siRNA and Paclitaxel Loaded PEG-PCL-PEI Tri-block Polymeric Micelle for Gene and Chemo-therapy in Multidrug-resistance Cancer Cells

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ABSTRACT

An annoying problem of chemotherapy is the drug resistance of cancer cells which express of multidrug-resistance associated proteins and thereby resulting in ineffective treatment. To conquer this problem, tri-block copolymer, polyethylene glycol-polycaprolactone-polyethylenimine forming micelle was utilized to encapsulate the chemotherapeutic drug, paclitaxel (PTX), in the core of micelle and to attract the small interfering RNA (siRNA) on the corona of micelle and to make drug resistance cancer cell sensitizing to chemotherapeutic drug. Gratifyingly, dual agents (PTX and siRNA) combination polymeric micelles were showed to increase the intracellular accumulation of paclitaxel and were observed to kill doxorubicin resistant (MCF-7 ADR) cells by silencing the expression of multidrug-resistance associated proteins, P-glycoprotein. This result suggested that the tri-block polymeric micelle as a potential carrier for gene and chemo-therapy against the multidrug-resistance cancer cell.

Keywords: multidrug-resistance, gene therapy, chemotherapy, micelle

1 INTRODUCTION

The multidrug resistance (MDR) phenotype has been a tormented problem in tumor chemotherapy and has been observed both *in vitro* and *in vivo* [1]. MDR is frequently associated with the overexpression of a 170-KDa membrane protein, known as P-glycoprotein (P-gp) [2]. This protein belongs to the superfamily of ATP binding cassette (ABC) transporters and pumps the xenoboiotic compounds as well as anticancer drugs, such as paclitaxel [3] out of cell. Therefore, the intracellular drug accumulation was decreased by this ATP-dependent drug-efflux pump, P-gp.

There have been many studies on reversing the drug-resistance of cancer cell. For example, Pluronic micelle [4] or Vitamin E TPGS nanoparticles [5] were used to solve the problem. Except using a functional inhibitor and chemical compound, drug resistance could also be overcome by silencing the expression of the efflux transporter through RNA interference. In this approach, gene silencing is triggered by using small interfering RNA (siRNA) molecules, that are about 20–25 base pairs long. Once siRNA into cell, siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands guide the RISCs to complementary RNA molecules where they cleave and destroy the target mRNA [6]. In previous report, silencing the MDR1 gene to inhibit P-glycoprotein expression by using MDR-1 siRNA can sensitize P-gp overexpressed cancer cells to chemotherapy [7].

Because siRNA is a charged hydrophilic macromolecule, it is not easy to penetrate across the cellular membrane. Thus, carriers which can efficiently deliver siRNA and co-deliver chemotherapeutic drug into tumor cells were developed in this study. In addition, the cytotoxicity of the dual agents (gene and chemotherapeutic drug) loaded carriers were also evaluated. In this study, we attempt to synthesis mPEG-PCL-PEI tri-block polymer as carriers which can co-delivery gene and chemotherapy drug. The mPEG-PCL copolymers can form nanocarriers (~100nm) have been considered as potential carriers for hydrophobic drug [8]. After PEI was conjugated on the mPEG-PCL, the PEI segment was as a complexion site with gene through electrostatic interactions. After administration of gene complex with drug loaded micelle to form polyplexes, the drug resistance cells (MCF-7 ADR) were sensitive to drug again.

2 MATERIAL AND METHOD

2.1 Materials

Pyridine, ε-Caprolactone and p-Toluenesulfonyl chloride were purchased from ACROS ORGANICS (Belgium). Methoxypolyethylene glycol 5,000, polyethylenimine, branched 25,000, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and ethidium bromide were purchased from Sigma-Aldrich (USA). Potassium dihydrogenphosphate, potassium chloride and sodium hydroxide were purchased from Showa (Japan). Triethylamine was purchased from J.T Baker (USA) ,stannous(II) octoate (Sn(Oct)2) was purchased from FLUKA (USA), paclitaxel (PTX) was purchased from SIPHAR (Taiwan) and MDR-1 siRNA was purchased from Santa Cruz biotechnology (USA). Sodium phosphate dibasic, sodium chloride and all of other solvents were purchased

fronm TEDIA (USA). Materials for cell culture were purchase from Gibco BRL (USA).

