



Cholesterol-conjugated polyethylenimine 25K for safe gene delivery into human carcinoma cells

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ABSTRACT

Low cytotoxicity is a major requirement of a safe gene delivery vehicle. In this study, cholesterol-conjugated polyethylenimine 25K (PEI25K-CL) was used to evaluate the buffer capacity, the complexing ability with plasmid DNA, pEGFP, colloidal properties, pEGFP expression and transfection efficiency, and cytotoxicity in HeLa cervical carcinoma cells. The buffer capacity of PEI25K-CL was close to that of PEI25K. The PEI25K-CL/pEGFP and PEI25K/pEGFP complexes were completely retained at the polymer/DNA ratios of above 0.5:1. The particle size of PEI25K-CL/pEGFP complexes was 220 ± 7 to 402 ± 34 nm, depending on the polymer/DNA ratio. The pEGFP expression and transfection efficiency of the PEI25K-CL/pEGFP complexes were higher than those of pEGFP and lower than those of the PEI25K/pEGFP and LipofectamineTM2000/pEGFP complexes. The cytotoxicity of the PEI25K-CL/pEGFP complexes was significantly lower than that of the PEI25K/pEGFP complexes. This study concluded that PEI25K-CL could be an efficient and safe gene delivery vehicle.

INTRODUCTION

The viral vector has been an effective vehicle and practical in clinics currently (Duan, 2018; Ong *et al.*, 2019). Among nonviral vectors, polyethylenimine 25K (PEI25K) has been reported as an effective gene delivery carrier among cationic polymers (Jiang *et al.*, 2017; Ryu *et al.*, 2018). Modification of PEI has been reported to improve the transfection efficiency of the gene into the cells (Lisha Mali *et al.*, 2021; Wu *et al.*, 2020).

Plasmid DNA is a gene-based strategy. It has potential to be an alternative therapy for many diseases including cancer (Shimada, 2018), inherited retinal disease (Zhang *et al.*, 2021), and cardiovascular disease (Gorabi *et al.*, 2018).

Safe gene delivery is a vital issue that serves the vehicle to be effective and practical for clinical application and treatment (Clanchy *et al.*, 2008; Ma *et al.*, 2019). The objective of this

current study was to investigate PEI25K-CL on the improved transfection efficiency of pEGFP and low cytotoxicity in HeLa cervical carcinoma cells.

MATERIALS AND METHODS

Materials

Polyethylenimine (branched, 25 KDa) was purchased from Sigma-Aldrich (St. Louis, MO). PEI 25K-CL (1:1 mole ratio) was provided by Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. The XLarge DNA Ladder was purchased from GeneDireX (Las Vegas city, NV). pEGFP encoding enhanced green fluorescent protein was purchased from Clontech (Palo Alto, CA). HeLa human cervical carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Plasmid DNA preparation

pEGFP was extracted and purified from DH5 α *Escherichia coli* containing pEGFP using the Geneaid Plasmid Maxi Kit (Taipai

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City, Taiwan). The plasmid DNA was extracted according to the manufacturer's instructions. The purified plasmid was dissolved in tris-ethylenediamine tetraacetic acid (EDTA) buffer, pH 8.0. DNA concentration was measured at 260 nm using a cuvette photometer (BioPhotometer, Eppendorf AG, Hamburg, Germany).

Preparation of PEI25K-CL/pEGFP complexes

PEI25K-CL (0.1 mg/ml) in sterile water and pEGFP (1 mg/ml) in tris-EDTA buffer, pH 8.0, were diluted in ultrapure water or in Minimum Essential Medium (MEM). The PEI25K-CL and pEGFP complexes based on weight ratios of 0.125:1 to 2.5:1 were formed by adding a pEGFP solution to a PEI25K-CL solution, pipetted up-down for 15 times, and allowing spontaneous complex assembling for 15–20 minutes at room temperature (Weecharangsan *et al.*, 2021).

