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SYNTHESIS OF POLYAMIDOAMINE DENDRIMER-PEG-DRUG CONJUGATES

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ABSTRACT : Star polymers offer promising multifunctional nanomaterials as carriers for the drug delivery system due to their well-defined size and tailor ability. Polyamidoamine (PAMAM) dendrimer is the most suitable star polymers for biomedical applications. In the present study, the effect of alterations of the chemical structure of PAMAM dendrimer on the conjugation of methotrexate (MTX) was investigated. Monomethyl poly (ethylene) glycol (MPEG) was used as spacer to facilitate the conjugation and reduce the toxicity of dendrimer. Two methods were studied to produce PAMAM-PEG-MTX and PAMAM-MTX conjugates. Characterization of the conjugates has been determined by multiple analytical methods such as UV Visible spectroscopy (UV-Vis), Fourier Transform Infra-red (FTIR), Nuclear Magnetic Resonance (NMR) and Differential Scanning Calorimetry (DSC). The conjugates efficiency of both methods was compared.

KEYWORDS : PAMAM dendrimer, PEG, MTX, conjugate

INTRODUCTION

Star polymers, known as dendrimers are a versatile class of branched nanomolecules, in which they resemble treelike molecular architectures since they are built from repetitive monomers with branching point units that are radially connected around a template core. Dendrimers are discrete molecules with a high degree of molecular uniformity and monodispersity. Anticancer chemotherapeutics are by far the main representative use of this dendrimers. In most examples, dendrimers are found in the concepts of cell targeting, drug solubilization, macromolecular prodrugs, and drug nanocarriers. Dendrimers are now perceived as a promising class of drug scaffolds because they are well defined, monodisperse, readily soluble in solvents, and well characterized. In contrast, it was reported that polydispersity and reproducibility in the preparation of functionalized hyperbranched or linear polymers may lead to irreproducible pharmacokinetic behaviors as a result of the variation in the molecular-weight distribution profile (Marc Gingras *et al.*, 2007).

Methotrexate (MTX) formerly known as amethopterin, is an antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases. It acts by inhibiting the metabolism of folic acid. Polymer-based drug delivery systems are designed to improve the pharmacokinetics and biodistribution of a drug and or provide controlled release kinetics to the intended target (Kumar, *et al.*, 2007). The ideal dendrimer carrier should exhibit high aqueous solubility and drug-loading capacity, biodegradability, low toxicity, favorable retention and biodistribution characteristics, specificity, and appropriate bioavailability. In dendrimer-based drug delivery, a drug is either non-covalently encapsulated in the interior of the dendrimer or covalently conjugated to form macromolecular prodrugs (Wolinsky & Grinstaff, 2008).

Considering the use of dendrimers for drug delivery, it is necessary that they are nontoxic and biocompatible. It has been demonstrated that widely used dendrimers, such as PAMAM dendrimers bearing primary amino group termini, are quite cytotoxic, and also these dendrimers were cleared rapidly from the circulation when administered intravenously (Kumar, *et al.*, (2007), Wolinsky & Grinstaff, (2008), Hong, *et al.*, (2004) and Diallo, *et al.*, (2004). Prodrug design can be highly effective for solving many of the stability, solubility, permeability and targeting problems in drug discovery and development (Hu, 2004). In addition to improving oral bioavailability, prodrugs are increasingly used for targeting purposes including site-specific activation and delivery of anticancer drugs to tumor tissues through transporters, tumor- or tissue-specific enzymes, and gene therapy (Hu, 2004). Polyethylene glycol (PEG) is a relatively non - reactive and non-toxic polymer that is frequently used to improve pharmacological characteristics, which include increased stability, prolonged half-life and reduced toxicity, for both potential and existing macromolecular therapeutic agents Hu, L. (2004) and Marc Gingras *et al.*, (2007). However, the method of conjugating the drug and PEG to dendrimer via covalent bonding is still a major challenge to the researcher.

Therefore, the present study was aimed at developing and synthesizing the PEGylated PAMAM dendrimer attached with methotrexate drug, to be used as nano drug delivery in tumor treatment. This method focused on attaching the PEG and MTX onto PAMAM dendrimer.

