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RESEARCH PAPER

Efficient Intracellular Gene Delivery Using the Formulation Composed of Poly (L-glutamic Acid) Grafted Polyethylenimine and Histone

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ABSTRACT

Purpose Inefficient endosomal escape and poor nuclear import are thought to contribute to low gene transfer efficiency of polycations. To overcome these drawbacks, we prepared multiple gene delivery formulations including low cytotoxic polycation, histone containing NLSs and chloroquine as the endosomolytic agent.

Methods Comb-shaped poly (L-glutamic acid) grafted low-molecular-weight polyethylenimine (PLGE) copolymer was synthesized by aminolysis of poly-γ-benzyl-L-glutamate using low-molecular-weight polyethylenimine (800 Da). The formation of DNA/histone/PLGE terplex was observed by atomic force microscope and gel retardation assay. The particle size and zeta potential of DNA complexes with varying content of histone were also measured to confirm the terplex formation. Cytotoxicity of vectors was assayed by MTT. Multiple gene delivery formulations were optimized to their best transfection efficiency that was monitored by fluorescence microscope and flow cytometry. *In vivo* gene delivery of the optimal formulation was evaluated by the GFP-expression levels in drosophila melanogaster.

Results The DNA/histone/PLGE terplex was successfully formed. The PLGE and histone together condensed DNA into small, discrete particles (less than 200 nm in diameter) in isotonic solution. Cytotoxicity of PLGE and histone were much lower than that of PEI 25 K. Either histone or chloroquine contributed to enhancing the levels of transfection activity of PLGE polymer. However, chloroquine and histone did not show a synergistic effect on the improvement of transfection efficiency. The optimal formulation was the DNA/histone/PLGE terplex at the N/P ratio of 15 and histone/ DNA weight ratio of 0.8. Compared with Lipofectamine 2000 and PEI 25 K, the optimal formulation showed significantly increased levels of GFP-expression both *in vitro* and *in vivo*.

Conclusion This formulation provided a versatile approach for preparing high efficiency of the polycation-based gene vectors. It also reinforced the finding of earlier studies that nuclear import and endosomal escape were rate-limiting steps for nonviral gene delivery.

KEY WORDS chloroquine · gene delivery formulation · histone · poly (L-glutamic acid) · polyethylenimine · transfection efficiency

ABBREVIATIONS

2-HP	2-Hydroxypyridine
DMSO	Dimethyl sulfoxide

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FBS	fetal bovine serum
GFP	green florescent protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NLS	nuclear localization signal
PBLG	poly-γ-benzyl-L-glutamate
pDNA	plasmid DNA
PEI 25K	polyethylenimine with molecular weight of 25KDa
PLGE	poly (L-glutamic acid) grafted low-molecular-weight polyethylenimine

INTRODUCTION

Current gene therapy has not proven very successful in clinical trials. It is mainly hampered by the lack of safe and efficient vectors to deliver therapeutic genes to the patient's target cells (1,2). The most common vectors are the recombinant viruses, including adenoviruses, retroviruses and adeno-associated viruses. These viral vectors deliver their genes to the target cells with high efficiency but in a pathogenic manner (3). Moreover, the host's immune response and malignant transformation are the limitations to the clinical development of viral gene therapy (4,5). Unlike virus-mediated gene-delivery systems, nonviral vectors based on polycations offer a safe alternative for gene delivery. The polycations are able to condense DNA into nano-sized polyplex by electrostatic interaction and release DNA into the cytoplasm by polymer ionization (6). This approach diminishes the risk of pathogenic and immunological complications, and has significant clinical potential. However, most of the polymeric vectors show much lower transfection efficiency than the viral vectors. The ineffective intracellular trafficking is thought to be mainly responsible for this low efficiency (7).