Measurement of buffer capacity of PEI 25K-CL

PEI 25K-CL (1:1 molar ratio) was dissolved in ultrapure water at a concentration of 1 mg/ml and then diluted with 10 ml of a 0.9% sodium chloride solution to a concentration of 0.1 mg/ml. The PEI 25K-CL solution was then titrated with a 0.1 N hydrochloric acid solution of 0.5–8.0 μmol . Simultaneously, the pH of the PEI 25K-CL solution was monitored using a pH meter (Bench 700, Oakton Instrument, IL). The buffer capacity of PEI 25K-CL was calculated by dividing the amount of hydrochloric acid by pH change at a high resistance of pH change (Sinko, 2006).

Gel electrophoresis assay

To analyze whether pEGFP was compacted by PEI25K-CL, a gel retardation assay was performed by electrophoresis (Weecharangsan *et al.*, 2021). The PEI25K-CL/pEGFP complexes at polymer/DNA ratios of 0:1, 0.125:1, 0.25:1, 0.5:1, 0.75:1, and 1:1 were prepared and loaded with loading dye Invitrogen (Grand Island, NY) into the wells of 1% agarose gel prepared in tris-borate-EDTA buffer containing 0.74% SYBR Green. The sample-loaded gel was electrophoresed at the voltage of 100 V for 15–20 minutes. The DNA bands were visualized on ImageQuant LAS 4000 Mini (GE Healthcare Bioscience AB, Uppsala, Sweden).

Particle size, polydispersity index, and zeta potential

The particle size, polydispersity index, and zeta potential of the PEI25K-CL/pEGFP complexes in ultrapure water were determined using the Zetasizer Nano Series (Malvern Instruments Ltd., Worcestershire, UK) at the temperature of 25°C.

In vitro transfection and pEGFP expression

HeLa cells were plated in a 24-well plate at a density of 2×10^4 cells/well with MEM + 10% fetal bovine serum (FBS) for 24 hours. 1 μg /well of pEGFP was gently mixed with 0.5–2.5 μg /well of PEI25K-CL and incubated for 15 minutes at room temperature. The PEI25K-CL/pEGFP complexes were subjected to HeLa cell transfection for 4 hours, and the transfected cells were rinsed with phosphate buffered saline (PBS) pH 7.4 and incubated with a complete growth medium at 37°C for 24 hours. The Lipofectamine™2000/pEGFP complexes at liposome/DNA ratio of 0.92/1 (v/w) were used as control. The cells were observed under a fluorescence microscope (Eclipse TS100, Nikon, Tokyo, Japan). The number of GFP-expressed cells was counted. The transfection efficiency was defined as the ratio of GFP expressed cell number to the plate area (Paecharoenchai *et al.*, 2012).

Cytotoxicity of PEI25K-CL

HeLa cells were seeded at a density of 8×10^3 cells/well in a 96-well plate in MEM containing 10% FBS and grown for 24 hours. PEI25K-CL and PEI25K at the concentration of 0 to 20 $\mu\text{g}/\text{ml}$ were added to each well and incubated for 4 hours. Cells treated with a growth medium were used as control. After 4 hours, the cells were then rinsed with PBS pH 7.4 and incubated with a growth medium for 24 hours. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to detect the cytotoxicity of PEI25K-CL (Weecharangsan *et al.*, 2014).

Statistical analysis

The data were collected as mean \pm SD. The significant difference in the data was analyzed using Student's *t*-test and analysis of variance as $p < 0.05$.

RESULTS

Buffer capacity of PEI25K-CL

Buffer capacity of PEI25K-CL and PEI25K was calculated at the high region resistance of pH change 7.12 ± 0.05 to 7.76 ± 0.04 and 7.01 ± 0.07 to 7.77 ± 0.12 , respectively, at the amount of HCl of 1.5×10^{-3} μmol (Fig. 1). The buffer capacity of PEI25K-CL and PEI25K was 0.0023 ± 0.0002 and 0.002 ± 0.0002 , respectively. The buffer capacity of PEI25K-CL was not different from that of PEI25K. However, the amount of HCl needed for neutralization of PEI25K was higher than that of PEI25K-CL for the pH range of 7.0–7.7. This could be due to the amount of free NH_2 group of PEI25K being higher than that of PEI25K-CL.

