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Methotrexate-Incorporated Polymeric Micelles Composed of Methoxy Poly(ethylene glycol)-Grafted Chitosan

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Abstract: In this study, methotrexate (MTX)-encapsulated polymeric micelles using methoxy poly(ethylene glycol) (MPEG)-grafted chitosan (ChitoPEG) copolymer were prepared. The MTX-incorporated polymeric micelles of ChitoPEG copolymer has a particle size of around 50-100 nm. In 1H nuclear magnetic resonance (NMR) study, the specific peaks of MTX disappeared in heavy water (D_2O) and only the specific peak of MPEG was observed, while all of the peaks were confirmed in dimethyl sulfoxide (DMSO). These results indicated that MTX was complexed with chitosan and then formed an ion complex inner-core of the polymeric micelle in an aqueous environment. The drug contents of the polymeric micelle were around 4~12% and the loading efficiency of MTX in the polymeric micelles was higher than 60% (w/w) for all of the formulations. The cytotoxicity of MTX and MTX-incorporated polymeric micelle against CT26 tumor cells was not significantly changed.

Keywords: methotrexate, polymeric micelle, chitosan, ion complex, MPEG

Introduction

Chitosan, a natural polysaccharide derived from chitin, is regarded as biocompatible, biodegradable, non-toxic and polycationic polymer. Chitosan has been extensively investigated in the drug delivery, gene delivery, and biomedical area due to its amino functional groups.[1-4] It has been reported that chitosan enhance drug delivery across the mucosal layer without damage.[5] Furthermore, cationic characteristics may offer valuable properties for drug delivery systems and gene delivery systems, i.e. ion complex or polyelectrolyte formation between chitosan and anionic drug or DNA can be formed and these intrinsic properties are amplifying the value of chitosan.[6-8]

Core-shell type polymeric micelles have been extensively investigated for drug delivery application.[9-12] Since polymeric micelles are composed of hydrophobic inner-core and hydrophilic outershell, it has several advantages for the drug delivery application as follows: hydrophobic cores as a microreservoir of hydrophobic drugs, hydrophilic outershell as a defense layer against attack of the reticuloendothelial system (RES), long blood circulation of drug, and passive targeting against tumor.[9-12] Thunemann *et al.*[12] have reported that ion complex between poly(ethylene oxide)-*b*-poly(*L*-lysine) and all-trans retinoic acid was formed and they showed formation of core-shell type polymeric micelles.

Methotrexate is widely used in the treatment of various malignancies including childhood acute lymphocytic leukemia, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, breast cancer, psoriasis, choriocarcinoma and related trophoblastic tumors.[13] However, undesirable side-effects of MTX has been reported such as toxic side-effects to normal cells, drug resistance, nephrotoxicity, bone marrow suppression, acute and chronic hepatotoxicity, interstitial pneumonitis and chronic interstitial obstructive pulmonary disease.[14] Various delivery systems for antitumor therapy using MTX were proposed to solve this problem. MTX-conjugated block copolymers composed of PEG/poly(2-hydroxyethyl L-aspartamide) formed polymeric micelles for sustained release of MTX[15] and pH-sensitivity of MTX-conjugated polymeric micelles.[16] Kang *et al.*[17] reported that MTX was entrapped into self-aggregates of poly(2-hydroxyethyl aspartamide) by physical entrapment and chemical conjugation. ABA triblock copolymer were also proposed to entrap MTX and its loading efficiency was 7~30% (w/w) according to the copolymer.[18] Conjugates of MTX/dextran showed antitumor effect in accordance with the molecular weight of dextran.[19] Yang *et al.*[20] reported self-aggregated nanoparticles of MPEG-modified chitosan and encapsulated MTX for controlled release. In their report,

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loading efficiency were varied about 20~95% (w/w) according to the copolymer composition and nanoparticle/drug weight ratio.