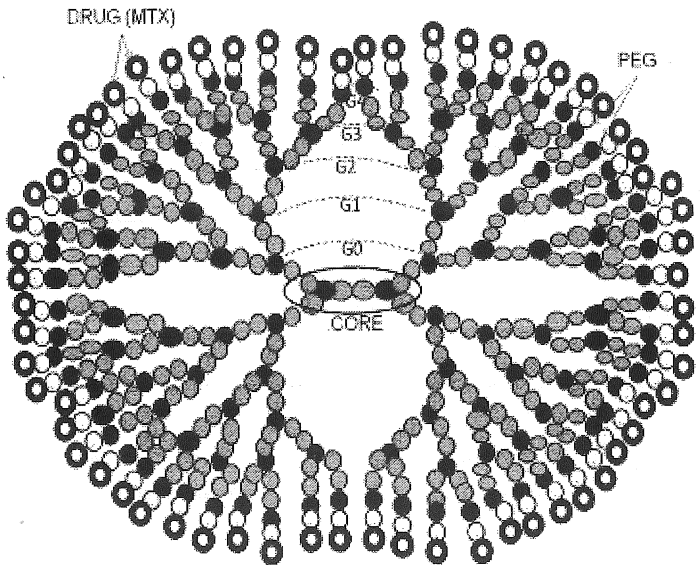

Figure 1. *PEGylated- Dendrimer G4-Drug conjugate, reproduced from reference 3.*

MATERIALS AND METHOD

Materials

PAMAM-G4-NH_2 terminal dendrimer was purchased from Sigma-Aldrich. Monomethyl ether poly-ethylene glycol 2000 (M-PEG 2000), 4-nitrophenyl chloroformate and triethylamine, were supplied from Sigma-Aldrich. Solvents such as dimethyl sulphoxide (DMSO) and tetrahydrofuran (THF) were purchased from Fisher. Methotrexate and dicyclohexylcarbodimide (DCC) were obtained from Fluka. Dialysis membrane of molecular weight cut-off of 12000-14000 Da and 3500 Da were purchased from Spectrapor.

Synthesis of M-PEG-4 nitrophenyl carbonate

M-PEG 2000 (3.6 mmol) was first dissolved in 360 ml of THF. Following that, 4-nitrophenyl chloroformate (7.2 mmole) and triethylamine (7.2 mmol) were added into the mixtures

slowly and stirred at room temperature for 48 hours. The M-PEG-4 nitrophenyl carbonate was recovered by evaporation of the reaction mixture. The crude product was purified by recyrstallization from chloroform and diethyl ether (15:1, 300 ml: 20 ml). Figure 2 summarizes the synthesis steps.

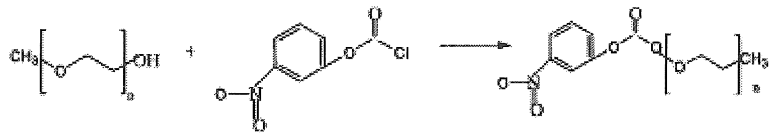

Figure 2. *Synthesis of M-PEG-4 nitrophenyl carbonate*

Synthesis of M-PEG attached onto PAMAM dendrimers

The M-PEG attached PAMAM dendrimers were synthesized as shown in Figure 3. Fourth generation (G4) of PAMAM dendrimers (7 μmol) was prepared in 10 ml DMSO. M-PEG-4 nitrophenyl carbonate (0.9 mmol) was added into the solution and was stirred for 5 days at room temperature. The reaction mixture was diluted with distilled water and dialyzed against distilled water for 24 h using a dialysis bag (molecular weight cut off 12000-14000 Da). The resulting product was lyophilized for 24 h and characterized using NMR and FTIR.

Synthesis of Polyamidoamine dendrimer-PEG-MTX Conjugate

Method 1 for Conjugate A. Conjugation of MTX to MPEG- PAMAM G4-NH_2. MTX and molar end equivalent of synthesized MPEG-PAMAM-G4-NH_2 were dissolved in 10 ml DMSO, followed by the addition of a molar equivalent of DCC. The reaction mixture was stirred continuously for 72 h at room temperature in the dark. After 72 h, it was filtered to remove dicyclohexyl urea (DCU) and the filtrate was dialyzed against an excess amount of DMSO using membrane (MW cutoff = 3500 Da) for 24 h to remove free MTX and unreacted DCC. The filtrate was vacuum-distilled at 180 °C to obtain MTX-dendrimer conjugate. This conjugate (conjugate A) was characterized using NMR, UV-Vis, DSC and FTIR analysis. Figure 4 (Method 1) summarizes the synthesis steps.