Electrophoresis of PEI25K-CL/pEGFP complexes

Gel electrophoresis was used to examine the PEI25K-CL/pEGFP complexes and PEI25K/pEGFP complexes (Fig. 2). The electrophoretic mobility of pEGFP of the PEI25K-CL/pEGFP and PEI25K/pEGFP complexes was completely retained at the polymer/DNA ratios of above 0.5:1. At the polymer/DNA ratio of 0.5:1, the PEI25K-CL/pEGFP complexes exhibited higher DNA band intensity than that of the PEI25K/pEGFP complexes. This suggested that PEI25K-CL had a lower ability to form a complex with pEGFP than that of PEI25K.

Colloidal properties of PEI25K-CL/pEGFP complexes

Figure 3 shows the particle size, polydispersity index, and zeta potential of the PEI25K-CL/pEGFP and PEI25K/pEGFP complexes performed in ultrapure water. The particle size, polydispersity index, and zeta potential of the PEI25K-CL/pEGFP and PEI25K/pEGFP complexes were 220 ± 7 to 402 ± 34 and 271 ± 12 to 462 ± 8 nm, 0.39 ± 0.05 to 0.51 ± 0.16 and 0.47 ± 0.07 to 0.6 ± 0.07 , and 32.9 ± 1.4 to 43.7 ± 1.7 and 37.2 ± 4.6 to 43.5 ± 1.8 mV, respectively.

The particle size, polydispersity index, and zeta potential of the PEI25K-CL/pEGFP and PEI25K/pEGFP complexes were dependent on the polymer/DNA ratio. At the polymer/DNA ratios of 0.5:1 and 2.5:1, the particle size of the PEI25K-CL/pEGFP complexes was significantly larger than that of the PEI25K/pEGFP complexes, while at the polymer/DNA ratios of 0.5:1 and 2.5:1 the particle size of the PEI25K-CL/pEGFP complexes was significantly smaller than that of the PEI25K/pEGFP complexes. At the polymer/DNA ratio of 0.5:1, the polydispersity index and zeta potential of the PEI25K-CL/pEGFP complexes were

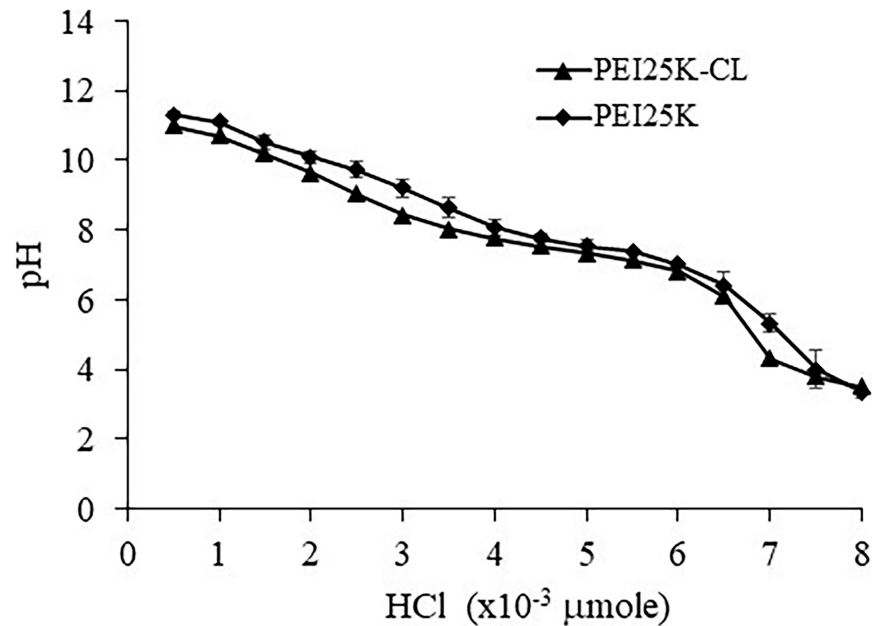


Figure 1. Buffer capacity of PEI25K-CL and PEI25K analyzed by acid-base titration.