In this study, we prepared MTX-incorporated polymeric micelle using MPEG-grafted chitosan (abbreviated as ChitoPEG) to develop an antitumor drug delivery system. Since both MTX and chitosan has ionic properties in aqueous solution, polyion complex formation between MTX and ChitoPEG copolymer are expected and this can form core-shell type polymeric micelle. We previously approved that ChitoPEG copolymer can form polymeric micelles or core-shell type nanoparticles through ion-complex formation with negatively charged drugs.[8,11] MTX-incorporated polymeric micelle of ChitoPEG copolymer was evaluated physicochemical properties using dynamic light scattering (DLS), ^{1}H NMR and TEM. Antitumor activity was evaluated using tumor cells at *in vitro*.

Experimental

Materials. Water-soluble chitosan (Molecular weight (M.W.) =10,000 Da, deacetylation degree = 97.0%) was a gift from Chittolife Co. Ltd. Korea. Methoxy poly(ethylene glycol)-*N* hydroxysuccimide (MPEG-NHS) (M.W. = 2,000 g/mol) was purchased from SunBio Co. Korea. Methotrexate (MTX), 4-dicyclohexylcarbodimide (DCC), and *N*-hydroxysuccimide (NHS) were purchased from Sigma Co. Ltd., USA. Dimethylsulfoxide (DMSO) and dichloromethane (DCM) as extra-pure grade were purchased from Aldrich Chemical Co. Ltd., USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DMSO (D-form for NMR study), and deuterium oxide (D_2O) were purchased from Sigma Co., USA. Dulbecco's modified-Minimum Eagle's medium (DMEM) was purchased from GIBCO (Invitrogen Co., USA). The Dialysis tube (molecular weight cut-off (MWCO) = 3,500 and 12,000 g/mol, respectively.) was purchased from SpectraPor Co. Ltd., USA. The Dialysis tube was treated with hot water (100 °C) for 30 min and then washed with tapped water for 2 h before use.

Synthesis of MPEG-*g*-Chitosan (ChitoPEG) Copolymer. ChitoPEG graft copolymer was synthesized as reported previously.[11] Briefly, 100 mg of chitosan was dissolved in 0.2 ml of deionized water and diluted with 9.8 mL of DMSO. To this solution, calculated amount of MPEG-NHS dissolved in 2 mL of DMSO was added and reacted for overnight at nitrogen atmosphere. After that, the resulting solution was dialyzed using dialysis tube against a plenty of deionized water for 2 days followed by its lyophilization. Generally, chitosan is not soluble in DCM while MPEG is freely soluble in it. Therefore, unreacted MPEG-NHS was removed by suspending lyophilized solid into a plenty of DCM for three times. After that, ChitoPEG copolymer was fractionated into deionized water followed by its lyophilization.

Measurement Using Gel Permeation Chromatography (GPC). The absolute molecular weight (M.W.) and M.W. distribution, represented as the polydispersity index (PD), of the ChitoPEG copolymers were measured by using a gel permeation chromatograph equipped with a multi-angle laser light scattering detector (GPC-MALLS, 18 angle detector, Wyatte, USA), as reported previously.[11] The samples were dissolved in 0.5 M ammonium acetate (pH 5.5, at more than 5 different concentrations ranging from 0~1.0 mg/mL), and the increments in the reflective index (*dn/dc*) were measured by means of a OPTILAB-DSP reflectometer (Wyatt, USA). Then, the absolute M.W. and M.W. distribution of the ChitoPEG copolymer were obtained from the GPC chromatogram with light scattering data (Debye plot regressions). The mobile phase was 0.5 M ammonium acetate buffer (pH 5.5), and the flow rate was 0.5 mL/min. The injection volume was 0.2 mL (10 mg/mL).

Preparation of MTX-Incorporated Polymeric Micelles. MTX-incorporated polymeric micelles of ChitoPEG copolymer were prepared as follows: 100 mg of ChitoPEG-2 was dissolved in 10 mL of deionized water. 5~20 mg of MTX dissolved in 0.1 mL of DMSO was dropped into this solution with mild magnetic stirring at 25 °C for 30 min. After that, the resulting solution was dialyzed against deionized water for 9 h. During dialysis procedure, deionized water was exchanged for every 2 h to remove organic solvent. After that, resulting solution was analyzed or lyophilized for 2 days.