Synthesis of Polyamidoamine dendrimer -MTX Conjugate

Method 2 for Conjugate B. Conjugation of MTX to PAMAM G4-NH_2. MTX and molar end equivalent of PAMAM-G4-NH_2 (based on 64 end functional groups) were dissolved in 10 ml DMSO, followed by the addition of a molar equivalent of DCC. The reaction conditions and

the purification steps were similar to those mentioned above. The conjugate B was formed as a result of amide bond formation between the $-NH_2$ groups of dendrimer and the -COOH group of MTX. This conjugate (conjugate B) was characterized using NMR, UV-Vis, DSC and FTIR analysis. Figure 4 (Method 2) summarizes the synthesis steps as follows.

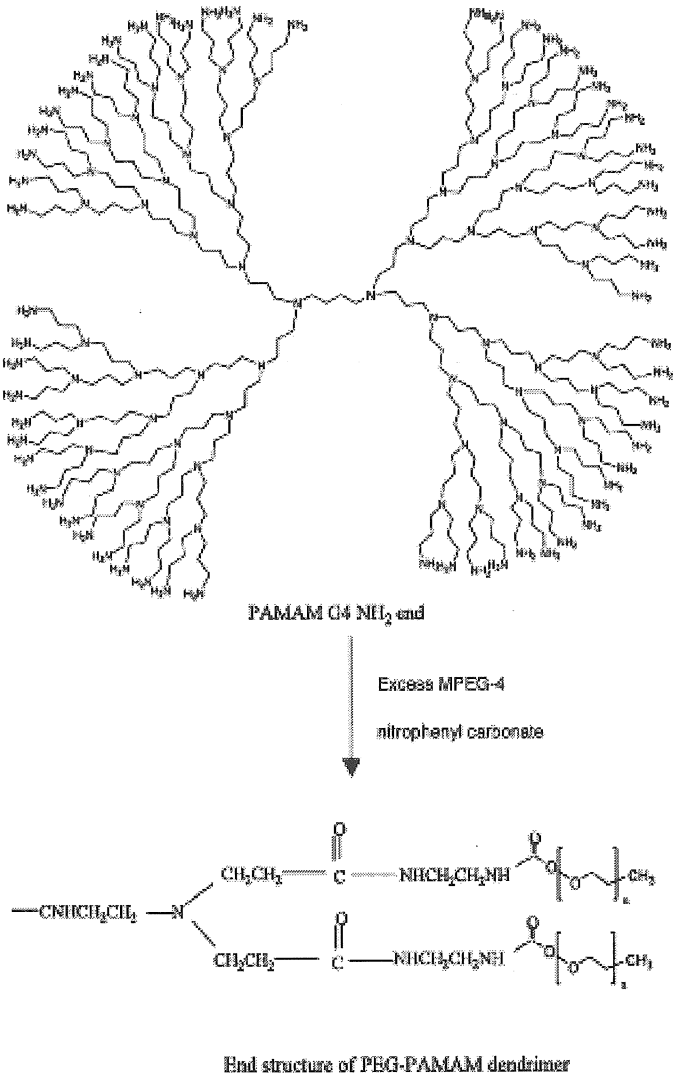

Figure 3. *Synthesis of M-PEG-attached PAMAM dendrimers*

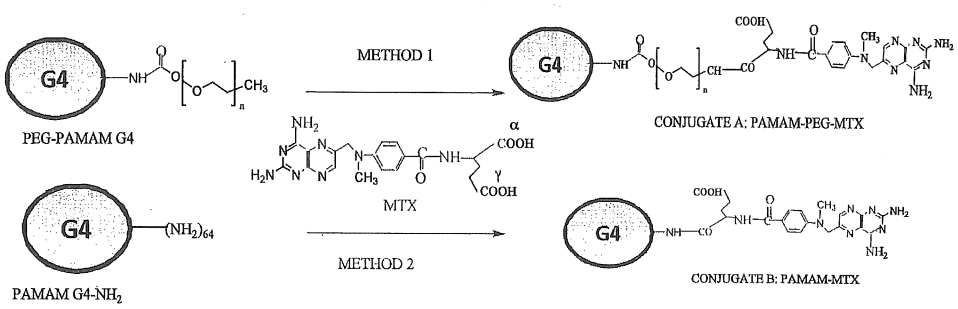

Figure 4. *Synthetic routes of conjugates via Method 1 and Method 2*

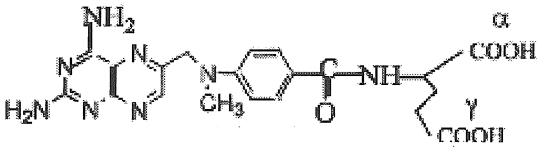

Figure 5. *Chemical structure of MTX*

Characterization of conjugates

FT-IR spectra were recorded using Perkin Elmer Spectrum 2000 Fourier Transform Infrared Spectrometer equipped with a diamond tip HATR (Horizontal Attenuated Total Reflectance). The absorbance at 372 nm was measured to determine the MTX using UV-Visible Spectroscopy from Perkin Elmer and the data was analyzed using Lambda 25 software. ^{1}H Nuclear Magnetic Resonance (H^1 NMR) spectra were recorded on a Varian (VNMRS – 300 MHz) using d_6-DMSO and d_8-CDCL3 as solvents. The melting point of MTX was determined using Differential Scanning Calorimetry analysis (DSC) of Metler Toledo DSC821E equipped with STARe software with temperature range of 0 - 250 °C and heating rate of 10 °C/min.