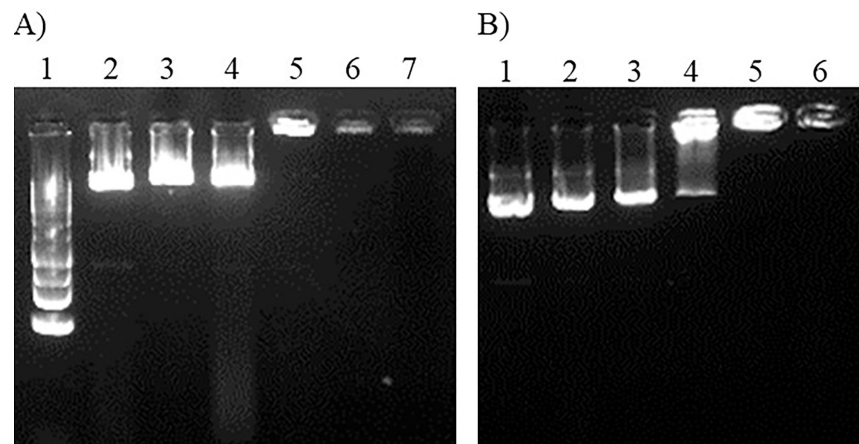


Figure 2. gel electrophoresis of PEI25K-CL/pEGFP complexes and PEI25K/pEGFP complexes on 1% agarose gel A) DNA ladder: lane 1, PEI25K/pEGFP complexes at polymer/DNA ratios of 0:1, 0.125:1, 0.25:1, 0.5:1, 0.75:1 and 1:1; lane 2-7, respectively (15 min electrophoresis); B) PEI25K-CL/pEGFP complexes at polymer/DNA ratios of 0:1, 0.125:1, 0.25:1, 0.5:1, 0.75:1 and 1:1; lane 1-6, respectively (20 min electrophoresis).

significantly lower than those of the PEI25K/pEGFP complexes while not significantly different at other polymer/DNA ratios.

pEGFP expression of PEI25K-CL/pEGFP and transfection efficiency

pEGFP expression of the PEI25K-CL/pEGFP and PEI25K/pEGFP complexes in HeLa cells analyzed by fluorescent microscopy is shown in Figure 4. PEI25K-CL/pEGFP exhibited lower pEGFP expression than that of PEI25K-CL/pEGFP. Transfection efficiency of pEGFP into HeLa cells of the PEI25K-CL/pEGFP complexes was 8 ± 4 to 679 ± 295 cells/cm² depending on the polymer/DNA (Fig. 5A). The transfection efficiency of pEGFP into HeLa cells of the PEI25K-CL/pEGFP complexes was significantly higher than in cells treated with pEGFP (Fig. 5A) while significantly lower than in cells treated with the PEI25K/

pEGFP complexes and Lipofectamine™2000/pEGFP complexes (Fig. 5B).

Cytotoxicity of PEI25K-CL

The cytotoxicity of PEI25K-CL and PEI25K in HeLa cells is shown in Figure 6. The cell viability of PEI25K-CL and PEI25K was $23.6\% \pm 0.8\%$ to $99.5\% \pm 1.5\%$ and $20.0\% \pm 0.9\%$ to $97.2\% \pm 3.4\%$, respectively. The cell viability of PEI25K-CL and PEI25K was not different at the polymer concentration of 0.1 $\mu\text{g}/\text{ml}$, while that of PEI25K-CL was significantly lower than that of PEI25K at the polymer concentration of all above 0.1 $\mu\text{g}/\text{ml}$.

