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Combining Gene and Chemo Therapy using Multifunctional Polymeric Micelles

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***Abstract*—**Non-viral gene carriers composed of biodegradable polymers or lipids have been considered as a safer alternative for gene carriers over viral vectors. We have developed multi-functional nano-micelles for both drug and gene delivery application. Polyethyleneimine (PEI) was modified by grafting stearic acid (SA) and formulated to polymeric micelles (PEI-SA) with positive surface charge for gene and drug delivery. Our results showed that PEI-SA micelles provided high siRNA binding efficiency. In addition, siRNA delivered by PEI-SA carriers also demonstrated significantly high cellular uptake even in the presence of serum proteins. The post-transcriptional gene silencing efficiency was greatly improved by the polyplex formulated by 10k PEI-SA/siRNA. The amphiphilic structure of PEI-SA micelles provided advantages for multifunctional tasks; where the hydrophilic shell modified with cationic charges can electrostatically interact with DNA or siRNA, and the hydrophobic core can serve as payloads for hydrophobic drugs, making it a promising multifunctional vehicle for both genetic and chemotherapy application.

***Keywords*—**polyethyleneimine, gene delivery, micelles, siRNA

I. INTRODUCTION

RNA interference (RNAi) is a process that induces sequence-specific and post-transcriptional gene silencing by using synthetic double-stranded small interference RNA (siRNA). This gene silence works by incorporating siRNA into RNA-induced silencing complex (RISC), which seeks out to bind and degrade complementary mRNA, leading to target gene silencing. Synthetic siRNAs have been reported to successfully knocking down various diseases *in vivo* such as hypercholesterolaemia, liver cirrhosis, hepatitis B virus, ovarian cancer and bone cancer [1]. Numerous genetic therapeutic approaches using siRNA have also been investigated attempting to suppress the malignant tumor growth or metastasis as anti-cancer therapy [2-3]. In particular, inhibition of vascular endothelial growth factor (VEGF) expression of the tumor cells by the VEGF targeted siRNA holds great promises for cancer therapy, due to the fact that VEGF appears to be a critical regulator for the angiogenesis of the tumor growth. Tumor growth often requires adequate blood supply to deliver oxygen and nutrition for the cancer cells. Therefore, through RNAi to silence VEGF expression, tumor-induced angiogenesis can be reduced which then leads to cancer cells proliferation down regulation and tumor growth suppression. However, once introduced into the system circulation, siRNA complex faces series of hurdles such as kidney filtration, uptake by phagocytes, aggregation with the serum proteins, and enzymatic degradation [4]. For these reasons, additional delivery vehicle system is often required to transmit the siRNA or even assist to enhance and facilitate the knock down efficiency.

In this study, we have designed a biodegradable cationic amphiphilic copolymer, which consists of stearyl side chains on a cationic polyethyleneimine (PEI) main chain. By a self-assembly process, this copolymer can easily form core-shell nanoparticles with a hydrophobic core and a cationic shell in aqueous environment. This carrier can bound VEGF-targeted siRNA with the cationic shell through electrostatic interaction, and further incorporates chemotherapeutic anti-tumor drug doxorubicin into the hydrophobic core for potential *in vivo* co-delivery application.

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II. MATERIAL AND METHODS

Materials

Polyethylenimine (MW 1.8 and 10 kDa) were purchased from Wako, Osaka, Japan. Stearic acid (SA), EDC (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide), and doxorubicin hydrochloride (DOX-HCl) were purchased from Aldrich-Sigma (NJ, USA). Small interfering RNA was purchased from MDBio, Inc., Taiwan, and the sequences were as following: FAM labeled negative siRNA (sense, 5'-UUC UCC GAA CGU GUC ACG UdTdT-3'; antisense, 5'-ACG UGA CAC GUU CGG AGA AdTdT-3'); Human VEGF siRNA (siVEGF) (sense, 5'-GGA GUA CCC UGA UGA GAU CdTdT-3'; antisense, 5'-GAU CUC AUC AGG GUA CUC CdTdT-3')

Methods

Synthesis of PEI-SA micelle

SA grafted PEI was synthesized by reacting the carboxyl group of SA with the amine group of PEI using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a linker. PEI-SA micelles were prepared using oil in water (o/w) solvent evaporation method as previously described [5]. Briefly, 30 mg stearic acid was pre-dissolved in 10 ml ethanol and added drop-wised to 18 mg PEI and 15 mg EDC in 20 ml aqueous solution under magnetic stirring of 800 rpm at 80℃.

