Original Article

Synthesis, Characterization, and Cytotoxicity Evaluation of Methotrexate-Polyethylene Glycol-Glutamic Acid Nanoconjugate as Targeted Drug Delivery System in Cancer Treatment

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Abstract

Introduction: Methotrexate (MTX) is used as a folic acid antagonist in the treatment of many human cancers. Attachment of hydrophilic ligands to MTX improves its efficacy due to reducing toxicity and enzymatic degradation and it also increases its *in-vivo* half-life. **Materials and Methods:** In the present study, pH-responsive nanoconjugates of methoxy poly(ethylene glycol)-glutamic acid methotrexate (mPEG-Glu-MTX) have been prepared and characterized using hydrogen nuclear magnetic resonance (^{1}H-NMR) and Fourier transform infrared (FT-IR). Glutamic acid is attached to the mPEG chain by the carboxylic group and to the MTX via an amide bond to the amine group. **Results:** The prepared nanoconjugate has the mean diameter ranging from 160 to 190 nm and, the drug release was significantly induced two times at the pH of 5.5 and 3.5 compared with pH 7.4 ($P < 0.05$). The prepared mPEG-Glu-MTX nanoconjugate showed toxicity similar to AGS, MDA, and MCF7 cell lines compared with the free form of MTX ($P > 0.1$), which indicates that the conjugation does not effect on the MTX cytotoxicity but is expected to be successful in the targeted delivery of MTX. **Conclusion:** The results show that manufactured nanoconjugates can be considered as an efficient drug delivery system in the treatment of cancer; however, further studies are needed on the targeting activity of this nanocarrier in *in-vivo* conditions.

Keywords: *Chemotherapy, drug delivery, glutamic acid, methotrexate, nanoconjugate*

Introduction

The application of natural polymers in drug delivery systems is considered for various reasons, such as their biodegradability, biocompatibility, hydrophilicity, and protective properties.[1]

Previous studies have investigated the conjugation of drugs to various soluble polymers such as polysaccharides, dextran, peptides, amino acids, and polyethylene glycols (PEGs). These small-sized nanoconjugates can be used as targeted drug carriers to tumor cells passively and to improve drug accumulation owing to the enhanced permeation and retention (EPR) effect.[2-5] Also, they can be synthesized for active targeted treatment by responding to external stimuli (e.g., magnetic force and phototherapy) and responding to internal stimuli (e.g., temperature, pH, and enzymes) in tumor cells.[6]

Glutamic and aspartic acids are well-known amino acids used to prepare the biodegradable and pH-responsive carrier systems. The presence of carboxylic acid functional groups in their side-chain loses hydrogen at the physiological pH and makes it negatively charged.[7]

Glutamic acid, which is a non-essential proteinogenic amino acid, has a prominent role in amino acid metabolism of organisms. This effective neurotransmitter is assumed to have a vital role in neural activation. Glutamic acid is converted to glutamine in the body by the reaction of this acid and ammonia and the enzyme glutamine synthase.[8] It also is a nitrogen donor in nucleotide synthesis, improving the essential amino acid uptake and TOR kinase activation. Glutamine is necessary for maintaining the potential and integrity of the mitochondrial membrane. Glutamate is a crucial compound in cellular metabolism. Cancer cells need more bioenergy than normal cells of the body owing to their high metabolic activity and proliferation, thus making cancer cells more dependent on glutamate. Cancerous cells consume glutamine on a large scale, leading to tumor survival. This amino acid is the respiratory fuel of tumor cells.[8]

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Polyethylene glycol is among the most popular used polymers used for drug conjugation and nanomedicine studies. PEG has several applications in advanced drug delivery regarding its neutral hydrophilic properties and has no significant cytotoxicity effect in the form of passive or active drug targeting.[2,9] Generally, using PEG in drug conjugation form increases the healing efficiency of active therapeutic ingredients.[9]

Methotrexate (MTX) has been widely applied to treat some human diseases, including psoriasis, non-Hodgkin's lymphoma, choriocarcinoma, osteosarcoma, sarcoidosis, neck Hodgkin's disease, and breast, head, and lung cancer.[10-14] However, chronic hepatotoxicity, bone marrow depression, nephrotoxicity, ulcerative colitis, and limited permissible dose restrict the use of MTX. Because this drug is effective in high doses, it is often associated with drug resistance and toxicity to normal and proliferative cells.[15] Adverse side effects could be effectively reduced by direct delivery of the drug into the tumor site.[16,17] Recently, some drug carriers, including nanoparticles (NPs) made from biodegradable and biocompatible compounds, have been developed to overcome these limitations.[18,19]

