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Technical Note

Synthesis, Characterization, and *in Vitro* Stability of Chitosan-Methotrexate Conjugates

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INTRODUCTION

The use of macromolecular carriers to optimize the delivery of therapeutic agents has been widely studied (1). Macromolecule-drug conjugates have been investigated for the systemic (2–4) and regional delivery of anticancer drugs (5). Physicochemical properties of macromolecular carriers such as size, electric charge, and lipophilicity significantly influence the properties of the conjugate and hence require careful consideration (6). Methotrexate (MTX) is one of the most widely used drugs for the treatment of various neoplastic diseases in humans (7). A major drawback with MTX therapy is its poor ability to cross the blood-brain barrier (BBB) (8). Attempts have been made to improve transport of MTX across the BBB by using osmotic disruption techniques (9). A variety of macromolecular carriers has been investigated as drug delivery systems for MTX (3,4,10). Chu and Howell (3) have reported an increased half-life of MTX when linked to bovine serum albumin, while Shen and Ryser (4) have reported an increased cellular uptake *in vitro* in MTX-resistant cells, using a poly(L-lysine)-MTX conjugate. Ghosh *et al.* have reported an immunoglobin-MTX conjugate for targeting the drug to tumor associated antigens. However, a MTX conjugate designed specifically to interact with the vascular endothelium and cross the BBB has not yet been reported.

Chitosan is a naturally occurring polysaccharide made up of aminosugar monomers and has been utilized in a variety of biomedical applications (11) including drug delivery systems (12). It is biodegradable (13) and has been demonstrated to have potential as a carrier for receptor-mediated endocytosis via anionic heparin-related receptors on the luminal surface of capillary endothelial cells (14). Chitosan has a structural feature in the form of free amino groups that would allow its conjugation with MTX. Thus it may be considered as a suitable macromolecular carrier for investigation in the site-specific delivery of MTX.

The primary objective of this investigation was to synthesize a chitosan-MTX (CMTX) conjugate as a potential site-specific delivery system for MTX, to determine its stability *in vitro*, and to evaluate its ability to complex with heparin. Heparin has been used as a model for the anionic glycosaminoglycan (GAG) receptors which are structurally related to heparin (14). Since methylene blue, as a model cation, will compete with other cations for the anionic binding sites on heparin, this method can be used to demonstrate the formation of macromolecular ionic complexes between heparin and the CMTX conjugate. Such studies have been reported for polylysine (15), chitosan (16), and chitosan microspheres (14).

MATERIALS AND METHODS

Chemicals

Chitosan (MW 200,000) was purchased from Protan Laboratories (Redmond, WA). Methotrexate was a gift from Lederle Laboratories (Pearl River, NY). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI), methylene blue, and heparin were purchased from Sigma Chemicals (St. Louis, MO). Spectrapor cellulose dialysis tubing (MW cutoff 12,000–14,000) was purchased from Fisher Scientific (Atlanta, GA). All analytical grade reagents and HPLC-grade solvents were obtained from J. T. Baker Inc. (Philipsburg, NJ). The internal standard, *N*-[4-[[(2,4-diamino-6-quinazolyl)methyl]amino]benzoyl]aspartic acid (MBAA) was provided by the National Cancer Institute (Bethesda, MD).

Equipment

A Caframo (Ontario, Canada) stirrer Type RZR1 was used for conjugate synthesis. An FTS (Stone Ridge, NY) Flexi-Dry freeze drier was used to lyophilize the purified conjugate. A Beckman (Fullerton, CA) DU-7 spectrophotometer was used to record ultraviolet scans and a Bausch and Lomb (Rochester, NY) Spectronic 200 spectrophotometer was used to determine the drug content of the conjugates. A Perkin–Elmer (Norwalk, CT) 684 IR spectrophotometer interfaced with a Perkin–Elmer (Norwalk, CT) 7500 Professional Computer was used to record infrared scans. A

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Fisher (Altanta, GA) Dyna-Mix stirrer was used for the dynamic dialysis studies. A Waters (Milford, MA) 510 pump and a Lambda Max 481 variable wavelength detector interfaced with a Kipp and Zonen (Delft, Holland) strip chart recorder and an Alltech (Deerfield, IL) Hypersil C_{18} reverse-phase column were used for HPLC analysis of MTX in dialysis samples.