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The final solution was then dialyzed against 10% ethanol solution using a dialysis membrane (MWCO: 12 – 14 kDa, spectrum Laboratories, CA, USA) for 16 hrs to remove by-products and unreacted reagents, followed by dialysis against distilled water for 24 hr with frequent exchange of fresh distilled water.

Particle size and zeta potential

The hydrodynamic diameters and zeta potentials of PEI-SA micelles were determined by photon correlation spectroscopy. Diameters measurements were obtained with dynamic laser light scattering (Zetasizer Nano ZS90, Worcestershire, UK) using He-Ne ion laser (633nm) as the incident beam. Data was obtained at a detection angle of 90° at 25 °C and was analyzed by a cumulant method to calculate the hydrodynamic diameters. The zeta-potentials of the micelles were evaluated by the laser-doppler electrophoresis method using Zetasizer Nano ZS90. The zeta potential was measured in angle of 90° detection angle at 25 °C. The results were expressed as mean values (±SEM) of three experiments.

Gel retardation assay

The binding efficiency of PEI-SA micelles with siRNA was evaluated by gel retardation assay with 1 % agarose gel. PEI-SA/siRNA complexes were formed at various weight ratios from 0 to 6 and incubated for 30 mins at room temperature. Two microliter sample buffer containing 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF in Tris-borate-EDTA (TBE) buffer was added to each 10 μl sample. These samples were then loaded onto the 1 % (w/v) agarose gel containing GelRed (Biotium, CA, USA) reagent by 25,000x dilution and electropheresis was performed with voltage of 100V for 30mins in 1xTBE buffer solution. The gel was then photographed under UV light system (UVB-1, Apices Scientific, Taiwan)..

Quantification of trnasfection efficiency by flow cytometry

Huh-7 cells were seeded in 6-well plates with density of 2x105 per well at 37 °C. PEI-SA was premixed with plasmid DNA (pEGFP) for 30 mins at room temperature in DMEM medium and subsequently added into each well with final plasmid concentration of 1 μg per well and incubated in complete medium for 24 hrs. Gene transfection efficiency was evaluated by Becton Dickinson Calibur Flow Cytometry (Franklin Lakes, NJ) in the selected gate region (2x102 < FL1-H < 104) normalized with untreated EGFP expressing Huh-7 cells. The data was analyzed with CellQuest Software (Becton-Dickinson).

Quantification of VEGF suppression

Suppression of VEGF by RNAi silencing mechanism was validated using PEI-SA as functional gene carriers. Huh-7 cells were precultured in 24-well plates with medium containing 10% FBS. VEGF targeted siRNA was complexed with PEI-SA polymers for 30 mins and added into each well accordingly followed by additional 48 hours incubation. siRNA complexed with PEI or PEI-SA/chloroquine were also performed as controls for efficacy comparison. The culture medium was then collected for VEGF ELISA assay. The VEGF level secreted by the cells was evaluated using human VEGF immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Statistical analysis was carried out by a Student's *t* test. Statistical significance was assigned for *p* values <0.05.

Encapsulation and release of doxorubicin

Doxorubicin (DOX, 10 mg/ml) was dissolved in DMSO as stock solution. The DOX-loaded PEI-SA micelles were then prepared by added dropwise 200 μg DOX stock solution to PEI-SA micelles solution under mild agitation. The solution was then dialyzed (MWCO: 12-14 kDa, Spectrum Laboratories, CA, USA) against distilled water for 24 hours with frequent exchange of fresh distilled water. The drug encapsulated content ($C_{encapsulated}$) was determined by dissolving the DOX-loaded PEI-SA micelles in 90% DMSO and measuring the fluorescence emission of DOX using a spectrophotometer (Perkin Elmer, MA, USA) at λ_{ex}: 485 nm and λ_{em}: 595 nm. Drug loading percentage (DL%) and encapsulation efficiency (EE%) were determined based on the following equations

$$\text{DL \%} = \frac{C_{encapsulated}}{C_{encapsulated} + C_{PEI-SA}} \times 100\% \quad (1)$$