The polymeric NPs with PEG grafts of MTX (mPEG-NP-MTX) were introduced as a novel drug delivery system in a previously published work. The result was an optimum formulation of nanocarrier, which increased cell cytotoxicity. The average IC_{50} values for MTX in poly(lactic-co-glycolic acid) (PLGA) nanoparticles for different cell lines were twice as much as values in PLGA-PEG nanoparticles ($P < 0.05$).[20]

In the present study, it was tried to improve the physicochemical and biopharmaceutical properties of MTX. Therefore, in the design of the new targeted nanocarrier, two ligands, PEG and glutamine, due to their hydrophilic properties, pH responsibility, and high uptake by cancer cells, were used. To this end, we characterized, prepared, and modified PEG-Glu-drug nanoconjugate, and their cytotoxicity was evaluated on different cancer cells.

Materials and Methods

Materials

Monomethoxy PEG 2000D, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), and glutamic acid were bought from Sigma-Aldrich (St Louis, MO, USA). MTX was purchased from Hermann PCS Company (Feucht, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), triethylamine (TEA), and dialysis membrane (MWCO 3500 and 6000–8000 Da) were obtained from Fisher Scientific Co. Other used materials were of analytical or HPLC grade. Distilled water was prepared in our laboratory by using the RO instrument.

Synthesis and characterization of mPEG-Glu-MTX nanoconjugate

mPEG-Glu conjugation is as follows. In brief, 6.41×10^{-2} mmol of each of glutamic acid and methoxy-PEG was separately dissolved in distilled water and subsequently 1eq of TEA was added as a catalyst to the reaction medium under nitrogen gas for 72 h at room temperature. The mixture was then stirred, and a white precipitate was formed after 3 days. The precipitate was concentrated and purified by a speed vac 2 concentrator at 60°C.[21-23]

The prepared mPEG-Glu was dissolved in DMSO. MTX (6.41×10^{-2} mmol) was added to DMSO and stirred until maximum dissolution was reached. Then this solution was reacted with NHS (3 mmol) and DCC (19.9 mmol). Activated MTX was carefully introduced to the solutions of mPEG-Glu, followed by stirring for 4 days. This product was purified by dialyzing against water for 24 h, using a dialysis membrane (cutoff 12,000 Da). To obtain a fine powder of mPEG-Glu-MTX nanoconjugate, the as-prepared product was lyophilized using a lyophilizer (ZibrusVaco 10-II-E; Germany),[21-23] as shown in Figure 1. Conjugation of MTX to mPEG-Glu has been determined by Fourier transform infrared (FT-IR) and nuclear magnetic resonance (^{1}H-NMR). FT-IR spectra were obtained in a transmittance mode in the spectral region of 450–4000 cm^{-1} by a Shimadzu IR-prestige 21 FTIR spectrometer.

Encapsulation degree characterization

The percentages of MTX encapsulation degree (ED) and drug loading (DL) were calculated using the spectrophotometric determination at 303 nm employing a spectrophotometer (Shimadzu, Japan). The obtained formulation was dialyzed overnight against water, using a cellulose membrane bag with a molecular weight cutoff of 12,000 Da. The measured absorbance values are used to determine drug concentration via standard calibration curve. The MTX encapsulation was calculated by using Equation (1):

$$ED\% = \left[(Wa - Ws) / Wa \right] \times 100 \quad (1)$$

where Ws and Wa are the analyzed weight of drug in the medium and weight of drug added into the system, respectively.[24]

Particle characterization

The particle size and size distribution of the as-prepared nanoconjugates were determined using dynamic light scattering (Zetasizer Nano ZS, Malvern, UK).

The physical state of MTX in the nanoconjugate carrier was investigated by using differential scanning calorimetry (DSC) experiment. Thermograms of MTX, mPEG polymer, amino acid, optimum formulation, and physical mixture were performed with a DSC-60 (Shimadzu, Kyoto, Japan). For this purpose, 5 mg of each sample was put in an aluminum pan and completely sealed. The heating rate was 10°C/min, and the heat flow was recorded from 10°C to 260°C.