Synthesis of CMTX Conjugates

CMTX conjugates were synthesized in a biphasic reaction of 5 ml of a chitosan gel (10 mg/ml prepared in 1% acetic acid and adjusted to pH 7.4 with 1 *N* NaOH) with 2 ml of a MTX solution (20 mg/ml in 0.02 *N* NaOH) in the presence of ECDI dissolved in 2 ml of phosphate-buffered saline, pH 7.4 (PBS). The reaction was conducted at four levels using starting ratios of ECDI to MTX of 2:1, 4:1, 6:1, and 8:1. The reaction mixture was stirred for 3 hr at 275 rpm and 25°C and stored overnight at 4°C. The conjugate was then purified by dialysis using PBS (1000 ml changed every 12 hr) for 48 hr, followed by deionized distilled water for 12 hr. The purified conjugate was lyophilized and then stored in a desiccator for further studies. The above procedure was repeated without adding ECDI to the reaction mixture.

Characterization of CMTX Conjugates

The identity of the conjugate was confirmed using ultraviolet (UV) and infrared (IR) spectroscopy in combination with the control reaction, conducted in the absence of the cross-linking agent, ECDI. UV spectra of chitosan, MTX, and the conjugates were recorded in a 1% acetic acid solution between 200 and 400 nm. IR spectra of chitosan, MTX, the conjugates, and a physical mixture of chitosan and MTX were recorded using KBr disks. The conjugates were analyzed for drug content using UV absorbance at 305 nm.

In Vitro Stability of CMTX Conjugates

The stability of the CMTX conjugates was determined *in vitro* in various media using a dynamic dialysis technique (17). The dialysis cell was a modified version of the one used by Gupta *et al.* (17) and consisted of a stirred buffer reservoir, maintained at 37°C, in which dialysis tubing was suspended. The CMTX conjugates were placed in the test medium within the dialysis tubing and stirred at 475 rpm. The test media used were a pH 7.4 phosphate-buffered saline (PBS), a pH 5.6 citrate buffer, rat serum, and a lysosomal subfraction of rat liver, prepared by a method similar to that used by Fleischer and Kervina (18). In the studies using PBS and rat serum, the external buffer reservoir contained PBS, whereas in those using the pH 5.6 buffer and the rat liver subfraction, the external buffer reservoir contained the pH 5.6 buffer. The external buffer reservoir was sampled periodically for 24 hr. The samples were analyzed for MTX content using high-pressure liquid chromatography (HPLC) with UV detection at 313 nm. A 190-μl reservoir sample was mixed with 10 μl of MBAA solution by brief vortexing. A 100-μl aliquot of this was injected onto the HPLC system. The mobile phase consisted of 20% (v/v) methanol in water with 40 m*M* dibasic potassium phosphate at pH 7.0.

Competitive Binding Displacement Study

The cationic character of the CMTX conjugates was examined in dilute acetic acid (pH 3.0) and at a neutral pH, by a competitive binding displacement technique (14). The displacement of methylene blue (MB) from heparin binding sites by the CMTX conjugate would indicate the formation of a CTMX:heparin complex. The cationic nature of the CMTX would be due to the ammonium ion ($-NH_3^+$) on the chitosan. Spectra in the visible range (500–700 nm) were recorded at each pH for the following mixtures: MB (5.7 μg/ml); MB (5.7 μg/ml) and heparin (0.225 mg/ml); and MB (5.7 μg/ml), heparin (0.225 mg/ml), and CMTX conjugate, levels 1 and 2 (0.2 mg/ml).

RESULTS AND DISCUSSION

Synthesis and Characterization of CMTX Conjugates

Carbodiimides have been used with great success in the synthesis of drug-macromolecule conjugates, to cross-link the carboxyl group of a carrier or drug to the amino group of the counterpart (6). The water-soluble carbodiimide ECDI has been used to cross-link MTX with a variety of macromolecules such as albumin (3), polylysine (4), and immunoglobin (10), through covalent amide bonds. ECDI was chosen as the cross-linking agent in this investigation because it was considered suitable for bond formation between the carboxyl group of MTX and the amino group on chitosan.

Figure 1 shows the UV absorption spectra of CMTX conjugates and MTX between 200 and 400 nm. The spectra were found to be identical, while chitosan itself showed no

