1:1 and 1.3:1, respectively. G-MTX-H has molar ratio of 24:1. Concentrations represent MTX equivalent. Values are mean ± S.D. of three replicates.

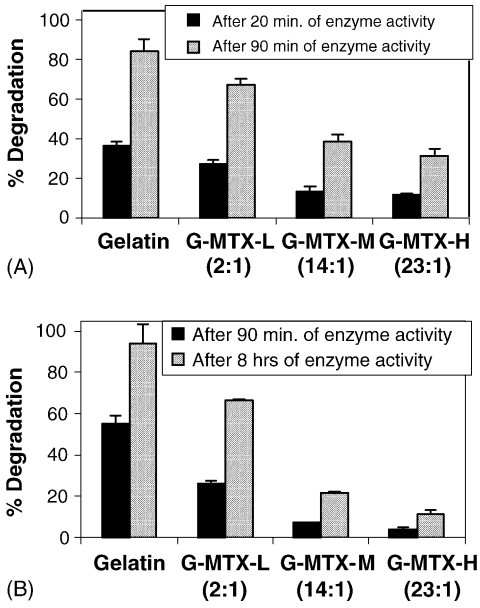

Fig. 5. Effect of MTX:gelatin molar ratio on the ability of Cathepsin B to degrade the conjugate at 37 °C in (A) in vitro lysosomal, pH 4.8, and (B) in vitro tumor, pH 6.5 environments. Values are mean ± S.D. of three replicates. Degradation represents degraded gelatin. See text for details.

cells with PI staining and the absence of PI staining in cells with GF uptake.

3.3. Conjugate stability in cellular conditioned growth medium

Fig. 4 shows free MTX release from conjugates incubated in cell conditioned media. Controls of MTX with and without gelatin ranged from 83 ± 9% to 100 ± 6% of the original MTX and showed no evidence of gelatin interference or MTX degradation. MTX release increased over the 2–48 h exposures. By 48 h, MTX release was 6.1 ± 0.4% for M-GEL, 5.9 ± 0.4% for G-MTX-L, and 4.2 ± 0.1% for G-MTX-H (0.04 mg/ml). The virtually identical MTX release from M-GEL and G-MTX-L conjugates indicates no difference due to conjugate bond polarity. All three G-MTX-H conjugate samples had no statistically significant release differences at each time point which indicates no effect from conjugate concentration, nor from the presence or absence of gelatin. However, all three conjugates with a high molar ratio released about 1/3 less MTX compared to conjugates with a low molar ratio. The effect of conjugate bond polarity was not examined further because no effect was observed in the growth inhibition and conjugate stability studies.

3.4. Degradation and release under in vitro lysosomal and intra-tumor conditions

3.4.1. Enzyme degradation of conjugate

Cat B degradation of the conjugate was measured by assay of degraded gelatin. The effect of molar ratio on enzymatic degradation of the conjugate was examined. Fig. 5 shows conjugate degradation by Cat B for three different molar ratios at the lysosomal pH of 4.8 and at the intra-tumor pH of 6.5. There is a clear trend of decreasing degradation with increasing molar ratio. At lysosomal pH, degradation after 90 min of enzyme activity was 85 ± 6%, 67 ± 3%, 40 ± 3%, and 30 ± 4% for the gelatin control, and conjugates of molar ratio values of 2:1, 14:1, and 23:1, respectively. The same trend was observed for 20 min of enzyme activity at this pH. At intra-tumor pH, gelatin degradation after 8 h of enzyme activity was 94 ± 10%, 67 ± 0.6%, 22 ± 0.6%, and 11 ± 2% for the gelatin control, and conjugates of molar ratio values of 2:1, 14:1, and 23:1, respectively.

3.4.2. MTX release

Fig. 6 shows MTX release under in vitro lysosomal and intra-tumor conditions. Less MTX was released at pH 4.8 than at pH 6.5 regardless of the effect of enzyme or molar ratio. MTX release by 72 h at pH 4.8 ranged from 1.8 ± 0.1% to 3.4 ± 0.2%, while release at pH 6.5 ranged from 3.1 ± 0.1% to 7.1 ± 0.3%. An additional release experiment was conducted using a succinate buffer at these two pH values to verify the stronger conjugate bond at pH 4.8 and to check for specific buffer effects. The same release trend was observed: 5.1 ± 0.3% release at pH 4.8 and 7.9 ± 0.3% release at pH 6.5. At pH 4.8 the enzyme had no effect for the medium molar ratio conjugate. At pH 6.5, the medium and high molar ratio released the same MTX by 72 h without enzyme (∼3.1%) and with enzyme (∼4.6%). The lowest release occurred from the medium and high molar ratio conjugates without enzyme. The highest release was from the low molar ratio conjugates at both pH values.

