Influence of conjugating folic acid and methotrexate on physicochemical properties of P(CL)-mPEG nanoparticles

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ABSTRACT

The objective of this study was to investigate an effect of conjugating folic acid (FOL) as a targeting ligand and conjugating methotrexate (MTX) as an anticancer drug on the formation of nanoparticles (NPs). MTX-conjugated nanoparticles were prepared using 10, 20 and 30 % MTX conjugated poly(ε-caprolactone)-co-methoxy poly(ethylene glycol) (P(MTXCLCL)-mPEG) copolymers by dialysis method. Additionally, the targeted nanoparticles were obtained by the physical adsorption of FOL-conjugated (P(FOLCL)-mPEG) copolymer on the surface of P(MTXCLCL)-mPEG nanoparticles. The particle size of P(MTXCLCL)-mPEG nanoparticles increased with an amount of conjugating MTX. After the adsorption of P(FOLCL)-mPEG, the nanoparticles became larger and the zeta potential turned to more negative by approximately two times. All nanoparticles were spherical in shape and uniform size distribution. The % yield of nanoparticles ranged between 52-58%. The release profile showed higher release of MTX in acidic pH medium.

Keywords: methotrexate, folic acid, targeted nanoparticles, poly(ε -caprolactone)-co-methoxy poly(ethylene glycol)

1 INTRODUCTION

Generally, various nanoparticulate carriers have been exploited in drug delivery research including micelles, dendrimers, liposomes, polymeric nanoparticles and solid lipid nanoparticles. Among these carriers, the polymeric nanoparticles have gained much attention as a promising delivery system because they possess several essential advantages for drug delivery system such as sub-cellular size, biocompatibility with tissues and cells, and sustained- and controlled-release properties [1-2]. Nowadays, the polymeric nanoparticles are preferably developed from biodegradable materials which are non-toxic and removable from the body. In addition, the surface properties of polymeric nanoparticles can be modified with targeting molecules for chemotherapy. These nanoparticles exhibit the versatility for targeting delivery of small drug molecules as well as macromolecular therapeutic agents to the tissues of interest.

In general, the drugs are encapsulated in the polymeric nanoparticles by physical interaction; however, the initial burst release and uncertain amount of drug payload are frequently encountered. Hence, the polymer-drug conjugates is introduced to overcome these problems. The nanoparticles made of polymer-drug conjugates have received much attention in drug delivery due to their advantages such as increasing drug stability and improving pharmacokinetics of drugs, leading to diminish toxicity and multi-drug resistance [3-4].As approved by U.S. FDA, P(CL) have been increasingly investigated as biomaterials for pharmaceutical and biomedical applications. To increase the use of P(CL) in biomedical application, poly(ethylene glycol) (PEG) has been employed to copolymerize with P(CL) resulting in amphiphilic copolymer or PEGylated P(CL). In the previous report, small bioactive molecules could be successfully conjugated onto the P(CL) backbone at various amounts by click reaction. Therefore, it is of our interest to further investigate the conjugation of cytotoxic drugs and targeting ligands on the PEGylated P(CL) copolymers for chemotherapy.

In our previous study, P(CL)-methoxy-PEG or P(CL)-mPEG were synthesized and conjugated with methotrexate (MTX) (P(MTXCLCL)-mPEG) and folic acid (FOL) (P(FOLCL)-mPEG) as anticancer drug and targeing moiety, respectively [5]. MTX was conjugated at 10, 20 and 30 % mole fraction whereas FOL was fixed at 5 mole per polymer chain. Therefore in this study, the targetd and non-targeted NPs using these conjugated copolymer are prepared. The characteristics of NPs were charcterized in term of particle size, size distribution, surface charge, % yield, morphology and release characteristics.

2 MATERIALS AND METHOD

2.1 Materials

MTX- and FOL-conjugated P(CL)-mPEG copolymers defined as P(MTXCLCL)-mPEG and P(FOLCL)-mPEG, respectively, were synthesized according to our previous report [5]. MTX was obtained from Suzhou Rovathin, Pharmatech Co., Ltd., China. FOL was purchased from Fluka (Steinhheim, Germany). All solvents of analytical grade. Acetoniltrile (ACN), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were purchased from RCI Labscan Ltd., Bangkok, Thailand.

2.2 Preparation of MTX-conjugated NPs

Briefly, MTX-conjugated copolymers were dissolved in DMF. Then, the polymeric solution was dropped into deionized water as an aqueous phase. The mixture was equilibrated by magnetically stirring and then transferred into a dialysis bag (MWCO 6000-8000 Da, Boron GmbH, Ludwigshafen, Germany). The colloidal dispersion was dialyzed against deionized water to remove an organic solvent. The dialysis medium was changed every 3 h. The NPs were kept as a dispersion form for further analysis.