For the measurement of drug contents and loading efficiency, 5 mg of MTX-incorporated polymeric micelles was distributed into 0.5 mL of water and 9.5 mL of DMSO was added. The contents of MTX were estimated using UV spectrophotometer (303 nm, UV-1601, Shimadzu Co., Ltd., Japan). An equivalent amount of ChitoPEG copolymer was used as a blank test. All procedures were performed at dark condition and repeated for three times.

$$\text{Drug contents} = \frac{\text{Amount of MTX in the polymeric micelles}}{\text{Weight of polymeric micelles}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of MTX in the polymeric micelles}}{\text{Feeding amount of MTX}} \times 100$$

^{1}H Nuclear Magnetic Resonance Spectrometry (NMR) Measurements. The ^{1}H NMR spectra of the copolymer and polymeric micelles were measured in D_2O or DMSO (*d*-form) using a 400 MHz NMR spectrometer (AVANCE 400FT-NMR 400 MHz, Bruker).

Measurement of Particle Size. The particle size and the zeta potential of the polymeric micelles were measured with an ELS-8000 electrophoretic LS spectrophotometer (NICOMP 380 ZLS zetapotential/particle sizer, Otsuka electronics Inc., Japan) equipped with a He-Ne laser beam at a wavelength

of 632.8 nm at 25 °C (scattering angle of 90°). To analyze particle size, lyophilized MTX-incorporated polymeric micelles of ChitoPEG copolymer was reconstituted into deionized water. This sample solution prepared was used for the particle size measurement (concentration: 1.0 mg/mL).

TEM Observation. For the TEM observations, a drop of polyion complex micelle suspension was placed onto a carbon film coated on a copper grid. The observation was done using a JEOL JEM-2000 FX at 80 kV.

Cytotoxicity of MTX-Incorporated Polymeric Micelle Against Tumor Cell. To evaluate antitumor effect, CT26 colon carcinoma cells were maintained at 5% CO_2 incubator at 37 °C. The effect of MTX-incorporated polymeric micelle on the tumor cell proliferation was determined using an MTT assay. MTX dissolved in 100% DMSO was diluted 100 times using DMEM (supplemented with 10% serum) and then this solution was added to tumor cells. MTX-incorporated polymeric micelles was reconstituted in DMEM (supplemented with 10% serum) and diluted to adjust the equivalent concentration of the free MTX. The tumor cells were seeded at a density of 5×10^3 per well in 96-well plates using 100 μL of DMEM supplemented with 10% serum in a CO_2 incubator (5% CO_2 at 37 °C) for 12 h. After that, 100 μL of DMEM (supplemented with 10% serum) containing free MTX or MTX-incorporated MTX was added. In the case of free MTX, the final DMSO concentration was 0.5% (v/v). After 1 day of incubation, MTT was added to the 96 wells and incubated for 4 h in a CO_2 incubator (5% CO_2 at 37 °C). After that, the supernatant was discarded and 100 μL of DMSO was added to the 96 wells. The absorbance was measured at 560 nm using a microtiter plate reader (Thermomax microplate reader, Molecular Devices).