RESULTS AND DISCUSSION

Modification of the surface of the dendrimer through attachment of PEG was carried out in view of reducing the cytotoxicity of the higher generation of cationic amine-terminated PAMAM. Firstly, the PEG was coupled with 4-nitrophenyl chloroformate forming MPEG-4- nitrophenyl carbonate before attaching it onto the dendrimers. Secondly, the drug was conjugated straight onto the dendrimers without utilizing any linkers. Drug loading efficiency

was monitored through various spectroscopic means which include UV, FTIR, DSC and NMR. There are three possibilities that should be considered when investigating the loading process. First, the drug could possibly be attached to the linker and is covalently bonded to PAMAM. Second, the drug is encapsulated in the PAMAM's cavity. Third, the drug could possibly be coexisting with the dendrimer without being encapsulated or conjugated.

UV Spectroscopy

Both method of incorporating MTX to PAMAM with or without linker showed the existence of the drug used. Figure 6 demonstrates that free MTX absorbs at λ_{max} of 388.29 nm and a slight shift occurred for both MTX-PEG-PAMAM and MTX-PAMAM of 383.68 nm and 383.80 nm respectively. This insignificant shifting process indicates the possibility of the drug being encapsulated in the dendrimer without any covalent bonding with the dendrimer itself. Dhanikula (2007) reported that when there is no major shifting process, it advocates the absence of π-π complexation or interaction of the dendrimer with the chromophore of MTX.