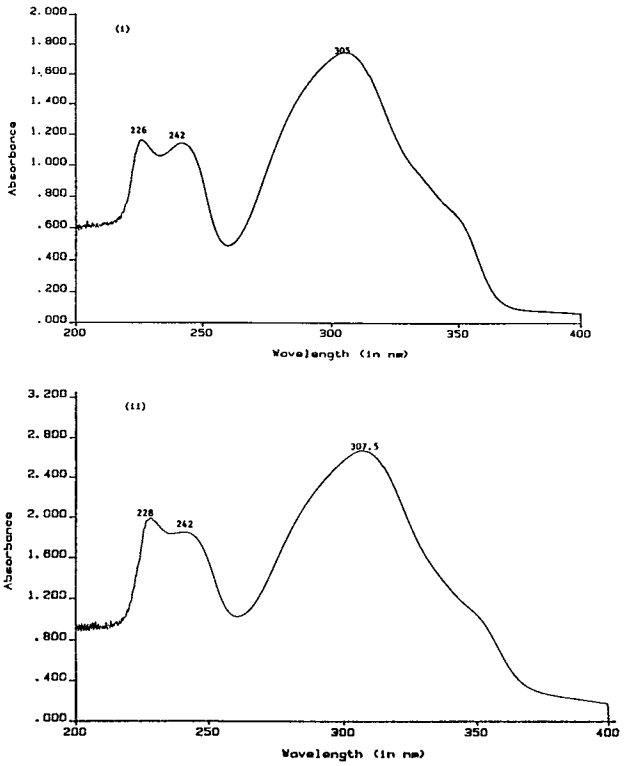

Fig. 1. Representative UV absorption spectra of (i) MTX and (ii) CMTX conjugate (9%, w/w, MTX) in 1% acetic acid.

UV absorption in this range. Since all free MTX had been previously removed by extensive dialysis, only MTX linked to chitosan would lead to the identical spectra. IR spectra were used to establish that the conjugates were a chemical identity different from the polymer and a physical mixture of the polymer with MTX. Computer additions of the individual spectra of chitosan and MTX were found to be identical to a physical mixture of chitosan and MTX but different from the CMTX conjugate scan. The synthetic reaction conducted in the absence of ECDI did not yield a stable conjugate but rather a mixture, from which the MTX could be rapidly dialyzed out using PBS. This fact along with the spectroscopic evidence helped establish the formation of a covalent linkage between chitosan and MTX when reacted in the presence of ECDI.

Drug content analysis showed that the percentage (w/w) MTX loading on the polymer increased as the ratio of cross-linking agent, ECDI to MTX was increased. Conjugates at levels 1 and 2 had loadings of 9 and 16%, respectively, or approximately 44:1 and 77:1 MTX:chitosan molar ratios, respectively. Conjugates at levels 3 and 4 were not investigated further, as their aqueous solubility at acidic pH was severely retarded. Chitosan dissolves in aqueous solutions of organic acids such as acetic acid due to the presence of free amino groups (13). Since these groups are also involved in the covalent linkage with MTX, drug loading above a certain limit would reduce the solubility of the polymer backbone.

In Vitro Stability Studies

Over a 24-hr period in the dynamic dialysis study, a maximum of about 2.8% (w/w) of MTX was released (see Fig. 2) in the various test media, indicating that the conjugate is stable. Lysosomal enzymes are known to act on a large variety of macromolecules *in vivo* and are also instrumental in releasing drug from their macromolecular conjugates (6). We studied the lysosomal test medium in order to determine its effect on drug release from the CMTX conjugates. The lack of significant differences in such a medium, compared to the others studied, suggests that the drug may be released by slow hydrolysis of the covalent bond rather than by enzymatic degradation. Pangburn *et al.* (13), have reported that chitosan itself is susceptible to degradation by lysozyme. This leads to the possibility that lower molecular weight derivatives of the conjugate may be released in the lysosome and these may be sufficient to cause a cytotoxic effect at the tumor site.

Competitive Binding Displacement Studies

The results of the binding displacement study (see Fig. 3) in acidic medium indicated that the conjugate had retained some of the properties of the polymer. Thus, the conjugate due to its cationic character was able to displace MB from its complex with heparin, yielding a UV scan similar to that of free MB. The formation of a cation:anion complex was considered significant since it is directly dependent on the availability of free amino groups as is the case with its solubility. Based on a chitosan monomer weight of 200 and a percentage deacetylation of 85.3, about 853 free amino groups are available per mole of chitosan. Based on a molar drug loading of 44:1 and 77:1 for the conjugates, 776–809 (91–95%) of those amino groups still exist in a free state in the conjugate. However, due to the random-coil structure of the polymer chain, all of these groups may not be accessible when in solution (19). Thus, the availability of a significant number of free amino groups on the conjugate will potentially enable the CMTX conjugate to interact with GAG receptors on the vascular endothelium. A chitosan:heparin complex did not occur at a neutral pH and indicates a potential limitation to the *in vivo* interaction of the conjugate with endothelial receptors. However, a suitable formulation that allows the conjugate to retain its cationic character could overcome this limitation.