Results and Discussion

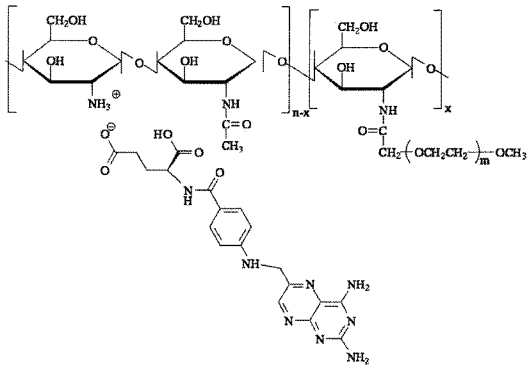
We previously showed that ionic complexes can be formed between anionic drug and chitosan, and then polymeric micelles were prepared by simple mixing of drug and chitosan copolymer.[11] Polymeric micelles have been extensively investigated in drug targeting objectives due to their potential as nano-carriers.[10] Recently, polymeric micelle can be formed through ion complex formation between ionic drug and ionic block copolymer. This types of polymeric micelles are considered as a promising anticancer drug delivery system.[11,12,21] At structure of polyion complex micelles, inner-core is composed of ion complexes between ionic domain of block copolymer and ionic drug, and the outershell is composed of non-ionic hydrophilic domains such as MPEG.

We used the graft copolymer composed of chitosan backbone and MPEG side chains to make the MTX-encapsulated core-shell type polyion complex micelles. It is expected that polycationic backbone, chitosan, and MTX may composed core of the micelle while non-ionic polymer, MPEG, compose hydrophilic outershell.

Table I. Characterization of ChitoPEG Copolymer

	M. W. by GPC			DS of MPEG
	M_n	M_w	Polydispersity	
ChitoPEG-1	14,300	17,000	1.19 ± 0.294	2.5
ChitoPEG-2	19,600	25,000	1.27 ± 0.685	5.15
ChitoPEG-3	32,300	44,600	1.38 ± 0.265	11.5

*M_n, M_w, and polydispersity of LMWSC was 9,300, 12,200, and 1.31 ± 0.918, respectively.
*Degree of substitution (DS) of MPEG=[(M_n of ChitoPEG copolymer – M_n of chitosan)/2,000]×100



Figue 1. Scheme of polymeric micelle formation between MTX and ChitoPEG copolymer.

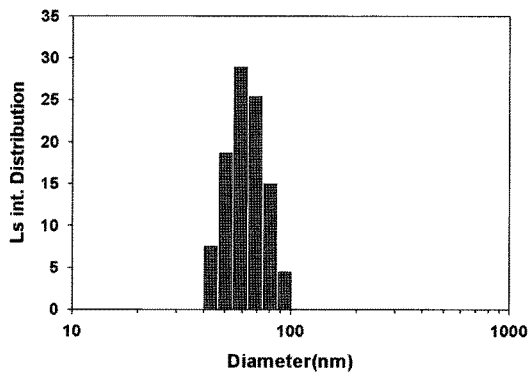


Figure 2. Typical particle size distribution of MTX-incorporated polymeric micelle of ChitoPEG-2 copolymer.

Characterization of ChitoPEG Copolymers and Its Polymeric Micelles. The ChitoPEG copolymer was synthesized as previously reported[11] and the characteristics of copolymer was summarized in Table I. ChitoPEG copolymer is composed of a non-ionic hydrophilic MPEG and a cationic chitosan as shown in Figure 1. The DS of MPEG and the molecular weight of ChitoPEG copolymer were evaluated by GPC measurements. The estimated M.W. of Chi-