In-vitro release studies

The pH-dependent drug release: The *in-vitro* release profile of the drug has been evaluated by applying the dialysis membrane method in a two-compartment Franz-type diffusion cell. Regarding sink condition, the donor compartment was filled with 1 mL of NPs suspension in phosphate-buffered saline

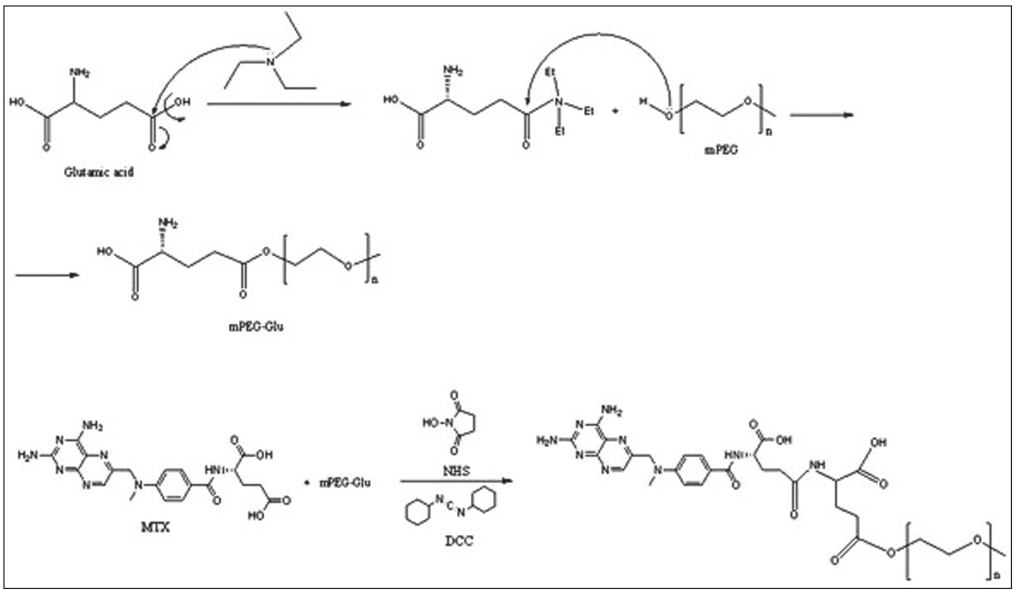

Figure 1: Synthetic routes for methoxy poly(ethylene glycol)-glutamic acid-methotrexate (mPEG-Glu-MTX) synthesis

(PBS) (0.1 M, pH 7.4). The receiver compartment was filled with 25 mL of PBS, incubated at 37°C under constant magnetic stirring. At specified time intervals, 1 mL of the receptor compartment was sampled, and the same volume of fresh PBS was replaced.[25] Afterwards, MTX concentration was measured using the spectrophotometer. The cumulative percentage of released MTX (%) was plotted as a function of time (h).

Cell culture and cytotoxicity study

In order to assess the viability of the cells, the *in-vitro* cytotoxicity of the as-prepared MTX nanoconjugate was determined by the MTT assay.[26] The ER-negative MDA-MB-453, ER-positive MCF-7 (breast cancer cells), and ER-positive gastric adenocarcinoma (AGS) were seeded in a 96-well plate (Nalge Nunc International, Rochester, NY, USA) at a density of 1.5×10^4 cells/well and incubated overnight at 37°C in 5% CO_2. Different concentrations of mPEG, Glu, free and nanoconjugate drug (1–18 µg/mL) were introduced to the grown cells and then incubated at 37°C for 24 h. Then the cells were carefully washed with PBS. Next, 20 mL of MTT dye solution was added. The medium was discarded, and formazan crystals were solubilized by stirring in DMSO. The optical density of each well was measured at 570 nm in a microplate reader (Bio-Tek, ELX 800, Winooski, VT, USA).[26]

Results

Synthesis and purification of mPEG-Glu-MTX

MTX is a chemotherapy agent and immune system suppressant by two aliphatic COOH and aromatic NH_2 groups. These groups can be used by performing some chemical modifications. Although MTX is a dicarboxylic acid, which can be esterified by the hydroxyl group of mPEG, the α-group is more active. So, in the equimolar ratio, α-carboxylic group is preferred for esterification.[21,22] In the present work, the –COOH group of MTX was activated, in a 1:1 molar ratio, using DCC and NHS for increasing the reaction efficiency. The encapsulation efficiency of 48 ± 5% was obtained for the chemical reaction of MTX coupling.

The percentage yield of the mPEG-Glu-MTX synthesis method was 32%.

The FT-IR spectrum of mPEG, mPEG-Glu, glutamic acid, and mPEG-Glu-MTX is shown in Figure 2. The mPEG-Glu-MTX esters show two strong peaks in 1100 and 2800 cm^{-1} that were not observed in the MTX spectrum. ^{1}H-NMR spectra for mPEG-Glu-MTX are illustrated in Figure 3.