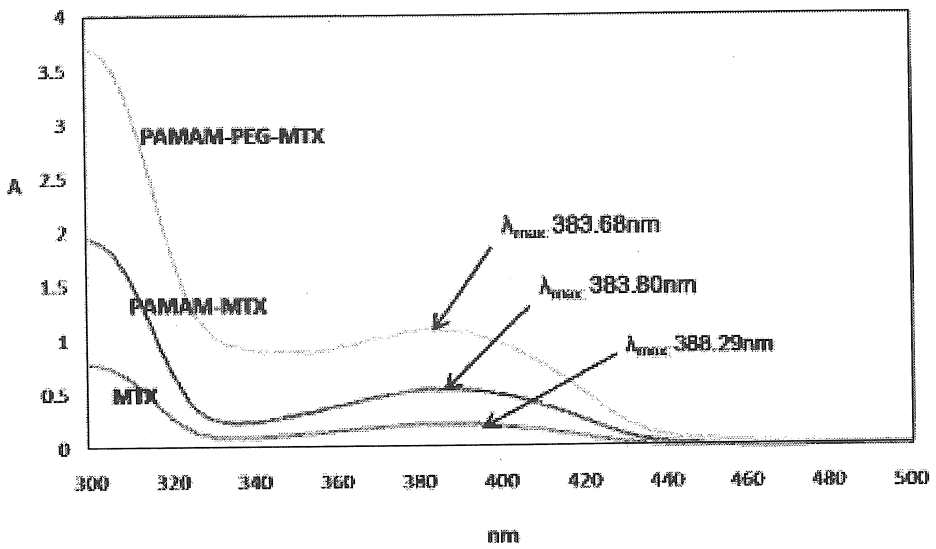

Figure 6. *UV-Vis absorbance of different samples*

FTIR studies

The FTIR spectra of both conjugated compounds produced from both methods can help to explain the bonding formation in both compounds. The FTIR of PAMAM-PEG-MTX (Figure 7) did not show any indication of the drug and the dendrimer at the final product. The final product exhibited the absorbance of peaks related to only PEG, which renders the possibility of the failure in synthesizing the PAMAM-PEG-MTX. It is thought that the absorbance of

MTX observed in the UV spectra could be true due to the drug located at the periphery of the dendrimer without any attachment. Once the compound was dissolved in the solvent for UV testing, the drug was immediately being washed off and can still be observed in the UV spectra. The drug encapsulation within the dendrimers are driven by the driving force based on covalent bond formation, electrostatic interactions, complexation reactions, steric confinement, various types of weaker forces (van der Waals, hydrogen bonding) and the combinations thereof (Crooks, 2001).

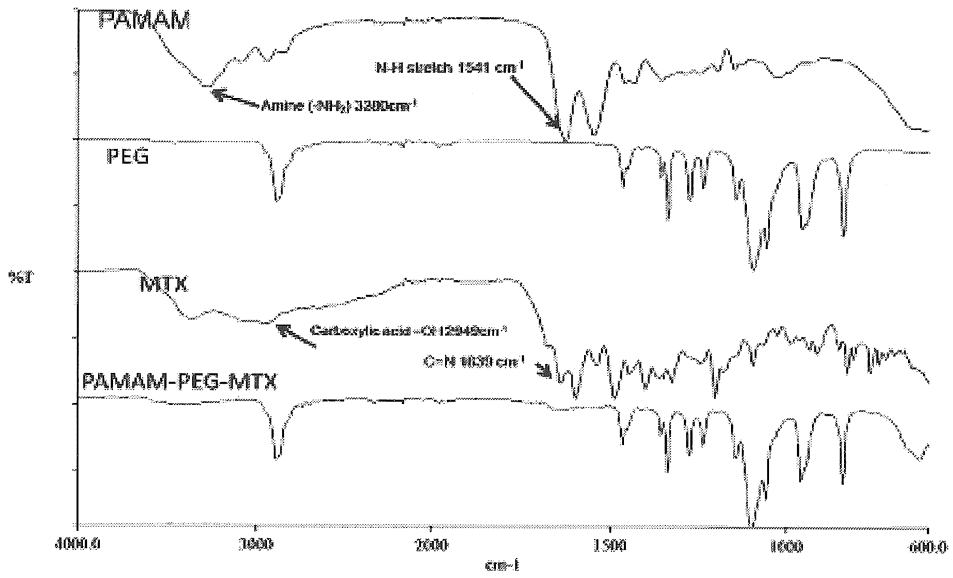

Figure 7. *Comparison of FTIR spectrums of PAMAM-PEG-MTX conjugates and raw materials*

Several factors could be considered when describing the failure of synthesizing PAMAM-PEG-MTX. It is at large due to the steric hindrance posed by the bulkiness of the dendrimer and high molecular weight of PEG used in this work. Elvira *et al.* has mentioned that modification of the PEG is based on the replacement of the hydroxyl end group, and the molecular weight of the PEG chains and the site of conjugation that influence the final properties of the conjugates. PEG with average molecular weight of several thousand has generally been used. Additionally, PAMAM-G4 NH_2 dendrimer is a star dendrimer with 4 branches, 64 amine groups at the surface and has a high average molecular weight as shown in Figure 1. MTX, the drug itself also has a long chain and high average molecular weight as shown in Figure 5. The conjugation of these three materials may form very big star structure with a long chain and higher average molecular weight, which limits the efficiency of the MTX drug to conjugate to the PEGylated-PAMAM dendrimer. Vast size and extended chain of dendrimer and PEG stand a slim chance for the MTX to get attached to the PEG. Even if some conjugated compounds have been successfully achieved, it may loss during the reaction and purification process.