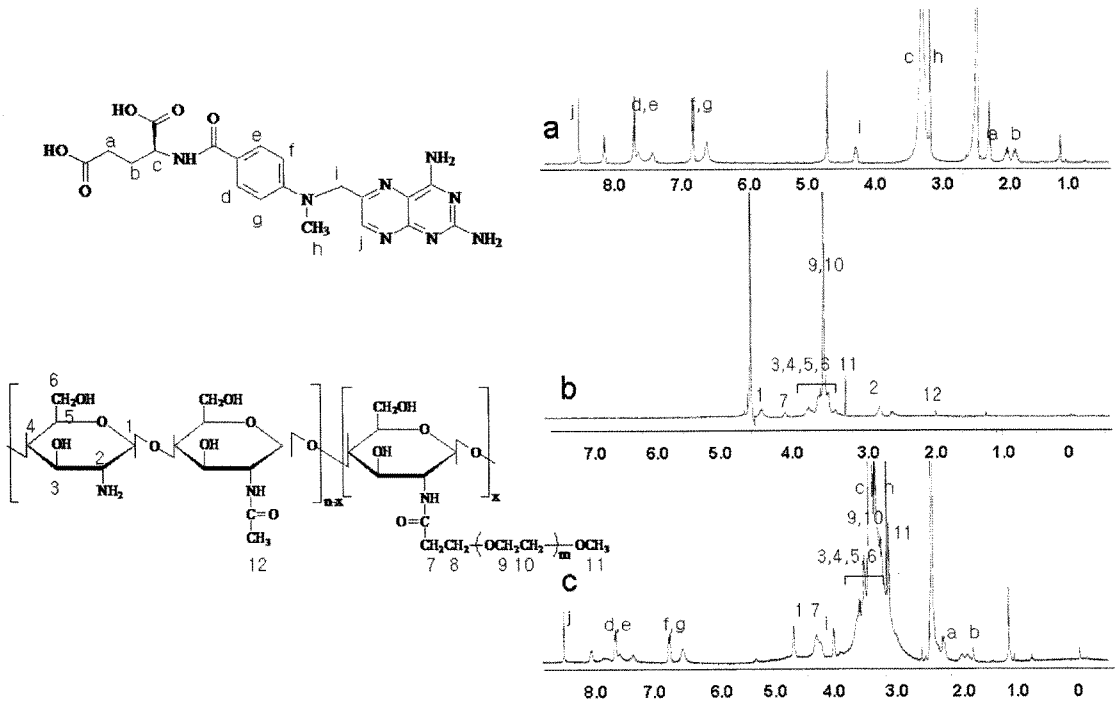


Figure 3. ^{1}H NMR spectra of MTX-incorporated polymeric micelles of ChitoPEG-2 copolymer (ChitoPEG-2/MTX weight ratio=100/10). MTX in DMSO (a); Polymeric micelles in D_2O (b); polymeric micelles in DMSO (c).

toPEG copolymer was summarized in Table I. The feeding ratio of MPEG was increased to synthesize higher DS of MPEG of ChitoPEG and then DS of MPEG was varied between 2.5 and 11.5.

Figure 2 shows the typical particle size distribution of MTX-incorporated polymeric micelles of ChitoPEG copolymer. Size distribution of MTX-incorporated polymeric micelles was narrowly distributed and the average particle size was below 100 nm. MTX-incoporated polymeric micelles has spherical shapes at morphological observations (data not shown). This result indicated that ion complex between MTX and ChitoPEG copolymer formed nano-aggregates with size lower than 100 nm.

Figure 3 showed ^{1}H NMR spectra of MTX-incorporated polymeric micelles of ChitoPEG copolymer. As shown in Figure 3(a), free MTX showed intrinsic specific peaks at 1.0~8.5 ppm. Specific peaks of glucosamine ring (C1~C6) of LMWSC were confirmed at 3.5~4.0 ppm even if their peaks were hardly classified due to the overlapping with the peaks of ethylene group of MPEG. When polymeric micelles were dissolved in D_2O, specific peaks of MTX were disappeared as shown in Figure 3(b) while its specific peaks were appeared again at DMSO (Figure 3(c)), indicating that core-shell structure has been formed by ionic complex between chitosan and MTX. These results showed that ChitoPEG copolymer is able to fabricate as polymeric micelle by using ion complex formation between chitosan main chain and anionic drug.

Table II. Characterization of MTX-Incorporated Polymeric Micelle of ChitoPEG Copolymer

	ChitoPEG/MTX Weight Ratio (mg/mg)	Drug Contents (%, w/w)	Loading Efficiency (%, w/w)
ChitoPEG-1	100/10	8.2	89.0
ChitoPEG-2	100/5	4.4	92.0
	100/10	7.5	81.0
	100/20	12.4	71.0
ChitoPEG-3	100/10	6.0	64.0