DISCUSSION

Our study demonstrated that PEI25K-CL had the ability to deliver plasmid DNA and yielded an efficient transfection efficiency and low cytotoxicity. The low cytotoxicity and

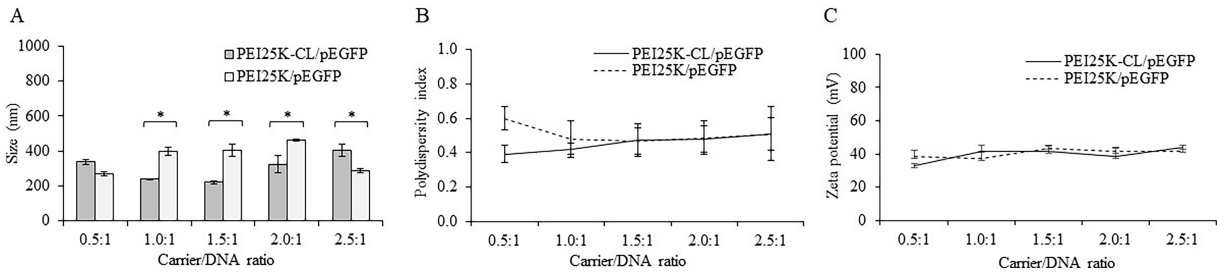


Figure 3. Particle size (A), polydispersity index (B) and zeta potential (C) of PEI25K-CL/pEGFP and PEI25K/pEGFP complexes analyzed at polymer/DNA ratios of 0.5:1, 1:1, 2:1 and 2.5:1. *, significantly different.

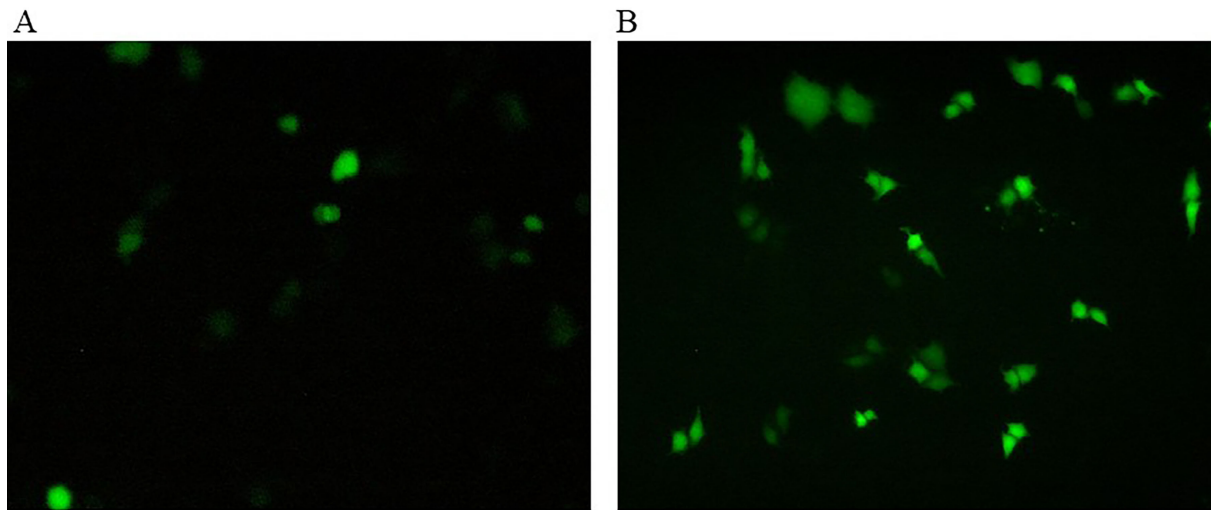


Figure 4. pEGFP expression of PEI25K-CL/pEGFP (A) and PEI25K/pEGFP (B) complexes in HeLa cells at polymer/DNA ratio of 1:1 analyzed by fluorescent imaging.