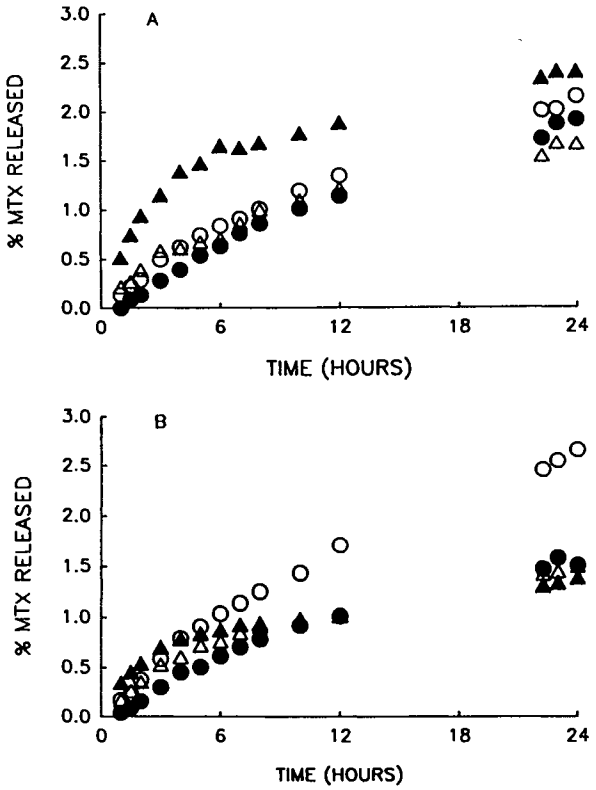

Fig. 2. Drug release profiles from dynamic dialysis studies of (A) CMTX conjugate level 1 and (B) CMTX conjugate level 2 in (○) PBS, (●) rat serum, (△) pH 5.6, buffer, and (▲) rat liver subfraction.

Heparin was used as a model molecule for the anionic GAG receptors on the luminal surface of the capillary endothelium (20). Gallo and Hassan (14) have demonstrated the potential of chitosan to be used as a receptor-mediated drug delivery system. Molecular chitosan and magnetic chitosan microspheres were able to displace MB through the formation of a chitosan:heparin complex. In the current investigation, the use of CMTX conjugates extended this previous work by demonstrating that conjugation of MTX to chitosan did not interfere with the ability of chitosan to displace MB from its complex with heparin.

There is considerable potential for target-specific or regional delivery of drugs using conjugates that bind to and are endocytized by capillary endothelial cells. An important aspect in the use of such conjugates will be to increase the residence time of the conjugate in the capillaries, allowing

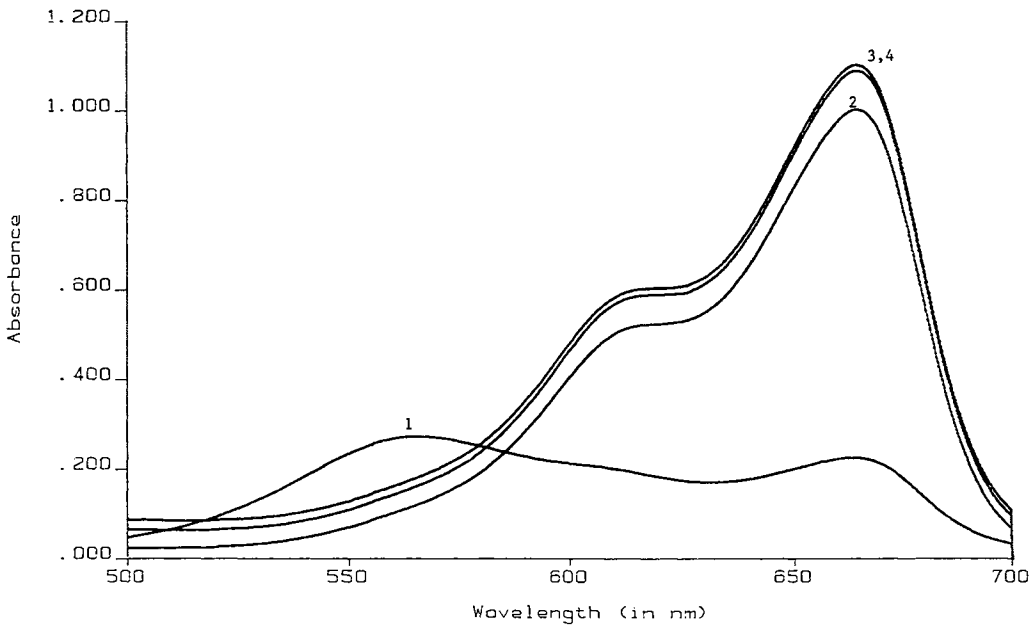

Fig. 3. Binding displacement study in 1% acetic acid. UV spectra of (1) MB + heparin, (2) MB, (3) MB + heparin + CMTX conjugate level 1, and (4) MB + heparin + CMTX conjugate level 2.

the conjugate to interact with the GAG receptors. A number of blood flow reduction techniques such as degradable starch microspheres (21) have been used to increase drug uptake following arterial infusions. These techniques should be applicable to enhance the efficacy of target organ delivery of macromolecular conjugates.

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