2.2 Synthesis and characterization of PEG-PCL-PEI tri-block polymer

mPEG-PCL amphiphilic block copolymers were synthesized by ring opening polymerization of ε-Caprolactone in the presence of methoxypolyethylene glycol (Mw=5000) as an initiator with $Sn(Oct)_2$ as a catalyst [9]. ε-Caprolactone (2 mL) was drop into mPEG (1g) and $Sn(Oct)_2$ containing flask and was reacted at 140℃ for 6h. The crude polymer solution was precipitation into cold ether twice and vacuum-dried to evaporate water.

After the mPEG-PCL diblock copolymer was synthesis, the tosylation of copolymer was executed by using p-Toluenesulfonyl chloride [10]. mPEG-PCL (1mmol) and pyridine (30mmol) were dissolved in dry tetrahydrofuran (THF) (10 mL) and placed in necked flask with stirring under a dry nitrogen atmosphere for 10 minutes. p-toluenesulfonyl chloride (30mmol) was dissolved in dry THF and was dropped into flak slowly. After 48 hours reaction, the resulting white powder was collected on a filter and re-dissolved in THF washed several times to remove other residual chemical.

To obtain the mPEG-PCL-PEI tri-block polymer, amino groups of hyperbranch Polyethylenimine was conjugated to tosylate block copolymer. The tosylate copolymer (0.1 mmol) and PEIs (Mw=25000) (0.02mmol) were dissolved in dry DMF in the presence of triethylamine (TEA) and were mixed at 60℃ for 48h under a dry nitrogen atmosphere. After the reaction, the polymer was dialyzed (MWCO= 50000) against methanol/acetone 1:1 for 48h and dialyzed against water for further 48 h to removed un-reactive polymer and was dried in vacuum.

The tri-block polymers were characterized by GPC and ^1H NMR. The tri-block polymer compositions were determined by BRUKERAVANCE-300 MHz FT- NMR at 300MHz using $CDCl_3$ as solvent. The average molecular weights and distribution were measured by gel permeation chromatography (WATERS, USA) using HPLC grade THF as elution solvent and a flow rate of 1 mL/min at 35℃.

2.3 Preparation of paclitaxel loaded polymeric micelle and siRNA/paclitaxel polyplexes

PTX loaded polymeric micelle was prepared using O/W emulsion-solvent evaporation method. Briefly, PTX and mPEG-PCL-PEI tri-block polymer were dissolved in 2 mL Acetone/ethanol 1:1 co-solvent and then were sonicated at 60℃ hot water bath until the formation of a clear solution. Different drug to polymer weight ratio from 1:5 to 1:20 were used. This solution was slow dropped into 10 mL d.d. H_2O under ultrasonic cell crasher sonicated at 70℃ hot water bath for 30 min to evaporated organic solvent. Finally, the micelle solution was filtered through a 0.45 μm cellulose acetate filter membrane to removed non-incorporated drugs.

siRNA incorporated PTX loaded polyplexes (siRNA/PTX polyplexes) were prepared as following description.Various drug loaded polymeric micelle solution, which the concentration of PTX from 1 μ/mL to 0.0001 μg/mL, were mixed with 200 nM of MDR-1 siRNA in a total volume 500 μL and were stilled at room temperature for 20 min.