Drug contents and loading efficiency of MTX aginst polymeric micelles was summarized in Table II. Drug contents was 4~12% (w/w). At all formulations, loading efficiency was higher than 60%. The higher the drug feeding, the higher the drug contents, and the lower the loading efficiency. The drug contents and loading efficiency was decreased when DS of MPEG was increased. Particle size of MTX-incorporated polymeric micelles was increased according to the increased drug contents as shown in Figure 4. Furthermore, zeta potential was decreased according to the increased drug

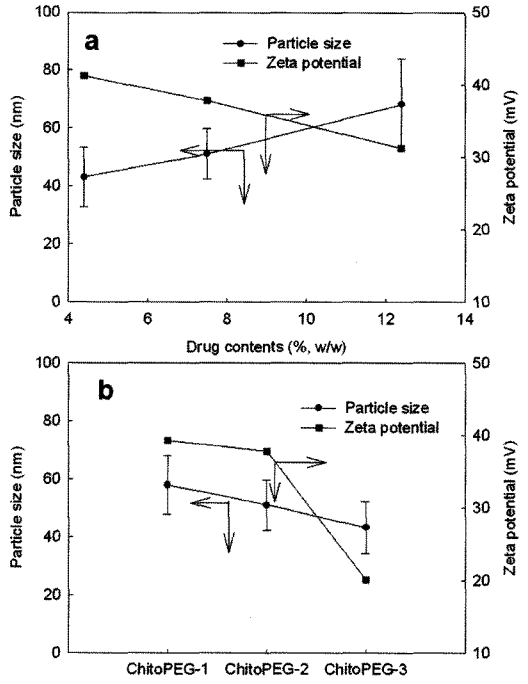


Figure 4. The changes of particle size and zeta potential of MTX-incorporated polymeric micelles. The effect of drug contents (ChitoPEG-2 copolymer) (a) and series of copolymers (b).

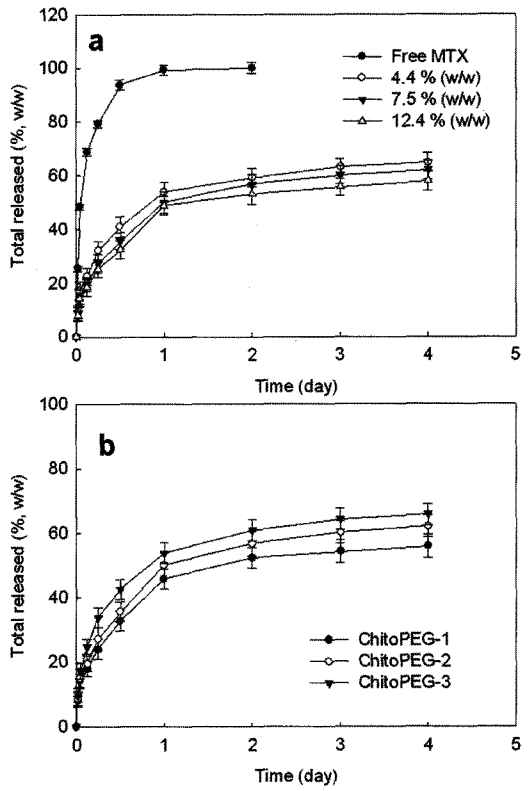


Figure 5. Drug release from polymeric micelle of ChitoPEG-2 copolymer. The effect of drug contents (a) and M.W. of ChitoPEG copolymer (b).

contents as shown in Figure 4. As shown in Figure 4, zeta potential of polymeric micelles showed still positive value even if their value was decreased. The reason of these results may be due to the strong positive charge of LMWSC and polymeric micelle showed positively charged even after ion complex formation. Furthermore, zeta potential was significantly decreased from ChitoPEG-2 to ChitoPEG-3. These results may be due to the higher content of PEG of ChitoPEG-3 copolymer than that of ChitoPEG-2 copolymer. And the other factor might be decreased particle size of ChitoPEG-3, i.e. decreased particle size might be induced that positively charged chitosan chain was less exposed on the particle surface. These results indicated that MTX was incorporated into the polymeric micelles of ChitoPEG copolymer and chitosan main chain was complexed with MTX, i.e. the increased complexation between MTX and chitosan resulted in decreased zeta potential. Furthermore, it suggested that ion complex formation between chitosan and MTX is a main driving force for the formation of polymeric micelle.