$$\text{EE \%} = \frac{C_{encapsulated}}{C_{total}} \times 100\% \quad (2)$$

The drug release profiles from nanoparticles were investigated using water, pH 7.4 phosphate buffer solution (PBS), or pH 4.0 buffer solutions as dissolution medium. In each experiment, DOX-loaded PEI-SA nanoparticle solution in 3.78 mg/mL concentration was dispersed into dissolution medium and placed in a 37℃ incubator and stirred at 100 rpm. At certain time intervals, the DOX fluorescence released in the medium was measured by a luminescence spectrometer at excitation and emission of 485 /595 nm, respectively, and the released content was calculated based on previously established standard.

III. Results

In this study, stearic acid was first grafted to 1.8k or 10k PEI by EDC as linker, and micelles were formulated by oil in water solvent evaporation technique. The average hydrodynamic diameter and zeta potential were determined by photon correlation spectroscopy. The physicochemical characteristics of the formulated PEI-SA micelles were summarized in Table 1. TNBS (sodium 2,4,6-trinitrobenzene sulfonate) analysis indicated that 1.8k PEI-SA and 10k PEI-SA micelles contained 0.61 ± 0.02 nmole and 1.15 ± 0.05 nmole amine groups on the hydrophilic corona per microgram PEI-SA, respectively.

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TABLE I
PHYSICAL CHARACTERISTICS OF PEI-SA NANOPARTICLES

Nanoparticle	Size (nm)	ζ–potential (mV)	PdI	Amine group content (nmole/μg)
1.8kPEI-SA	303.8±3.8	63.3±2.4	0.157	0.61 ± 0.02
10kPEI-SA	149.6±1.2	64.1±1.5	0.119	1.15 ± 0.05

PEI-SA polyplex were prepared to investigate the size and zeta potential variation resulting from different polymer:DNA ratios. Figure 1 (a) shows the particle size and zeta potentials of 10k PEI-SA/pDNA with weight ratios from 0.3 to 9. The particle size increased dramatically as weight ratio increases from 0.3 to 0.75. However a sudden decrease of particle diameter also observed from weight ratio of 0.75 to 2. The particle size remained stable at 150 nm with weight ratio from 2 to 9. In contrast, the zeta potential increased as the weight ratio increased, and reached to a plateau of approximately 50 mV from weight ratio of 0.75 to 9. Figure 1 (b) shows the particle size and zeta potential variation of 1.8k PEI/pDNA from weight ratios of 0.3 to 9. The particle size was fairly stable as the weight ratio increased from 0.3 to 9. However, a sudden increase of size was observed from weight ratio of 0.75 to 2, and dropped back to 300 nm. The zeta potential behaved similarly to the 10k PEI-SA/pDNA, where it increased steadily from weight ratio of 0.3 to 2, and begin to reach to a stable plateau of 50 mV from weight ratio of 2 to 9.

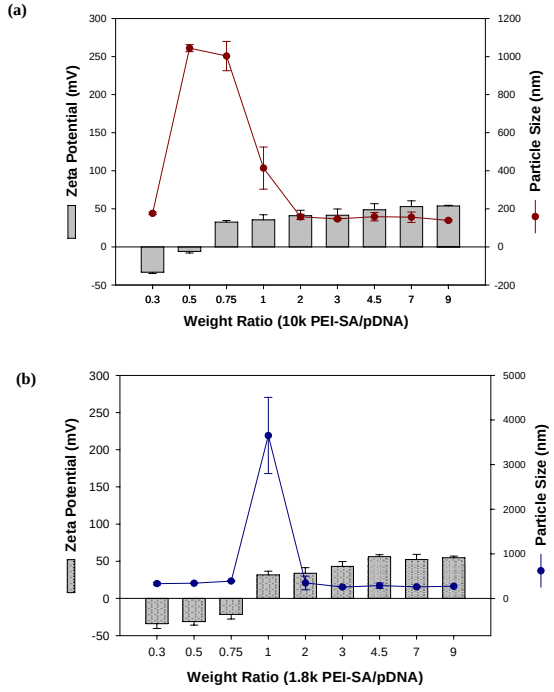


Fig. 1 Zeta potential and size analysis of PEI-SA micelles at various polymer/DNA ratios