Conjugates also released low molecular weight gelatin fragments containing MTX from the original macromolecular conjugate. Fig. 7 compares fragment and free MTX release at pH 4.8. The combined total of free and fragment release from low molar ratio conjugates at this pH is greater with enzyme than without enzyme (6.4 ± 0.2% and 3.5 ± 0.2%). Of the total release

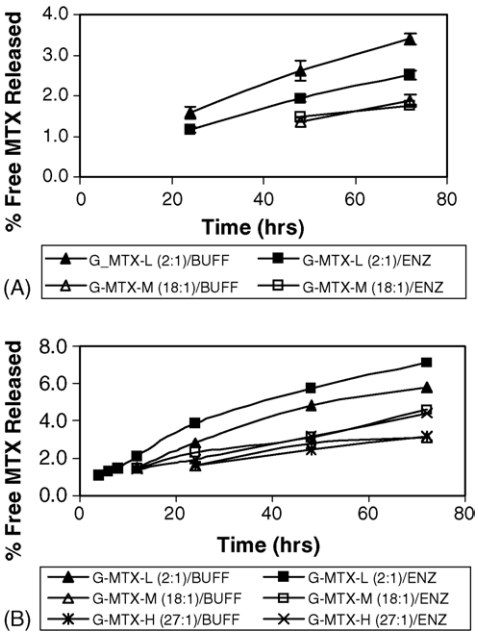

Fig. 6. MTX release in (A) in vitro lysosomal, pH 4.8, and (B) in vitro tumor, pH 6.5 environments of aqueous buffer vs. enzymatic degradation for low (2:1), medium (18:1) and high (27:1) MTX:gelatin molar ratios. Enzyme is Cathepsin B. Values are mean ± S.D. of three replicates.

with enzyme, 39% is free MTX; in the absence of enzyme, 97% is free MTX. MTX release at this pH with the enzyme from medium molar ratio conjugates (Fig. 7B) was the same as fragment release (1.8 ± 0.1% and 1.6 ± 0.6%). The combined total release is 3.4 ± 0.6%. Fig. 8 compares fragment and free MTX release at pH 6.5. Release from low molar ratio conjugates

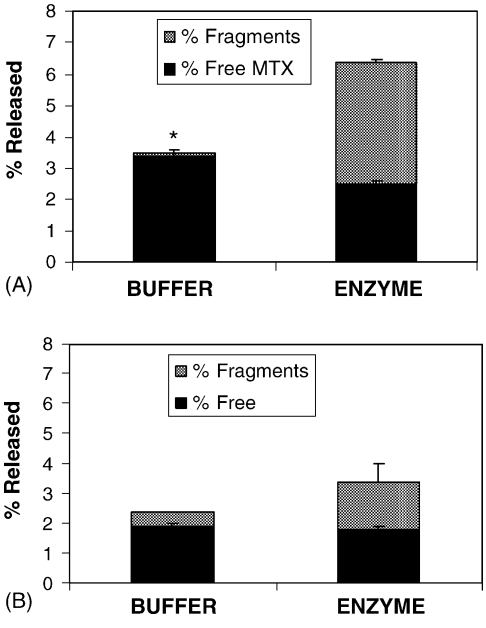

Fig. 7. Gelatin–MTX fragment and free MTX release in an in vitro lysosomal, pH 4.8, environment at 37 °C in the absence (buffer) and presence (enzyme) of Cathepsin B at 72 h for (A) low MTX:gelatin molar ratio conjugate (2:1) and (B) medium MTX:gelatin molar ratio conjugate (18:1). Values are mean ± S.D. of three replicates. *Estimate of % fragments; value below quantitation limit.