Particle characterizations

The particle size and the polydispersity index (PDI) of the formulation were determined by photon correlation spectroscopy. The particle size and PDI of 86 nm and 0.12 have been obtained, respectively [Figure 4]. It was calculated by extrapolation and ranged from a value of 0.01 for monodispersed particles up to the values of 0.5–0.7.[24]

The DSC thermograms of pure drug, mPEG, glutamic acid, physical mixtures, and nanoconjugates are indicated in Figure 5. The DSC curve of glutamic acid displays an endothermic peak at around 208°C. The mPEG thermogram has conducted an endothermic peak at 55°C, which showed a good agreement with mPEG transition temperature. The DSC thermogram of the drug showed an endothermic peak corresponding to its melting point with an onset temperature of 141°C and a peak at 149°C. Such a peak is also observed in the physical mixture. However, there was no separate MTX melting endotherm in the nanoconjugate. This might be attributed to the overall amorphization of the MTX in the nanoconjugate.[27]

In-vitro drug release evaluation

Figure 6 shows the *in-vitro* release profile of MTX in the sink condition. The drug release profile has not shown a significant

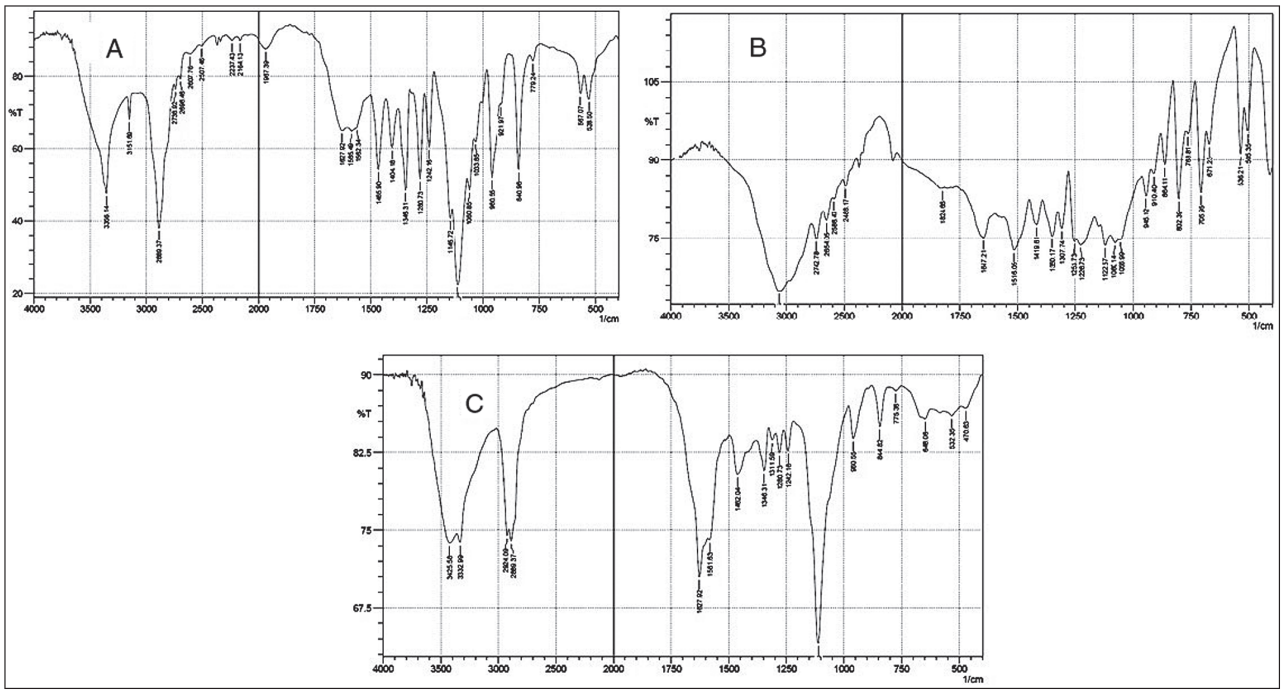

Figure 2: The infrared spectrum of mPEG-Glu-MTX nanoconjugate

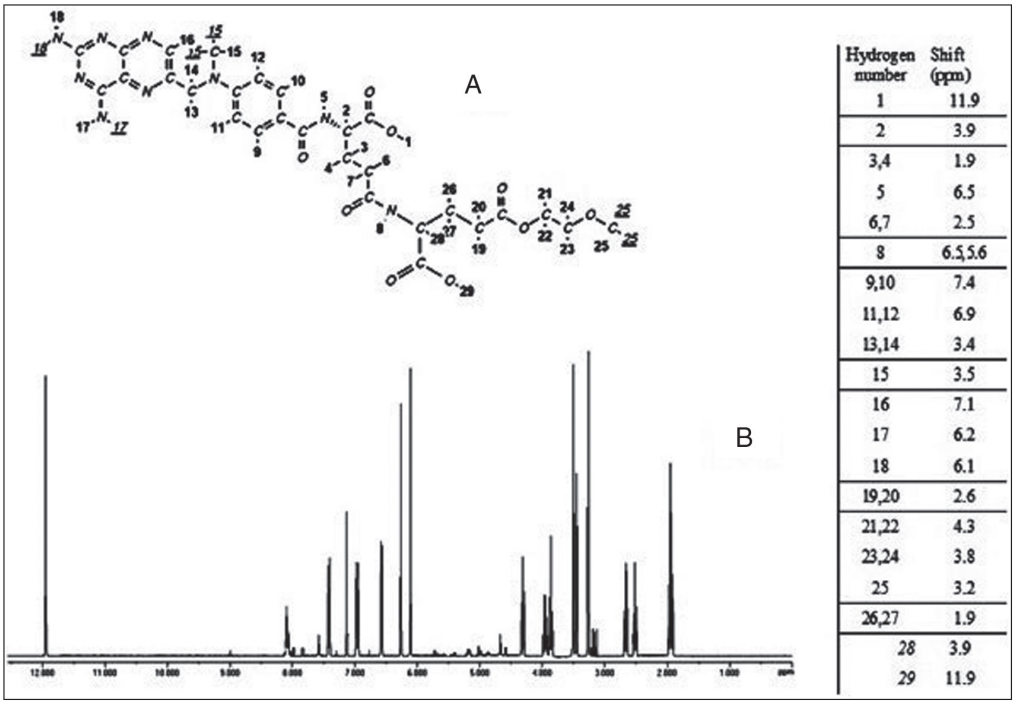

Figure 3: (A) The chemical structure of synthesized formulation and (B) the 1H-NMR spectrum of mPEG-Glu-MTX

burst effect during the first hour of release and was significantly induced at the pH of 5.5 and 3.5 compared with pH 7.4, which corresponds to the pH of late endosome. The sustained release pattern is due to the slow hydrolysis of the glutamic acid moiety of nanoconjugate, which should be exaggerated in a lower pH medium.[24] This phenomenon happened in our experiment, and a significant difference in the percent of released drug in different pH has been found ($P < 0.05$).

In-vitro cytotoxicity

The cytotoxic activity of mPEG-Glu-MTX was studied using the MTT assay on the MCF-7, AGS, and MDA-MB-453 cell lines after 24 h of exposure to various concentrations of loaded MTX. The IC_{50} value is calculated from the dose–response curve. From Figure 7, the results indicated that none of the glutamic acid and m-PEG showed any cytotoxic effect on cell lines within the measured concentrations.