Figure 5 shows the release of MTX from polymeric micelles of ChitoPEG copolymer. As shown in Figure 5, MTX release was decreased according to the decrease of drug contents and the higher DS of MPEG induced increased drug release rate from the polymeric micelles. These results indicated that polymeric micelles of ChitoPEG copolymer can be considered as a controlled release vehicles of MTX.

Cyrtotoxicity of MTX-Incorporated Polymeric Micelles of ChitoPEG Copolymer. Figure 6 showed cytotoxicity of MTX-incorporated polymeric micelles of ChitoPEG copolymer against CT26 tumor cells. As shown in Figure 6, free MTX was observed dose-dependent cytotoxicity against CT26 tumor cells. Polymeric micelles showed slightly lower cytotoxicity compared to MTX itself. The reason of these results might be due to that, in the case of polymeric micelles, delayed liberation of MTX from polymeric micelles can be affected to the lower cytotoxicity against tumor cells. Since MPEG is exposed on the particle surface in aqueous environment as shown in Figure 3, stealth properties of polymeric micelle may delayed the uptake of particles into the cells and much of drug-incorporated particles may remained in the media. These facts might be one of the reason for the lower cytotoxicity of polymeric micelle. As shown in Figure 6, ChitoPEG copolymer itself did not affected to the survivability of tumor cells.

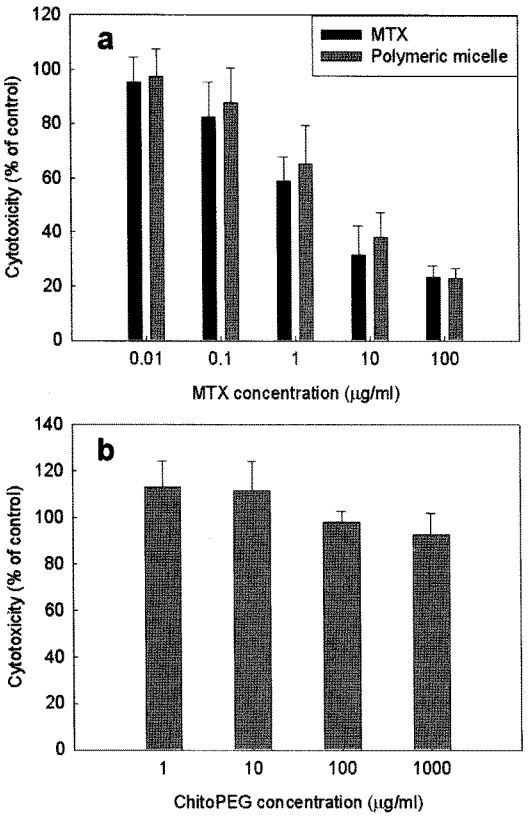


Figure 6. Cytotoxicity of MTX-incorporated polymeric nanoparticles (ChitoPEG-2 copolymer, MTX contents: 14.0 % (w/w)) against CT26 cells.

Conclusions

MTX-encapsulated polymeric micelles using ChitoPEG copolymer were prepared. MTX-incorporated polymeric micelles of ChitoPEG copolymer has around 50-100 nm in particle size. At ^1H nuclear magnetic resonance (NMR) study, the specific peaks of MTX were disappeared at D_2O and only the specific peak of MPEG was observed, while all of the peaks were confirmed at DMSO. These results indicated that MTX was complexed with chitosan and then formed ion complex inner-core of the polymeric micelle at aqueous environment. Drug contents of the polymeric micelle were around 4~12% and loading efficiency of MTX in the polymeric micells was higher than 60% (w/w) at all of the formulation. Cytotoxicity of MTX and MTX-incorporated polymeric micelle against CT26 tumor cells was not significantly changed.

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