The particle size distribution was evaluated by dynamic light scattering (DLS). The surface charge of nanoparticles was measured by Zetasizer (Malvern Instruments Ltd Nano ZS 90). Drug loading efficiency was determined by a high performance liquid chromatography system with WATERS C_{18} analytic column [11]. The amount of PTX was detected by UV detector at 227 nm. The drug loading efficiency and encapsulation efficiency were calculated by equations (1) and (2), respectively.

$$\frac{\text{Weight of drug in the nanoparticles}}{\text{Weight of nanoparticles}} \times 100\% \quad (1)$$

$$\frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of feed drug}} \times 100\% \quad (2)$$

2.4 Cell culture

Doxorubicin resistant cells (MCF-7/ADR) were used for the cellular studies. Cells were grown in T75 culture flask using MEM culture medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 0.1% amphotericin B, and 1% Penicillin-streptomycin-neomycin solution at 37 ℃ in 5 % CO2 incubator.

2.5 *In vitro* cytotoxicity of polymeric micelle and polyplexes

For evaluating the cytotoxicity pacltiaxel loaded polymeric micelles, MCF-7 ADR cells were seeded into 96 well plates at a density of 8000 cells per well in 100 μL MEM medium supplemented with 10% FBS and incubated for 24 hours at 37 ℃ in 5% CO_2. The free PTX and PTX loaded micelles were added with PTX concentration from 10 μg/mL to 0.00001 μg/mL then cells were incubated for 72 hours After incubation, cells were washed with 100 μL PBS and survival rate was determined by adding 100 μL MTT solution (0.5 mg/mL) to each well. After 3.5 hours incubation, the MTT solution was replaced with DMSO to dissolve the formazan compeletely. Percentage of cell viability was calculated from the absorbance at 570 nm using a multi-well ELISA reader (SpectraMaxs M2; Molecular Device).

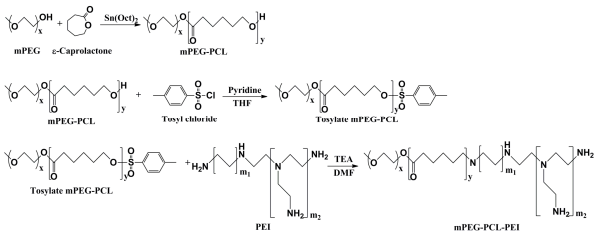
Similarly, the cytotoxicity of siRNA/PTX polyplexes were also evaluated in MCF-7 ADR cells. Briefly, MCF-7 ADR cells were seeded into 24 well plates at a density of

30,000 cells per well in 500 μL MEM medium supplemented with 10% FBS and incubated for 24 hours at 37 ℃ in 5% CO_2. The asprepared siRNA/PTX polyplexes were added to cells for 72 hours. After incubation, cells were washed with 500 μL PBS and survival rate was determined by adding 500 μL MTT solution (0.5 mg/mL) to each well. Percentage of cell viability was evaluated by MTT assay as above description except that the final formazan solution was transferred to 96 well plates to measure the absorbance.

3 RESULTS AND DISSCUSION

3.1 mPEG-PCL-PEI tri-block polymer

mPEG-PCL-PEI tri-block polymer (scheme 1) was synthesis through a serial reaction between the tosylated mPEG-PCL block copolymers and the amino groups of PEI polymers. First, mPEG-PCL block copolymer was synthesized by ring-opening polymerization using mPEG as a macroinitiator under $Sn(Oct)_2$ catalysis and characterized by ^1H NMR in $CDCl_3$. In the ^1H NMR ($CDCl_3$, δ in ppm, 300 MHz) spectrum, the characteristic resonances of both PCL δ1.368 (δ-H), δ1.65 (β-H and γ-H), δ2.286 (α-H) δ4.033 (ε-H) and mPEG δ3.375 (-OCH_3), δ3.637 (-OCH_2CH_2O-) were observed. The molecular weight of PCL was determined by comparing the peak intensities of the methylene protons of the oxyethylene units of mPEG to the methylene Protons of PCL [12]. The molecular weights and polydispersity of copolymers were also determined by GPC (Table 1). The copolymers were further modified, activated and then conjugated to PEI to form the desired mPEG-PCL-PEI tri-block polymer (Scheme 1). The mPEG-PCL-PEI tri-block polymer final molecule weight was determined by using mPEG Protons signal at 3.367 ppm compare to PEI Protons signal at 2.5-3.0 ppm. From the PEI ^1H NMR sings appearance, indicated the tri-block polymer was successfully synthesized and the molecular weights of tri-block polymer was also shown in Table 1.