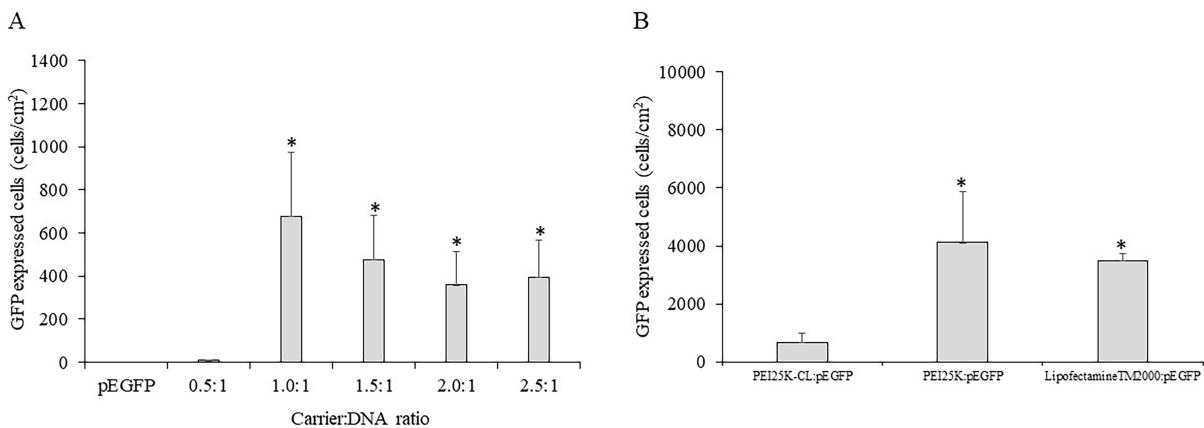


Figure 5. GFP expressed HeLa cells A) PEI25K-CL/pEGFP complexes at polymer/DNA ratios of 0.5:1, 1:1, 1:1.5, 2:1 and 2.5:1. *, significantly different from cells treated with pEGFP; B) PEI25K-CL/pEGFP complexes and PEI25K/pEGFP complexes at polymer/DNA ratio of 1:1, and Lipofectamine™2000/pEGFP complexes at liposome/DNA ratio of 0.92/1 (v/w). *, significantly different from cells treated with PEI25K-CL/pEGFP complexes.

efficient transfection efficiency of PEI25K-CL could be due to the amine groups of PEI25K partially substituted by cholesterol (Weecharangsan *et al.*, 2021).

PEI25K-CL had a buffer capacity similar to that of PEI25K and a lower neutralizing ability of HCl at the pH range of 7.12 ± 0.05 to 7.76 ± 0.04 . Wang *et al.* (2002) showed that PEI1.8K-CL and PEI10K-CL had a high buffer capacity in the pH

range of 5–7. Weecharangsan *et al.* (2021) showed that the buffer capacity of PEI25K-CA was not different from that of PEI25K.

PEI25K-CL had the ability to condense plasmid DNA, pEGFP. Jiang *et al.* (2010) exhibited that polyethylenimine-cholesterol cationic lipopolymer could condense plasmid DNA. Wang *et al.* (2002) showed that PEI1.8K-CL and PEI10K-CL could condense DNA to the nanosized and positively charged complexes.