2.3 Preparation of FOL-decorated MTX-conjugated NPs

To prepare the targeted NPs, P(MTXCLCL)-mPEG NPs were decorated with P(FOLCL)-mPEG copolymer. The colloidal suspension of P(MTXCLCL)-PEG NPs was initially formed by slowly dropping the polymeric solution into an aqueous phase. Subsequently, a solution of P(FOLCL)-mPEG in DMSO was added to the dispersion and magnetically stirred for 2 h to allow the adsorption of P(FOLCL)-mPEG onto the surface of nanoparticles. Then, the rest procedure was similarly performed as mentioned in section 2.2.

2.4 Characterization of NPs

2.4.1 Particle size and size distribution

The particle size (z-ave) and polydispersity index (PI) were determined by ZetasizerNanoZS (Malvern Instrument, Malvern, UK) withHe-Ne laser at a wavelength of 633 nm, 25 °C. The intensity of the scattered light was detected with a photomultiplier at a scattering angle of 173°. Each experimental was performed in triplicate.

2.4.2 Surface charge

The surface charge of NPs was determined by electrophoretic mobility of particles and calculated by the Helmholtz-Smoluchowsky equation. The measurements were conducted by ZetasizerNanoZS. The sample was diluted with deionized water to yield a conductivity of 50 µS/cm. Each experimental was repeated five times at 25 °C with a field strength of 20 V/cm.

2.4.3 Particle morphology

The morphological examination of NPs was conducted using scanning electron microscope equipped with field emission cathode using 15 kV upper detector(Hitachi S4500, Hitachi, Tokyo, Japan). The sample was dried and sputtered with gold before observation.

2.4.4 Yield of NPs

The yield of NPs was calculated according to Eq. (1). Briefly, the collected NPs after dialysis were transferred into 5 mL vial and then pre-frozen overnight. The frozen NPs were sequentially lyophilized for at least 24 h. Then, an actual weight of lyophilized NPs was recorded.

$$\% \text{ Yield} = \frac{\text{Actual weight of lyophilized NPs} \times 100}{\text{Initial weight of polymer}} \quad (1)$$

2.4.5 *In vitro* drug release study

The *in vitro* release measurement of MTX was performed in a 0.01 M phosphate buffer solution. The release of MTX from NPs was investigated by dialysis method. The 2 mL of NPs dispersion was put into a dialysis bag (MWCO 6000-8000 Da) and then introduced into 30 mL phosphate buffer solution pH 4.5 or 7.4. The release chamber was incubated at 37 °C with gentle shaking. At determined intervals, 2 mL of release medium was withdrawn and replaced with an equal volume of fresh release medium. The content of MTX was analyzed by HPLC method as described in the following section. The experiments were carried out in triplicate.

2.4.6 High performance liquid chromatography (HPLC) analysis

The amount of MTX in samples was quantified by HPLC method. The analysis was performed at 25 °C on a reverse phase Hypersil ODS column, 5 µm particle size, 250×4.6 mm (Thermo Fisher Scientific Inc., Massachusetts, USA) using a mixture of ACN and 0.05 M phosphate buffer pH 6 (10:90 v/v) as a mobile phase pumped through the column at a flow rate of 1.2 mL/min. A chromatogram was recorded by UV detector at a wavelength of 304 nm. The retention time of the drug was 15 min. The linearity and precision were also determined. The standard curve of MTX was linear with r^2 of > 0.9995 over the concentration range of 0.05-20 µg/mL. The intra- and inter-day precisions were less than 2 %. The recovery was 98 ± 1 % which is accepted for accuracy criteria (90-110%). Before analysis, all samples were filtered through 0.22 µm syringe filter.

2.4.7 Statistical analysis

The data were analyzed for statistical significance by one way ANOVA. The level of significance was at *p*-value < 0.05. All data are expressed as mean ± SD from at least three measurements. For the *in vitro* release results, the different and similarity factors (f_1 and f_2, respectively) were calculated and used for statistical comparison [6].

Typically, f_1 and f_2 values between 0-15 and 50-100, respectively, indicate define the similarity of two release profiles.

3 RESULTS AND DISCUSSION

3.1 Formation of MTX-conjugated NPs

Due to the di-block pattern of P(MTXCLCL)-mPEG, the feature of P(MTXCLCL)-mPEG nanoparticles could be formed as core-shell NPs by an aggregation of P(CL) segment inside the core of NPs whereas PEG segment presented as hydrophilic shell on the surface of NPs as demonstrated in Figure 1(a). The characteristics of all nanoparticles are summarized in Table 1.