On the contrary, the FTIR spectra for PAMAM-MTX conjugate demonstrates the existence of the MTX peaks in which the characteristics amine (-NH_2) peak was slightly shifted from 3280 cm^{-1} to 3282 cm^{-1}. Another shifting was also observed for the carboxylic peaks of MTX from 2949 cm^{-1} to 2934 cm^{-1}. The conjugation of MTX and PAMAM was confirmed through the amide bond HN-CO- peak at 1625 cm^{-1} which indicates the covalent bonding of MTX and PAMAM. It is thought that MTX has been successfully attached to the dendrimer via Method 2.

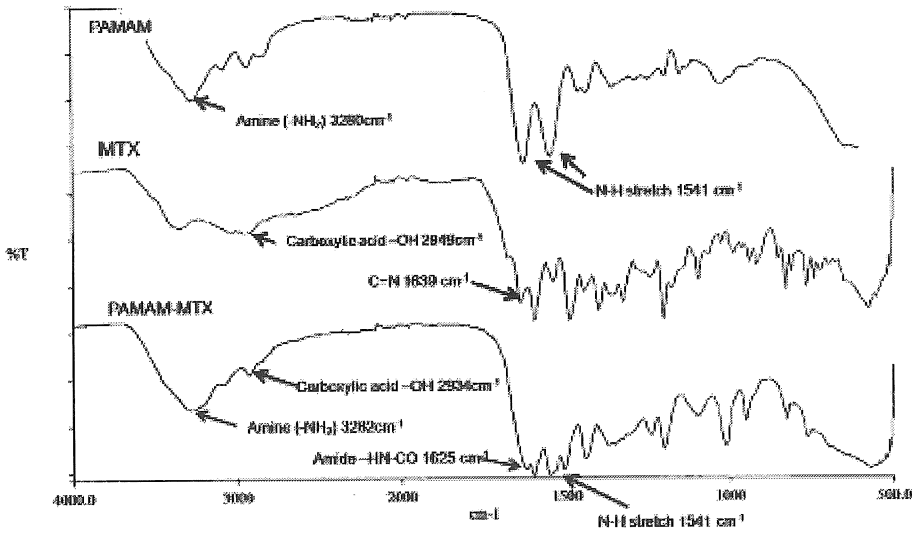

Figure 8. *Comparison of FTIR spectrums of PAMAM--MTX conjugates and raw materials*

MTX contains both α and γ-COOH groups in the glutamate moiety, as well as –NH_2 groups at the second and fourth positions in the 2, 4-diamino-6-pteridinyl group as shown in Figure 5. For conjugate B (Figure 4), MTX was successfully conjugated to PAMAM-G4 NH_2 dendrimers using DCC as a coupling agent. It is confirmed through FTIR result that MTX has been conjugated with –NH_2 terminals dendrimers specifically using –COOH groups. The two readily –COOH group on MTX make it possible for the amine –NH_2 group on dendrimer to covalently bond and form amide groups –HN-CO. Even though we did not confirm which of the two –COOH groups in the glutamate moiety of MTX interacts with the NH_2 groups of the dendrimer, it is most likely that the carboxyl group at the γ-position was responsible for the interaction and this is also reported by Sezen and co workers (Sezen Gurdag, *et al.*, 2006). Kono *et al.* showed that the conjugation of folic acid to hydrazide-terminated dendrimers could occur through its γ–COOH group, which is more reactive toward the amine groups in carbodiimide coupling reaction (Sezen Gurdag, *et al.*, (2006) and Kono K., *et al.*, (1999)). In addition, conjugation of MTX to human serum albumin occurred mainly through involvement of the γ-COOH group of MTX (Kono K., *et al.*, 1999).