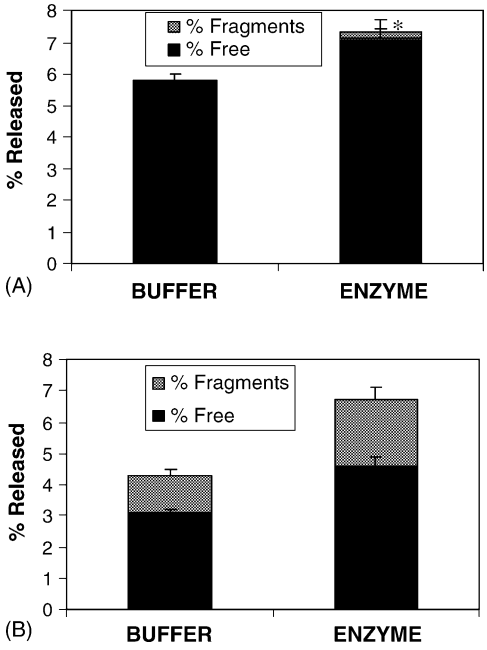

Fig. 8. Gelatin–MTX fragment and free MTX release in an in vitro intra-tumor, pH 6.5, environment at 37 °C in the absence (buffer) and presence (enzyme) of Cathepsin B at 72 h for (A) low MTX:gelatin molar ratio conjugate (2:1) and (B) medium MTX:gelatin molar ratio conjugate (18:1). Values are mean ± S.D. of three replicates. *Estimate of % fragments; value below quantitation limit.

is virtually all free MTX with and without enzyme; release is 7.1 ± 0.3% and 5.8 ± 0.2%, respectively. The combined total of free and fragment release from medium molar ratio conjugates is greater with than without enzyme (6.7 ± 0.2% and 4.3 ± 0.2%). Of this total ~70% is free MTX with and without enzyme.

4. Discussion

The effect of conjugate bond polarity was examined in these studies by comparing results from the M-GEL and G-MTX-L conjugates. The effect of drug load was examined by comparing results from the G-MTX-L, G-MTX-M, and G-MTX-H conjugates. The G-MTX conjugate (G-MTX-L) with a molar ratio that was comparable to the M-GEL conjugates was produced by lowering the amounts of MTX and EDC used in the conjugation reaction.

4.1. Cell growth inhibition

The inability to determine an MTX effect on the growth of HL-60 cells after a 24 h exposure (Fig. 2A) was not a surprise because the cell line had a 25 h doubling time under the study conditions, and because the growth inhibitory effect of MTX is dependent upon both the cell cycle and the depletion of cellular tetrahydrofolate levels (Jackson, 1984; Borsi and Moe, 1987). After a 48 and 72 h drug exposure, the effect of MTX upon HL-60 cell growth was more pronounced with maximum growth inhibitory effects of 80 and 95%, respectively (Fig. 2B and C). A maximum effect of 95% growth inhibition indicates that the

cell concentration after 72 h was relatively equivalent to the cell concentration at the beginning of the test period and that MTX had a growth inhibitory rather than a cytotoxic effect upon the HL-60 cells.

The similarity between the MTX and conjugate concentration–response curves (Fig. 2) suggests that the conjugated MTX is acting upon the HL-60 cells by the same mechanism as the free drug. However, this therapeutic effect requires a 10–25-fold higher concentration of conjugated MTX as compared to free MTX. This reduced in vitro conjugate effectiveness is not considered a disadvantage because of the expected conjugate effectiveness in MTX resistant cells (Ryser and Shen, 1980; Rosowsky et al., 1985) and the expected EPR effect of tumor accumulation.

The IC_{50} for MTX was $(1.3–2.6) \times 10^{-8}$ M (Table 1), which is comparable to the values reported for HL-60 cells using various assay methods (Bhalla et al., 1985; Chou et al., 1993; Rots et al., 1999). The IC_{50} values for the gelatin–MTX physical mixtures were not statistically different from the IC_{50} value for MTX and verified that the presence of gelatin in the cell media does not alter the effects of MTX upon the growth of HL-60 cells. However, conjugating MTX to gelatin resulted in a 10–25-fold higher IC_{50} value after 72 h (Table 1). The 24–30 h uptake delay of the conjugate suggests that a decreased uptake contributes at least in part to the higher IC_{50} values.

By the end of 72 h, conjugate bond polarity had no effect upon the resulting IC_{50} values of the conjugates; however, decreasing conjugate drug load resulted in a lower IC_{50} value (Table 1). Possible explanations for the lack of effect due to conjugate bond polarity include: (a) MTX is completely cleaved from each polarity conjugate within the cell, (b) any gelatin–MTX fragments produced by lysosomal degradation do not contribute to the conjugate effectiveness, or (c) fragments do have an effect but conjugate bond polarity within the gelatin–MTX fragments does not effect the MTX/DHFR interaction. The increase in conjugate effectiveness as drug load decreases has been reported in poly-L-lysine-MTX conjugates and was attributed to a lower net positive charge and a likely decreased uptake (Rosowsky et al., 1985). Alternatively, conjugating a greater amount of MTX to the gelatin may decrease the ability of lysosomal enzymes to degrade the conjugate and release MTX from the gelatin thereby lowering DHFR inhibition and conjugate effectiveness.