Moreover, gel retardation assay (Figure 2) was also performed to investigate the binding efficiency of PEI-SA with nucleotides at weight ratios of 0, 0.14, 0.28, 0.56, 1.13, 2.25, 4.5, and 6 (lane 1, 2, 3, 4, 5, 6, 7, 8, respectively). Figure 2 shows that mobility of siRNA was completely retarded when weight ratio above 2.25 for 10k PEI-SA (Figure 2 (a), lane 6), and weight ratios above 4.5 for 1.8k PEI-SA (Figure 2 (b), lane7). The migration of siRNA was hindered since the amine groups on the micelles provided positive charge to initiate electrostatic binding with siRNA containing negative charge phosphate. These physical characteristics of nano-sized micelles with positive charge provide the foundation for potential in vivo gene delivery application.

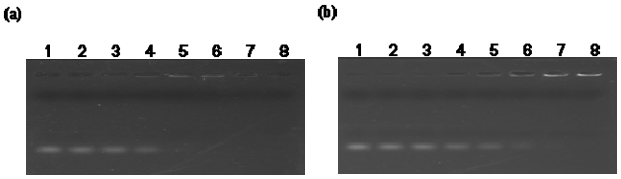


Fig. 2 Binding efficiency determined by gel retardation assay

Plasmid encoding Enhanced Green Fluorescence Protein (EGFP) was used to investigate the tranfection efficiency of PEI-SA/ pDNA complex by flow cytometry analysis in the selected gate region (2x102 < FL1-H < 104) normalized with untreated EGFP expressing cells. Huh-7 cells transfected with 10k PEI-SA/pEGFP showed more EGFP positive expressing cells than with 1.8k PEI-SA as carriers (Figure 3). Further analyzing of Huh-7 cells transfected with 10k PEI-SA/ pEGFP at weight ratio of 4.5 showed 62.9±2.2% cells with EGFP expression in 2.5 days comparing with treatment with pEGFP only.

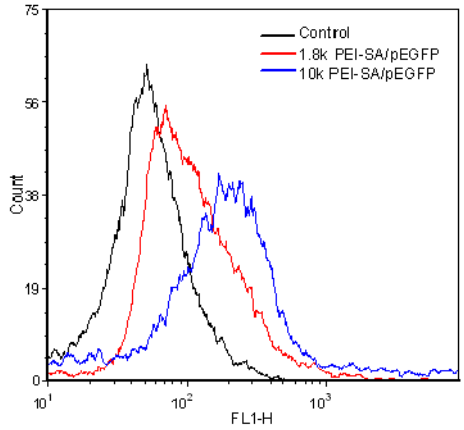


Fig. 3 Flow cytometry analysis for EGFP expression transfected with free plasmid DNA and 1.8k or 10k PEI-SA/pEGFP complex

To confirm the presence of the siRNA internalized into cells, fluorescence microscopic images of the cells were qualitatively evaluated by confocal microscopy after incubation with PEI-SA and FAM-labeled siRNA (FAM-siRNA). The fluorescence images provided information of the release profile for the endocytosed siRNA from the endosome. Figure 4b shows that the fluorescence intensity of internalized 10k PEI-SA/FAM-siRNA was significantly enhanced than that of

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1.8k PEI-SA/FAM-siRNA (Figure 4f), suggesting that 10k PEI-SA provided much higher cellular uptake than the treatment with 1.8k PEI-SA. In addition, endosomes/lysomes were stained with LysoTracker to reveal their presence and location within the cytoplasm (Fig 4c and Fig 4g). The overlapping images of endosomes/lysosomes to the siRNA fluorescence indicated that siRNA was uptaked and encapsulated within the endosomes/lysosomes. Yellow and green fluorescence each representing the endosome-ecapsulated siRNA and escaped-siRNA, respectively, in the cytosol were observed for both 10k PEI-SA/siRNA (Fig 4d) and 1.8k PEI-SA/siRNA (Fig 4h) complexes, suggesting efficient and rapid endocytosis and endosome/lysosome escape of both delivery systems. It is also worthy to note that the cellular endocytosis and endosome/lysosome escape at different phases can occur in same single cell and at same time point.