Scheme 1: Synthesis of mPEG-PCL-PEI tri-block polymers

Polymer	Mw[a]	Mw[b]	PDI[b]
mPEG-PCL	15624	16519	1.4
mPEG-PCL-PEI	87079		

Table 1: Characteristics of mPEG-PCL and mPEG-PCL-PEI. a. Determined by ^1H NMR in $CDCl_3$. b. Determined by GPC in THF using polystyrene as standards.

3.2 Paclitaxel loaded tri-block polymeric micelles and siRNA/paclitaxel polyplexes

The D/P ratio (D/P ratio = weight of PTX/weight of mPEG-PCL-PEI) range from 1/5 to 1/20 were tested in this study. After pacltiaxel loaded into tri-block polymeric micelles, the average size of each micelle was determined by DLS with various PDI showed in Table 2. The loading efficiency of PTX loaded tri-block polymeric micelles was about 76% when a D/P ratio of 1:10 was employed. However, the micelles became larger and exhibited a lower loading efficiency while the D/P ratio was reached the D/P ratio of 1/5 or 1/20 (Table 2). This is may be due to the mPEG-PCL copolymers were conjugated to a more hydrophilic polymer, PEI; thus the highly hydrophobic PTX was not easy to encapsulate into micelles and to reach good encapsulation efficiency.

The drug loaded micelles were further mixed with siRNA to form polyplexes at different N/P (nitrogen to phosphate) ratio. After the polyplexes were formation, the variation of particle size and zeta potential at different N/P ratio were evaluated. As N/P ratio was increased, the zeta potential changed from -10mV to + 45mV. The initial particle size was 300 nm at N/P ratio of 1/3 and increased to 400 nm at N/P ratio of 1. Continuously, the particle size decreased immediately to 238nm at N/P ratio of 3 and remained in the 210–200 nm range while the ratio was increased. These tri-block polymer could condense DNA to nanoparticles (~200 nm) after the N/P ratio reach 3.

These results showed that the tri-block polymeric micelles not only have the capability to prevent hydrophobic PTX from aggregation but have the ability to form stable polyplexes with siRNA. More importantly, the size of forming polyplexes was around 200 nm, which may an appropriate size for further application in tumor therapy via enhanced permeability and retention (EPR) effect [13].

D/P ratio	Particle size (nm)	PDI	Loading efficiency (%)	Encapsulation efficiency (%)
1/5	241.6	0.197	10.4	1.60
1/10	226.3	0.175	76.0	6.90
1/20	240.6	0.256	3.13	0.14

Table 2: Characteristics of paclitaxel loaded tri-block polymeric micelles

3.3 Cytotoxicity of paclitaxel loaded tri-block polymeric micelle and siRNA/paclitaxel polyplexes

It is known that multidrug resistant cells possess a higher palitaxel resistance capability than the no P-gp expression cells. Therefore, the cytotoxicity of PTX was evaluated in drug-sensitive (MCF-7 wt) and drug-resistance (MCF-7 ADR) cells. As Fig. 1 shown, the higher cell viability of

MCF-7 ADR cells at all tested concentration of PTX must be due to the overexpression of P-glycolprotein on the drug-resistance cellular membrane.

In the MDR siRNA/PTX polyplexes experiment, the cytotoxicity of PTX were showed significantly different at concentration of PTX from 0.01 to 1 μg/mL between drug loaded polymeric micelles and dual agents loaded polyplexes (Fig. 2). These results may result from the successfully inhibit the celluar surface P-gp expression. Although, the PTX loaded micelles did not increase the cytotoxicity of PTX in MCF-7 ADR cells which was compared with free PTX did in MCF-7 ADR cells, the cytotoxicity of paclitaxle was significantly increased through the MDR siRNA/PTX polyplexes treatment.