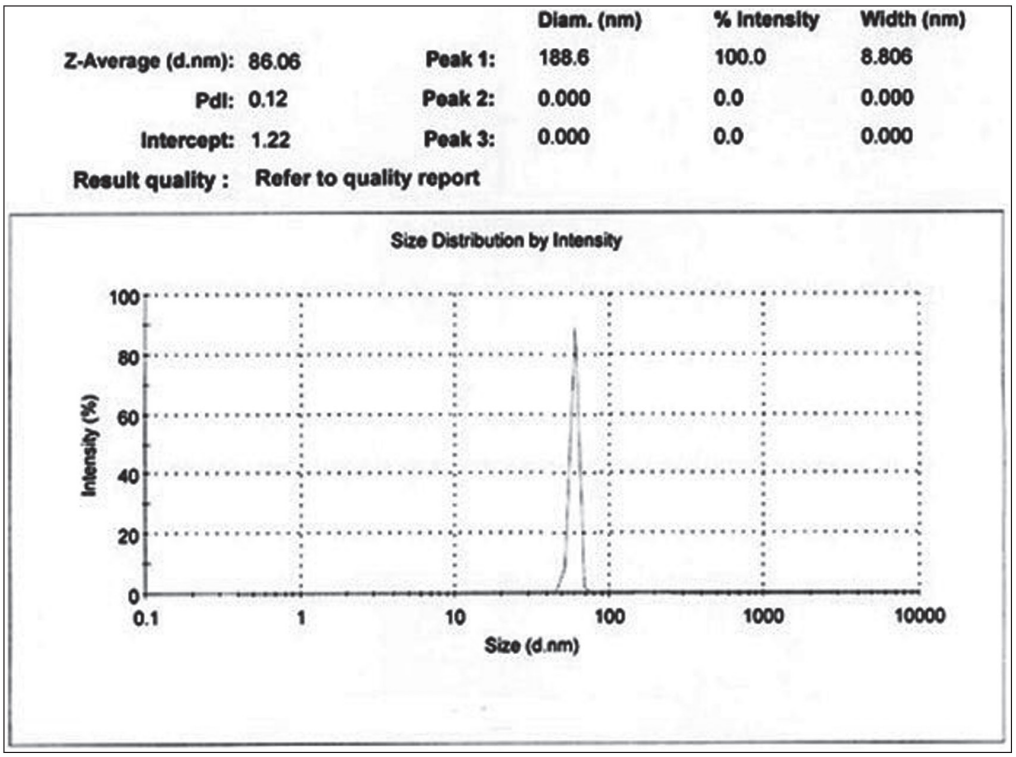

Figure 4: Particle diameter and size distribution of mPEG-Glu-MTX nanoconjugate

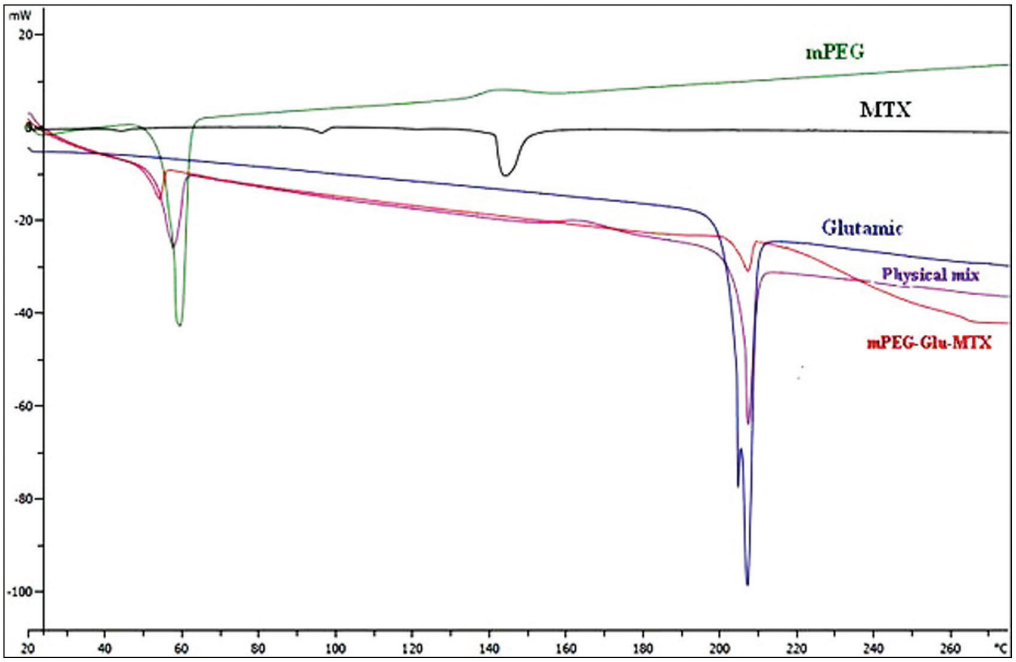

Figure 5: Differential scanning calorimetry thermograms of methotrexate, methoxy poly(ethylene glycol), glutamic acid, physical mixture of them, and nanoconjugate

The average IC_{50} values of MTX in nanoconjugates for MCF7, AGS, and MDA-MB-453 cells were 8.66, 14.52, and 15.04 µg/mL and those of free drug formulation were 7.62, 15.32, and 11.17 µg/mL, respectively. Based on this finding, there is no significant difference between nanoconjugate and free drug (P<0.05). It was shown that conjugated ligand has no destructive effect on MTX cytotoxicity on the cells after 24 h.[24,26]

Discussion

One of the main hypotheses of this work was to evaluate the cytotoxic effects of MTX in nanoconjugate formulation with PEG and glutamic acid.[28-30]

The pH-responsive nanoparticles with structural changes or solubility in tumor extracellular pH or lysosomal pH are

promising carriers for cancer drug delivery. An altered pH gradient across tumor cell compartments could trigger the release of the carried drugs in these nanoparticles. The glutamic acid moieties at the interface of prepared nanoparticles are the cause of pH responsibility. Also, the release of MTX in low pHs is higher than that of pH 7.4. The acidic environment inside the tumor environment resulting from the active metabolism of these cells is a favorable position for targeted drug delivery.[6,7]