4.2. Gelatin uptake into HL-60 cells

Charged macromolecules do not easily cross the lipid bilayer of cell membranes. A fluorescein labeled gelatin (145 kD) was evaluated for uptake into these HL-60 cells. Fig. 3 shows an apparent GF uptake into vesicle compartments which may be due to endocytosis. The conjugate stability of about 96% by 24 h and the first uptake observations at 24–30 h suggest that undegraded, high molecular weight gelatin molecules participate in this uptake. The first uptake observations indicate a substantial lag time for uptake. After this lag, the process continued and involved more cells to eventually take place in ~85% of the cells by end of the 96 h experiment. These results indicate that the large, anionic gelatin can be used as a macromolecular carrier for cellular drug delivery.

4.3. Conjugate stability in cellular conditioned growth medium

Cellular proteases, which might be secreted from exposure to gelatin conjugates, would obscure comparisons of cellular effects from conjugate and free drug. Conjugates were incubated in cell conditioned media in which cells were previously grown with gelatin to induce possible enzyme production that could degrade the conjugate. Conjugate stability, as measured by MTX release, was evaluated in the absence of cells, but in an environment that could contain cellular proteases. In the cellular conditioned growth medium at pH 7.4, 4.2% to 6.1% of MTX was released from the conjugate after 48 h. This conjugate stability is similar to a previously reported 93% stability value for a MTX-PEG conjugate in cell conditioned media for 3 days (Riebessel et al., 2002) and a 4.9% to 9.2% MTX release from these conjugates in PBS buffer after 48 h (Bowman and Ofner, 2000). MTX release in the PBS buffer is slightly greater than observed in the current cell conditioned medium study and suggests that release in the cell conditioned medium does not involve enzyme cleavage of the gelatin–MTX conjugate bond. These results also indicate good stability of the conjugates for cell culture studies.

The presence of gelatin, different conjugate concentrations, and conjugate bond polarity did not produce different MTX release, which indicates that these variables had no effect on conjugate stability under these conditions. However, increasing the molar ratio of MTX by about 20-fold reduced MTX release in three separate samples to about 1/3 less the release of MTX from low molar ratio conjugates (Fig. 4).

4.4. Degradation and release under in vitro lysosomal and intra-tumor conditions

Conjugates were evaluated under in vitro intra-tumor and lysosomal conditions to simulate conjugate fate after localization in the tumor interstitium and subsequent cellular uptake. The decreasing enzymatic degradation of the conjugate with higher MTX drug loads (Fig. 5) is clearly evident but the explanation is not completely understood. One possible explanation may be a non-specific and general steric hindrance of the Cat B enzyme by the larger number of MTX molecules. Regardless of the mechanism, this trend offers a partial explanation for the drug load effect observed in the growth inhibition study. The higher drug load appears to inhibit lysosomal degradation of the conjugate which reduces the availability and interaction of MTX with DHFR to inhibit growth. The reduced enzymatic degradation on gelatin at pH 6.5 indicates that the activity of this lysosomal enzyme is reduced at the higher pH of the tumor interstitium.

Free MTX release under these conditions is more complicated than enzymatic degradation because of the greater enzyme activity yet stronger conjugate bond at pH 4.8 than at pH 6.5. These results also show a drug load effect, but this effect is on MTX release.

Overall, free MTX release with enzyme is not largely different than in its absence (Figs. 6–8). This indicates that the enzyme has no specificity for the conjugate bond and that the conjugate bond is ultimately broken through aqueous hydrolysis. The lack of Cat B specificity for the conjugate bond is best seen in the low molar ratio results at both pH conditions (Figs. 7A and 8A) in which only slightly less and slightly more MTX release, respectively, occurs with enzyme. While the medium molar ratio results support this finding (Figs. 7B and 8B), release is impeded by the additional drug present.

While the enzyme does not act directly on the gelatin–MTX conjugate bond, the effects of molar ratio on MTX release can be seen. At both pH conditions, substantially more free MTX is released from the low molar ratio conjugates than from the medium and high molar ratio conjugates irrespective of enzyme effects (Figs. 6–8). This effect appears to reach a maximum with the medium (18:1) molar ratio because release is no greater for the high (27:1) molar ratio conjugate (Fig. 6).