The particle size of P(MTXCLCL)-mPEG NPs increased depending on the % conjugating MTX. 10 % P(MTXCLCL)-mPEG NPs had the smallest particle size 101±1 nm whereas 30 % P(MTXCLCL)-mPEG NPs had the largest size of 141±6 nm. It has been reported that the amount of drug molecules conjugated along the polymer chain had an effect on the formation of NPs [7]. The increasing % conjugating MTX led to the increased bulkiness of MTX molecules inside the core of NPs. However, these NPs were still smaller than 200 nm. This particle size range has been reported that the NPs can be passively targeted to the tumor tissue [8]. In addition, the particle size distribution of 10 % and 20 % P(MTXCLCL)-mPEG NPs was narrow with the PI value of 0.117-0.157. However, 30 % P(MTXCLCL)-mPEG NPs showed large PI due to more hydrophobicity of 30 % P(MTXCLCL)-mPEG copolymer leading to aggregation during dialysis. The surface charge of 10 and 20 % P(MTXCLCL)-mPEG NPs was negative and closed to zero because the PEG chain surrounded the surface of NPs. The ZP value of 30 % P(MTXCLCL)-mPEG NPs was slightly more negative indicating some of MTX molecules available on the surface of NPs. All formulations showed similar % yield ranging from 52.17-58.12 %. The NPs demonstrated spherical in shape and uniform size distribution as seen by SEM micrograph in Figures 2(a).

3.2 FOL-decorated MTX-conjugated NPs

FOL-decorated MTX-conjugated NPs (FOL-P(MTXCLCL)-mPEG NPs) were also prepared in order to develop the targeted nanocarriers by physical adsorption between PEG chains of both P(MTXCLCL)-mPEG and P(FOLCL)-mPEG. The anticipated architecture of FOL-P(MTXCLCL)-mPEG NPs is illustrated in Figure 1(b). The characteristics of FOL-P(MTXCLCL)-mPEG NPs are summarized in Table 1. All NPs after decoration had the significantly larger particles due to the adsorption of P(FOLCL)-mPEG on the surface of NPs. The mPEG chain of P(FOLCL)-mPEG inserted in the PEG shell of NPs tethering FOL molecules outside the NPs and thus enlarging the particles of FOL-decorated NPs. The ZP of FOL-P(MTXCLCL)-mPEG NPs became more negative being attributable to the presence of FOL molecules of P(FOLCL)-mPEG copolymer on the surface of P(MTXCLCL)-mPEG NPs. The % yield of FOL-P(MTXCLCL)-mPEG NPs were 53.89-56.98 %. These results showed that P(FOLCL)-mPEG had no impact on the yield of NPs. The morphology of FOL-P(MTXCLCL)-mPEG NPs also exhibited spherical in shape and uniform size distribution as shown in Figures 2(b).

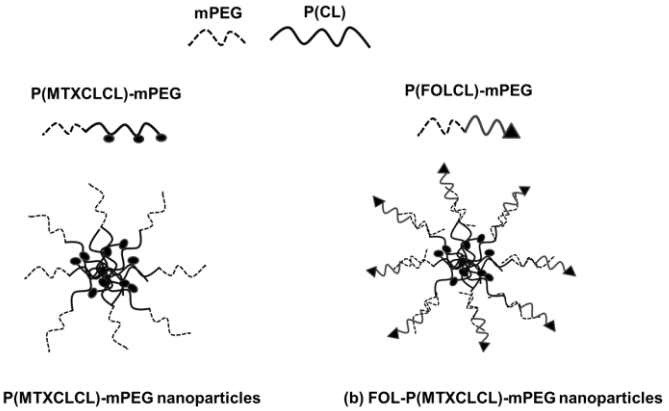

Figure 1 : The postulated architectures of P(MTXCLCL)-mPEG NPs (a) and FOL-P(MTXCLCL)-mPEG NPs (b).