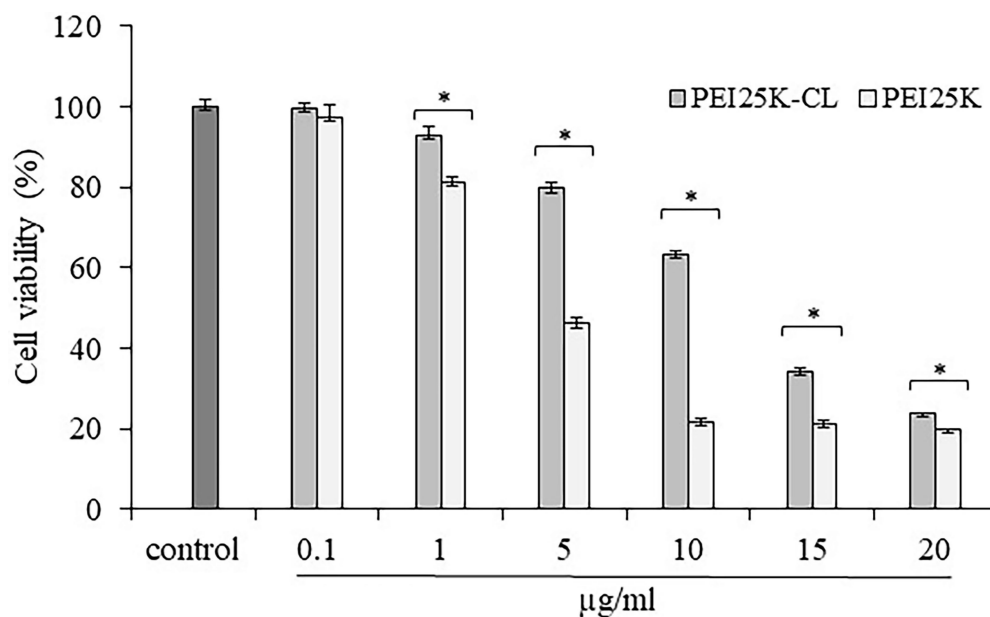


Figure 6. Cytotoxicity of PEI25K-CL and PEI25K at polymer concentration of 0.1 to 20 µg/ml in HeLa cells. *, significantly different.

The colloidal property often dictates the cellular entry mechanism of carrier/DNA complexes. The PEI25K-CL/pEGFP complexes yielded nanosized and positively charged particles. [Li *et al.* \(2018\)](#) demonstrated that polyethylenimine–cholesterol/miR was positively charged and nanosized.

PEI25K-CL improved pEGFP expression and transfection efficiency and low cytotoxicity in HeLa oral carcinoma cells. [Jiang *et al.* \(2010\)](#) exhibited that polyethylenimine–cholesterol lipopolymer/DNA had a good transfection efficiency in A549 and MCF-7 cells. [Wang *et al.* \(2002\)](#) showed that the PEI1.8K-CL/pCMS-EGFP and PEI10K-CL/pCMS-EGFP complexes had high GFP expression in Jurkat cells. [Remant *et al.* \(2020\)](#) demonstrated that cholesterol-grafted low-molecular-weight PEI enabled siRNA delivery into K562 myeloid leukemia cells depending on the degree of substitution of PEI backbone. [Sun *et al.* \(2014\)](#) reported that PEI-cholesterol liposomes improved cellular uptake and endosomal escape of cisplatin, as did [Wu *et al.* \(2020\)](#) in hydrophobization of PEI with cholesterol of siRNA.

PEI25K-CL had low cytotoxicity in HeLa oral carcinoma cells. [Gusachenko *et al.* \(2009\)](#) showed that lipophilic conjugated-PEI25K had low cytotoxicity at particular modification of amino groups of the polymer. [Jiang *et al.* \(2010\)](#) exhibited that the polyethylenimine–cholesterol cationic lipopolymer/DNA complexes were low cytotoxic in A549 and MCF-7 cells. [Wu *et al.* \(2020\)](#) exhibited that hydrophobization of PEI with cholesterol was low cytotoxic compared with PEI. [Wang *et al.* \(2002\)](#) showed that PEI1.8K-CL and PEI10K-CL had slight cytotoxicity in Jurkat cells. [Sun *et al.* \(2014\)](#) exhibited that PEI-cholesterol liposomes did not show noticeable systemic toxicity in H22 hepatoma-bearing mice. [Li *et al.* \(2018\)](#) demonstrated that polyethylenimine–cholesterol was nontoxic in CAL-27 cells at the concentration of 0–20 µg/ml. [Lin *et al.* \(2017\)](#) demonstrated

that PEI25K induced autophagy in mouse fibroblast cells. [Anwer *et al.* \(2013\)](#) showed that PEG-cholesterol lipopolymer/plasmid IL-12 did not exhibit worsened side effects in the chemotherapy treatment of a phase I trial.

CONCLUSION

This study concluded that PEI25K-CL had potential to be an efficient and safe gene delivery carrier.

AUTHORS' CONTRIBUTIONS

WW and BY contributed to the concept and design. WW contributed to the data acquisition and interpretation and the writing and final approval of the manuscript. PO contributed to the research supervision.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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