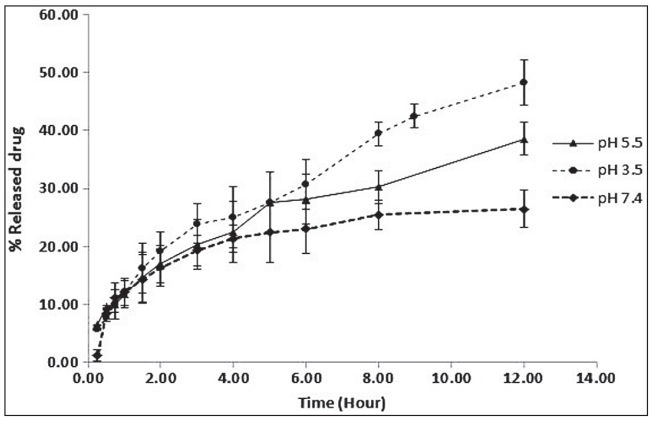

Figure 6: *In-vitro* release profile of methotrexate from nanoconjugate in phosphate-buffered saline and different pH

MTX has good prospects in clinical applications regarding its high impact on inhibiting the growth of cancer cells. Nevertheless, having a non-selective cytotoxic effect, low solubility, low bioavailability, and side effects are the treatment limitations with this drug.[27] To date, much research has been done on new drug delivery systems to trap or conjugate MTX to increase drug exposure time at the site of action, reduce drug entry into normal cells, and improve pharmacokinetic parameters and clinical efficacy. Several studies have investigated PEG-conjugated cytotoxic drugs and could overcome the problems of MTX in commercialized delivery systems.[2,29]

In the present study, we synthesized mPEG-Glu-MTX in two steps. In the first step, mPEG-Glu was conjugated and then MTX was attached to this conjugate via an esterification reaction of COOH groups of glutamic acid with the hydroxyl group of PEG. In the second step, the MTX conjugated to PEG-Glu was synthesized via an amide bond by carbodiimide activation.