DSC studies

Figure 9 shows the DSC thermogram of MTX and PAMAM-MTX under nitrogen during the heating scan. Three endotherm peaks were observed in DSC thermograms of MTX. The two first endotherm peaks appearing at 115 and 150 °C are associated with loss of free and bound water. The peaks that appear at 210 °C correspond to the melting point of the MTX drug. Similar peaks had also appeared at the DSC thermogram of PAMAM-MTX compound, which suggested that existence of MTX in the compound. The melting peak of MTX seems to appear in the PAMAM-MTX thermogram which indicates the conjugation of MTX onto PAMAM.

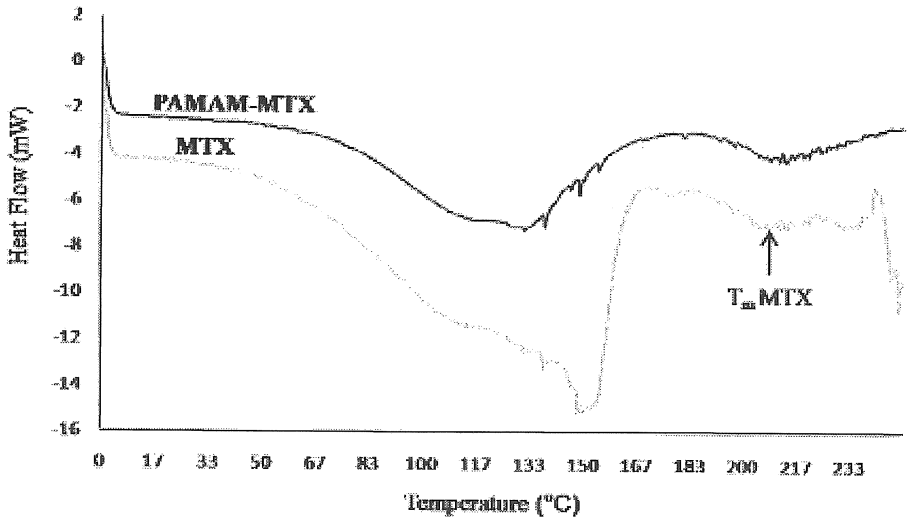

Figure 9. *DSC curves of PAMAM-MTX and MTX*

^{1}H NMR spectra

MTX showed peaks at chemical shift of 6.82 ppm and 7.82 ppm which are attributed to the aromatic protons (Figure 10). Chemical shift at 4.52 ppm corresponds to the CH_2-N in MTX.

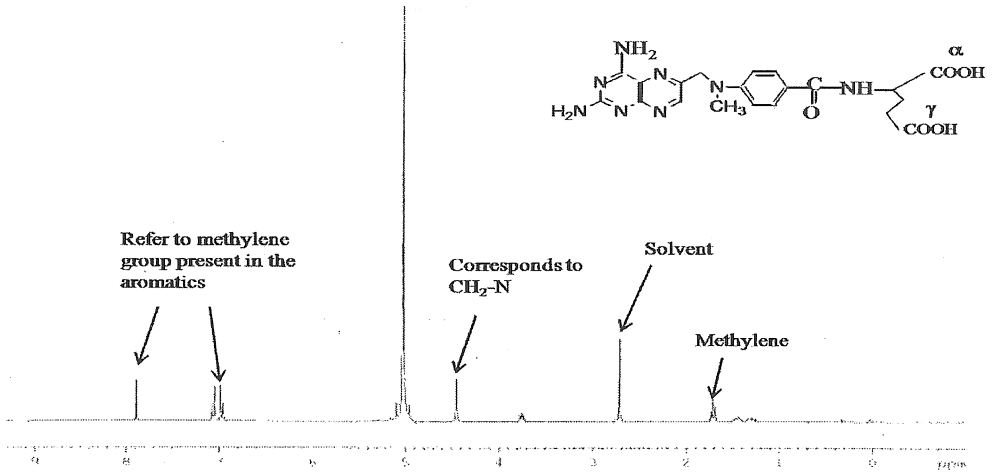

Figure 10. *^{1}H NMR spectra of MTX*

The ^{1}H NMR spectra of PAMAM-PEG-MTX (Figure 11) demonstrate only the existence of PEG in the final compound in which peaks appeared as multiplets at δ: 3.2 – 3.8 ppm were attributed to the CH_2 group of PEG. The aromatic protons of the drug could not be observed in the spectra. This observation agrees very well with the FTIR data which indicates the failure in attaching the MTX to PEG and followed by PAMAM.