MCF-7 ADR cells at all tested concentration of PTX must be due to the overexpression of P-glycolprotein on the drug-resistance cellular membrane.

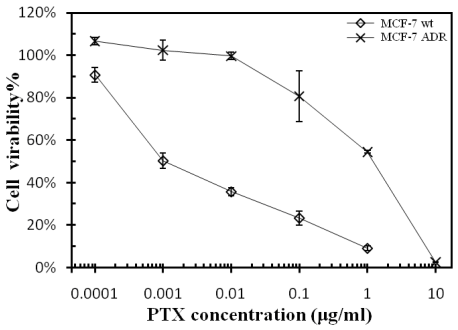


Figure 1: Cytotoxicity of free paclitaxel in MCF-7 wt and MCF-7 ADR cells

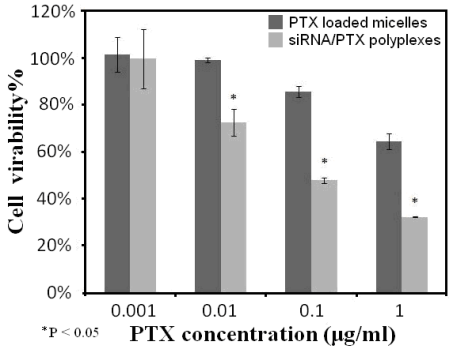


Figure 2: Evaluation the cytotoxicity of paclitaxel administered with and without siRNA polymeric micelles to MCF-7 ADR cells

4 CONCLUSION

In this study, mPEG-PCL-PEI tri-block polymer was synthesized successfully and was utilized to form dual functional nanocarriers. These nanocarriers can co-delivery gene (MDR-1 siRNA) and chemotherapeutic drug (paclitaxel) at the same time. The cytotoxicity of PTX was observed higher in multidrug-resistance (MCF-7 ADR) cells than in drug sensitive (MCF-7 wt) cells. Delightedly, after the PTX was encapsulated in the micelle and complex with siRNA to form the polyplexes, the MCF-7 ADR cells were sensitive to PTX again. This result suggested that the mPEG-PCL-PEI tri-block polymer as potential carriers for gene therapy and chemotherapy. Further, the targeting molecule modification polymers may be incorporated to render the nanocarrier for targeting therapy.

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REFERENCES

[1] Endicott JA, Ling V., Annual Review of Biochemistry. 58, 137, 1989.
[2] Shapiro AB, Ling V. Acta Physiol Scand Suppl. 643, 227, 1998.
[3] Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, et al. Clinical Cancer Research. 4, 389, 1998.
[4] Wang Y, Yu L, Han L, Sha X, Fang X. International Journal of Pharmaceutics. 337, 63, 2007.
[5] Collnot E-M, Baldes C, Wempe MF, Hyatt J, Navarro L, Edgar KJ, et al. Journal of Controlled Release. 111, 35, 2006.
[6] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Nature. 411, 494, 2001.
[7] Xiong X-B, Uludag H, Lavasanifar A. Biomaterials. 30, 242, 2009.
[8] Kim SY, Lee YM. Biomaterials. 22, 1697, 2001.
[9] Zhou S, Deng X, Yang H. Biomaterials. 24, 3563, 2003.
[10] Treetharnmathurot B, Ovartlarnporn C, Wungsintaweekul J, Duncan R, Wiwattanapatapee R. International Journal of Pharmaceutics. 357, 252, 2008.
[11] Carlisle RC, Read ML, Wolfert MA, Seymour LW. Colloids and Surfaces B: Biointerfaces. 16, 261, 1999.
[12] Hu Y, Xie J, Tong YW, Wang C-H. Journal of Controlled Release.118, 7, 2007.
[13] Maruyama K, Ishida O, Takizawa T, Moribe K. Advanced Drug Delivery Reviews. 40, 89, 1999.

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