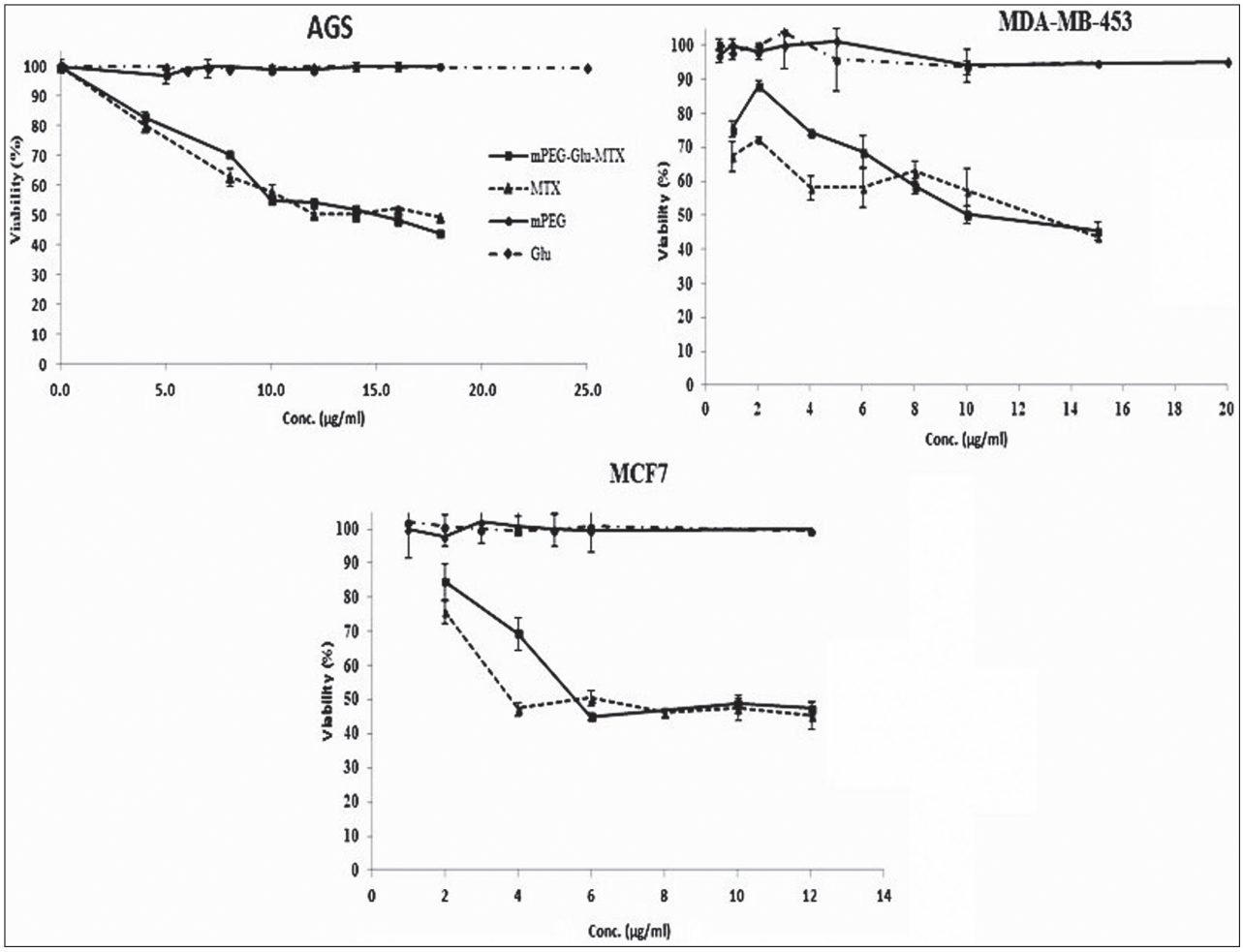

Figure 7: Cell viability of MCF-7, MDA-MB-453, and AGS cell lines after treatment with different concentrations of free methotrexate and nanoconjugate (mean ± standard error of the mean [SEM])

Table 1: IC_{50} cytotoxicity of MTX in different formulations and cell lines

Formulation / cell lines	AGS	MCF7	MDA-MB-453
Free MTX	15.32±2.24	7.62±2.06	11.17±2.75
mPEG-Glu-MTX	14.52±0.91	8.65±1.59	15.04±2.45

Values show the average result from three experiments (mean ± SD)

The result indicated that amidation with DCC and NHS is a proper strategy for the preparation of mPEG-Glu-MTX. Pre-activation of MTX by DCC is a simple and efficient method to increase reaction efficiency concerning ^{1}H-NMR confirmation. The cytotoxicity of MTX was investigated in three cancer cell lines. We observed *in vitro* that mPEG-Glu-MTX had the same effect as MTX on the cellular cytotoxicity in MCF-7 and AGS cell lines [Table 1]. However, the advantages of mPEG-Glu-MTX over MTX (as a targeted drug delivery) have been demonstrated through *in-vivo* studies.

Leng *et al*.[28] investigated poly(amidoamine) dendrimer-conjugated chitosan NPs for MTX delivery. This group showed that the *in-vitro* cytotoxicity of the prepared formulation was higher than the free form of the drug. The IC_{50} value of 0.8 mg/mL for MTX-CS-PAMAM is lower than that for MTX (~4.54 mg/mL).

In another experiment, MTX was coupled to human serum albumin (MTX-HSA). The results showed that effective drug uptake occurs through the HSA-mediated endocytosis process in the DU-145 cell line.[7]

Also, the radiolabeled concentration of MTX-HSA in the tumor tissue of Waxer-256 mice showed that MTX-HSA was accumulated in the tumor for a longer time.

MTX-HAS conjugate is an effective anti-tumor agent even in MTX-resistant tumors.[7]

Paclitaxel poliglumex (PPX) is a large macromolecular conjugate of paclitaxel and poly-L-glutamic acid, which is designed to increase the therapeutic index and efficiency of paclitaxel.