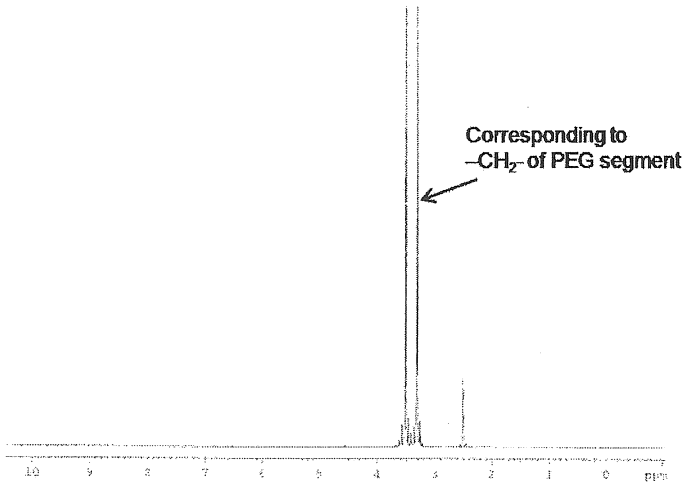

Figure 11. *H^1 NMR spectra for PAMAM-PEG-MTX conjugate*

^{1}H NMR spectra of MTX and MTX loaded PAMAM dendrimer were acquired to explore further the dendrimer and MTX interaction. It is obvious that the spectrum of the PAMAM-MTX conjugate contains signals originating from both the PAMAM-G4 dendrimer and MTX (Figure 12). The signals in resultant conjugates at 2.18-3.46 ppm corresponds to the CH protons of PAMAM, overlapping with aliphatic singlets of MTX. The signal at 7.7 ppm corresponds to the aromatic CH of MTX. Meanwhile signal at 7.95 ppm corresponds to the aromatic NH_2 of MTX. Low peak intensities in ^{1}HNMR spectrum of MTX loaded dendrimer suggested that MTX could be encapsulated inside the dendrimer and is not surface bound (conjugated). Though encapsulation could be the way of incorporating the drug as suggested by the ^{1}H NMR studies, the possibilities of conjugation could not be ruled out.

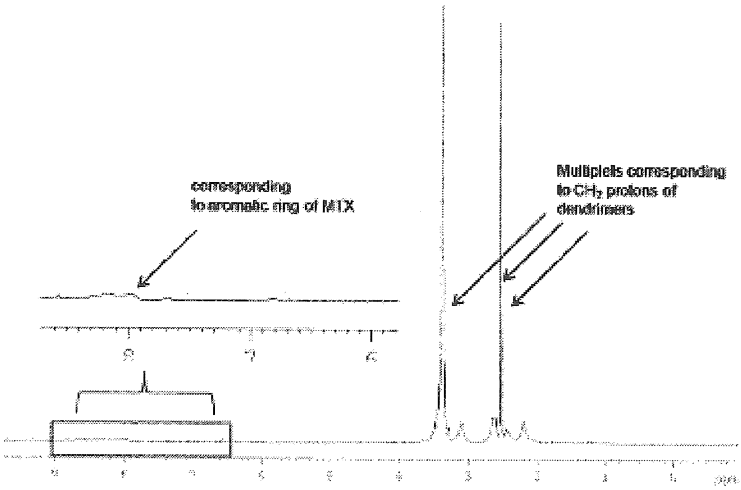

Figure 12. *H^1 NMR spectra for PAMAM-MTX conjugate*

CONCLUSION

The usage of PEG as a linker was not materialized as the compound produced, failed to indicate the presence of both the drug (MTX) and PAMAM. Direct conjugation of PAMAM and MTX showed two possibilities. First, both FTIR and DSC studies demonstrate the possible drug attachment to the dendrimer. Second, both UV and ^{1}HNMR indicate the possible encapsulation process.

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