Table 1 : Particle size (z-ave), polydispersity index (PI), zeta potential (ZP), and % yield of P(MTXCLCL)-mPEG NPs and FOL-P(MTXCLCL)-mPEG NPs

Formulation	z-ave (nm)	PI	ZP (mV)	% yield
10 % P(MTXCLCL)-mPEG	101±1	0.117±0.001	-3.7±0.8	58.12±2.35
20 % P(MTXCLCL)-mPEG	128±3	0.157±0.051	-4.7±1.1	53.23±3.21
30 % P(MTXCLCL)-mPEG	141±6	0.508±0.056	-10.7±1.1	52.17±2.24
FOL-10 % P(MTXCLCL)-mPEG	122±1	0.180±0.008	-12.8±0.1	56.98±1.23
FOL-20 % P(MTXCLCL)-mPEG	161±1	0.114±0.022	-8.1±0.1	55.16±0.58
FOL-30 % P(MTXCLCL)-mPEG	172±1	0.303±0.031	-18.0±0.9	53.89±1.08

3.3 *In vitro* release study

The release of MTX from P(MTXCLCL)-mPEG NPs was performed in buffers pH 4.5 and 7.4 representing the endosomal environment and in the blood stream, respectively [9]. From Figure 3, MTX showed the sustained release over 20 days by less than 50 % release of total drug.

At pH 4.5, the total MTX released from NPs increased with % conjugating MTX. The slow release of MTX at pH 7.4 suggested that the conjugating MTX may prevent the leakage of drug in the blood circulation before reaching the target site.

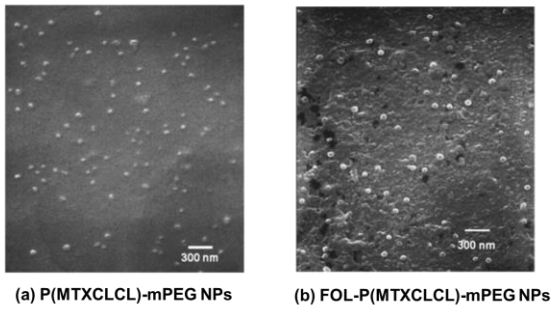

Figure 2 : SEM micrographs of P(MTXCLCL)-mPEG NPs (a) and FOL-P(MTXCLCL)-mPEG NPs (b).

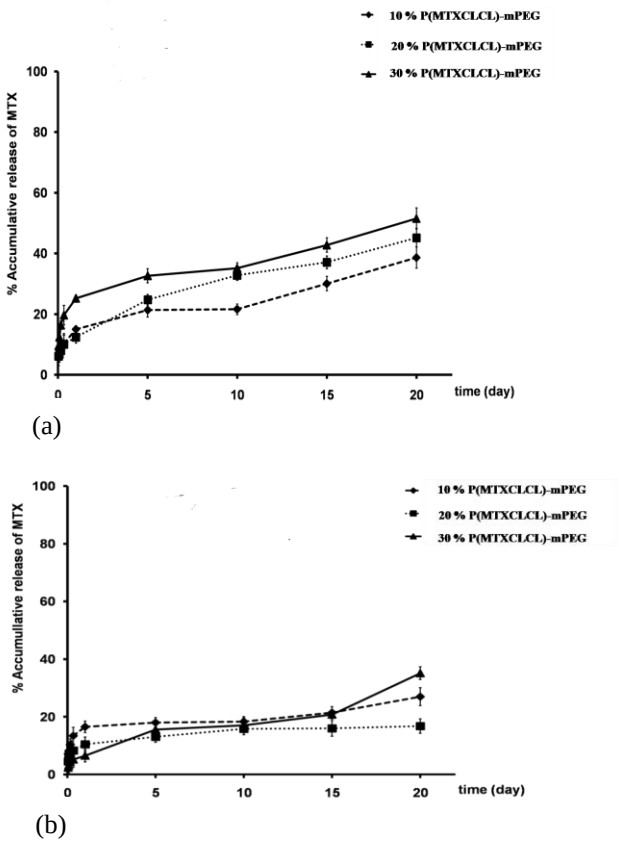

Figure 3: The release profiles of MTX from P(MTXCLCL)-mPEG NPs in phosphate buffer solutions pH 4.5 (a) and 7.4 (b).

4 CONCLUSIONS

The preparation of P(MTXCLCL)-mPEG NPs and FOL-P(MTXCLCL)-mPEG NPs were achieved by dialysis method. The particle size of P(MTXCLCL)-mPEG NPs relatively increased with the content of conjugating MTX. The targeted FOL- P(MTXCLCL)-mPEG NPs were successfully prepared by means of physically adsorptive interaction of PEG chains. The adsorption of P(FOLCL)-mPEG resulted in larger particle size and more negative surface charge whereas it did not affect PI and % yield. The release of conjugating MTX was pH-dependent with higher extent in acidic medium and could be sustained over 20 days. All of results led to the conclusion that P(MTXCLCL)-mPEG NPs and FOL-P(MTXCLCL)-mPEG NPs can potentially be used as targeted anticancer drug delivery system.

Acknowledgments

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