Animal tumor models have shown the higher effectiveness of PPX than the free drug. Moreover, it is associated with prolonged exposure to the drug and cells and reduction of side effects.[31]

Cisplatin combined with γ-poly α,l-glutamic acid (γ-PGA) is a water-soluble drug. γ-PGA-cisplatin conjugate, which was xenografted into nude mice, effectively inhibited human breast tumor cells. In addition to reducing the side effects of the free cisplatin, it improves the drug's pharmacokinetics and its anti-tumor effects.[32]

Camptothecins (CPTs) have the instability of active lactone, the therapeutic efficacy of which is limited in humans.[31,32] Binding of CPT to a high molecular weight anionic polymer such as poly(L-glutamic acid) increases the solubility, improves drug distribution in the tumor, and increases the permeability and retention of the drug in tumor cells (EPR effect).[33,34]

Overall, reports are in good agreement with our findings and explain the importance of glutamic acid nanoconjugate as a new drug delivery system.

Conclusion

This study designed and synthesized a new nanoconjugate platform to deliver the effective anticancer drug MTX.

The particle size of mPEG-Glu-MTX was about 160–190 nm and in spherical morphology. The experimental data indicated that nanoconjugate has about 48% loading value of the drug, and ^{1}H-NMR and FTIR confirmed its structure. Due to the increased release of the drug in an acidic environment, we can conclude the successful design of a pH-responsive drug delivery.

The results of cytotoxicity evaluation showed that MTX loaded in the modified nanoconjugates has a significant effect on the cellular cytotoxicity similar to free drug.

Based on the present results, it can be concluded that MTX nanoconjugate is a potential candidate for targeted drug delivery.

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Nil.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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