The hindrance effect from the drug load can also be seen from the formation of fragments through enzymatic degradation. At pH 4.8 the highly active Cat B degrades the gelatin backbone into fragments small enough (<10 kDa) to diffuse through the dialysis cassette into the release medium (Fig. 7A, enzyme). The medium molar ratio at this pH hinders the enzyme from degrading the gelatin backbone and less fragments are produced (Fig. 7B, enzyme). At pH 6.5, the enzyme is less active, but the conjugate bond is more labile. As Cat B degrades the gelatin backbone (even slightly), it alleviates the hindrance of MTX and makes the labile bond more susceptible to aqueous hydrolysis. This may even lead to virtually complete MTX release from fragments of the low molar ratio conjugate. Since fragments must have MTX present to be detected, such fragments would be undetectable. This might explain the absence of fragments from the low molar ratio conjugate at this pH (Fig. 8A). In addition, or alternatively, these fragments may not be detected because they are below detection limits due to the small amount of MTX on the fragment. In either case, the absence of fragments at this pH with enzyme is very unlikely because enzymatic degradation of gelatin clearly occurs at this pH and it occurs more on the low molar ratio than the high molar ratio (Fig. 5). When the molar ratio is increased at this pH, the conjugate bond in the fragments may be hindered from MTX release which would allow the fragments to be detected (Fig. 8B). Fragments susceptible to aqueous hydrolysis at pH 4.8 do not release more MTX due to a stronger gelatin–MTX conjugate bond.

The relatively low amount of MTX release in the absence of enzyme and at pH 4.8 (Fig. 7) raises the question of a potential MTX contribution from residual MTX left from conjugate preparation. A small amount of free MTX released at 2 and 4 h ($\leq$0.5%) in the release studies does indicate the presence of a small amount of residual free MTX. However, this early release and the complete release of MTX in the gelatin/MTX control mixtures, as well as the assay sensitivity to detect $\sim$0.5% of MTX, indicate that non-specific binding of free or residual MTX is unlikely to have more than a small contribution to MTX release.

4.5. Gelatin–MTX conjugate bond stability

MTX release results in buffer solutions (without enzyme) indicate a more stable peptide conjugate bond at pH 4.8 than at pH 6.5 (Figs. 6–8). More free MTX is released at pH 6.5 irrespective of molar ratio because the bond is less stable at this pH even though enzyme activity on the gelatin backbone is reduced. A similar peptide bond stability at lysosomal pH compared to physiological pH was reported for peptide spacers in polymeric prodrugs of 5-fluorouracil (Nichifor et al., 1997).

The molar ratio effect on aqueous hydrolytic release of MTX observed in the in vitro intra-tumor and in lysosomal conditions (and ascribed to results in the cell conditioned medium in Fig. 4) is noteworthy. A possible explanation for this reduced release may be a water of hydration sheath on the conjugate molecule that hinders free water access and hydrolysis of the gelatin–MTX conjugate bond. More MTX on the gelatin molecule increases the number of ionizable groups (but does not change the net charge) which increases this water of hydration and hinders hydrolytic release of MTX. The hydration sheath has been described as water molecules surrounding the polymer molecules in an orderly iceberg-like structure (Martin, 1993).

5. Conclusions

The growth inhibitory effects displayed by the gelatin–MTX conjugates used in this study with HL-60 leukemia cells indicate the feasibility of using gelatin as a soluble macromolecular carrier. However, these conjugates had less growth inhibition compared to MTX alone. The correlation between reduced growth inhibition and reduced in vitro lysosomal degradation by the high drug load conjugate compared to the low drug load conjugate suggests a hindered enzyme degradation as the number of MTX molecules increases on the gelatin carrier molecule. This molar ratio effect occurs at both lysosomal and tumor pH values. MTX release studies showed that Cat B is not specific for the conjugate bond and that aqueous hydrolysis ultimately governs MTX release. Formation of gelatin–MTX fragments and subsequent MTX release from these fragments are also influenced by molar ratio and pH. The gelatin–MTX conjugate peptide bond is stronger under these in vitro lysosomal conditions than under in vitro intra-tumor conditions. This may explain the small MTX release observed and probably contributes to the reduced growth inhibition of the conjugate compared to free MTX. Another factor contributing to the reduced effect of the conjugate could be the 24–30 h delayed gelatin uptake into the cell. The small MTX release might be suitable in vivo after passive localization in tumor tissue. The largest total MTX release from the combination of fragments and free MTX occurs under these in vitro intra-tumor conditions. Punctate images of fluorescently labeled gelatin after incubation with cells suggests that these conjugates could be taken into the cells by endocytosis. The stability results indicate that these gelatin–MTX conjugates should have good stability under different biological conditions during drug delivery.

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