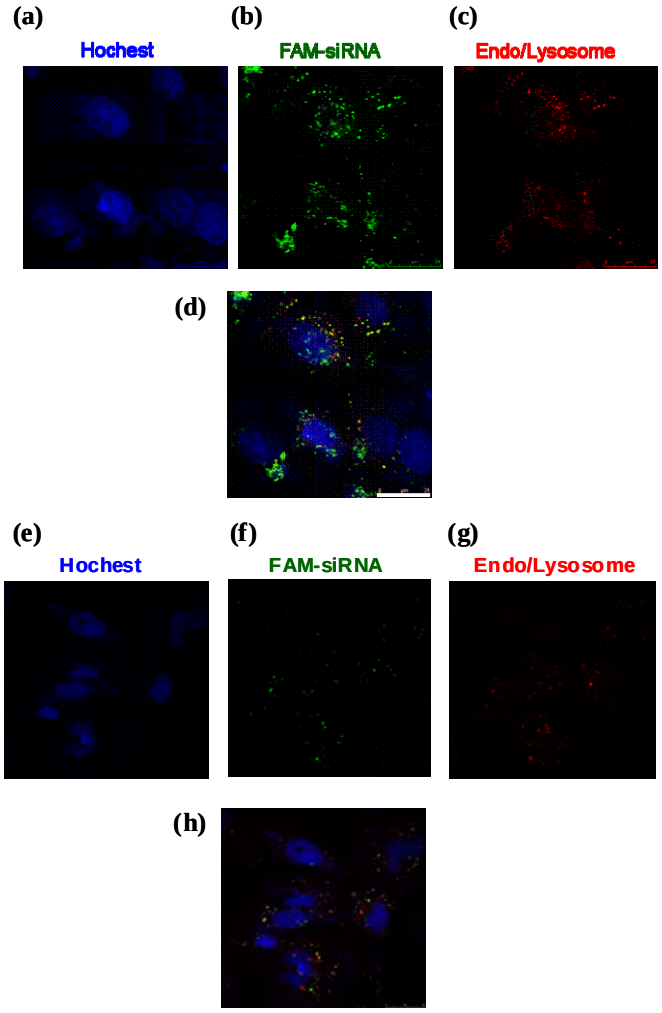


Fig. 4 Confocal microscopic images of FAM-siRNA with 1.8k or 10k PEI-SA after intracellular uptake by Hu-7 cells endocytosis upon 24 hour incubation

The development of anti-tumor macromolecular drugs has always been taking the advantage of the EPR effect originating from the leakiness of tumor vasculature [6]. These macromolecular drugs including various polymer-drug conjugates, polymeric micelles, and liposomes exhibit far better therapeutic efficacy and fewer side effects than the parent low-molecular-weight compounds [6]. In particular, polymeric micelle holds great potentials for its unique core-shell structure. The EE% and the DL% of the DOX loaded 10k PEI-SA nanoparticles were calculated based on equation (1) and (2) with 91.9% and 4.3%, respectively. The drug release profiles from the PEI-SA micelles were also investigated using pH 7.4 PBS, distilled water, and pH4.0 buffer as dissolution media. Figure 5 shows the drug release profile in all three dissolution medium. A maximum drug release percentage of 31% was observed for pH 4.0 buffer dissolution medium at day 52, suggesting the tight hydrophobic interaction between the PEI-SA micelles and DOX. The PBS dissolution showed faster drug release profile compared with both the distilled water and PBS. Our results demonstrated the possibility of of co-delivery of doxorubicin and VEGF siRNA by PEI-SA nanoparticles.

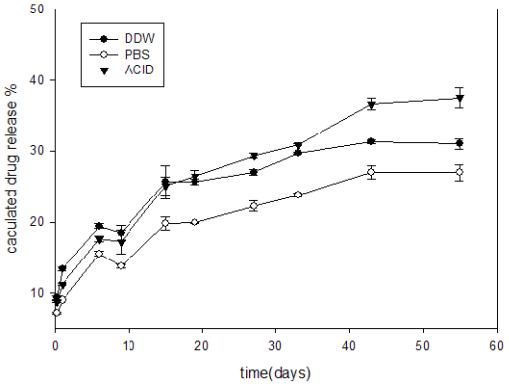


Fig. 5 Drug release profile of DOX in 10k PEI-SA nanoparticles in distilled water, pH 7.4 PBS, and pH 4.0 buffer

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