The intracellular trafficking of polymeric vectors includes binding to cellular membrane, uptake by endocytosis, translocation of DNA from the endosomes to the cytosol and, finally, nuclear transport of DNA (8). These barriers limit the transfection efficiency of polycation gene delivery systems. Different formulation strategies have been attempted to overcome these intracellular barriers. To enhance endosomal escape of DNA, fusiogenic peptides, such as KALA and GALA (9,10), and lysosomotropic reagents, such as chloroquine (11), have been employed. Chloroquine is known to disrupt the endosomal membrane by increasing the pH of the endosome environment and facilitating escape of the complexed DNA from the endosomes to the cytoplasm (12). Several studies show that chloroquine increases the rate of endosomal escape, resulting in improvement in transfection efficiency of polymeric vectors (13,14). Among the intracellular barriers, the rate-limiting step is reported to be the entry into the nucleus (15). Although the mechanism of nuclear transport is not clear, the trafficking of DNA from the cytoplasm to the nuclei is improved by using nuclear localization signal (NLS) to modify either the vector or the DNA itself (16). That suggests that NLS-mediated pathways are involved in the transport of exogenous DNA into the cell nucleus. The most commonly used NLS peptide is the first identified nuclear localization sequence PKKKRKV found in SV40 large T-antigen. The classical NLS of SV40 large T-antigen is transported into the nucleus via the formation of a NLS-substrate/importin alpha/beta complex (17). Histones are the chief nuclear protein components of chromatin. Five types of histone proteins have been identified, namely H1, H2A, H2B, H3 and H4 (18). They are enriched in positively charged residues of lysine and arginine, which may interact with negatively charged DNA via electrostatic interactions. Histones are known to contain at least one NLS in their amino-terminal tail. They have been used as a gene-transfer vector for the efficient delivery of DNA from cytoplasm into nucleus (19).

We have recently developed a method to synthesize a biodegradable comb-shaped copolymer, poly (L-glutamic acid) grafted low-molecular-weight polyethylenimine (PLGE), and demonstrate its potential applications in gene delivery (20). The PLGE has significantly lower cytotoxicity and higher transfection efficiency than that of PEI 25 kDa. In this study, we propose a new gene delivery formulation for improving the intracellular trafficking to further increase transfection efficiency. This formulation is composed of pEGFP-C1/histone/PLGE terplex and chloroquine. The histone and chloroquine were used to facilitate nuclear import and endosomal escape, respectively. The formation of pEGFP-C1/histone/PLGE terplex was observed by atomic force microscope and gel electrophoresis. MTT assay was performed to investigate the cytotoxicity of the compounds in the formulations. These formulations were specifically evaluated both *in vitro* and *in vivo* for their transfection efficiencies and transgene expression levels.

MATERIALS AND METHODS

Materials

Histone was obtained from Worthington Biochemical Corporation. Polyethylenimine with average molecular weight of 800 Da (PEI 800) and 25 kDa (PEI 25 K) was purchased from Sigma-Aldrich. Poly-γ-benzyl-L-glutamate (PBLG) was synthesized in our laboratory, and the detailed synthesis can be found in Refs (20) and (21). The pEGFP-C1 plasmid was a gift from West China University of

Medical Sciences. EndoFree Plasmid Kit was purchased from Tiangen. The HeLa cell line was obtained from the American Type Culture Collection. 2-Hydroxypyridine (2-HP) was purchased from Fluka. Lipofectamine 2000 was obtained from Invitrogen Corporation. RPMI-1640 medium, fetal bovine serum (FBS) and penicillin-Streptomycin were purchased from Gibco. Agarose was purchased from Biowest. Ethidium bromide was purchased from Invitrogen. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. All the other reagents were of analytical grade.

Synthesis of PLGE Polymer

PLGE was prepared by aminolysis of poly-γ-benzyl-L-glutamate (PBLG) using polyethylenimine with average molecular weight of 800 Da (PEI 800) as previously described (Scheme 1) (20). In brief, PBLG was dissolved in 20 ml of DMF and placed in the oil bath at 40°C. PEI 800 (10× mol ratio to the ester groups of PBLG) and 2-hydroxypyridine (2-HP) (5× mol ratio to the ester groups of PBLG) were added to the reaction mixture, and the solution was stirred for 48 h. The product PLGE was obtained after precipitation in cold ether, followed by dialysis against water for 48 h using a dialysis bag with a 3.5 kDa molecular weight cut-off (MWCO), and finally freeze-drying. The structure and molecular weight of PLGE were characterized by ^{1}HNMR and gel permeation chromatography.

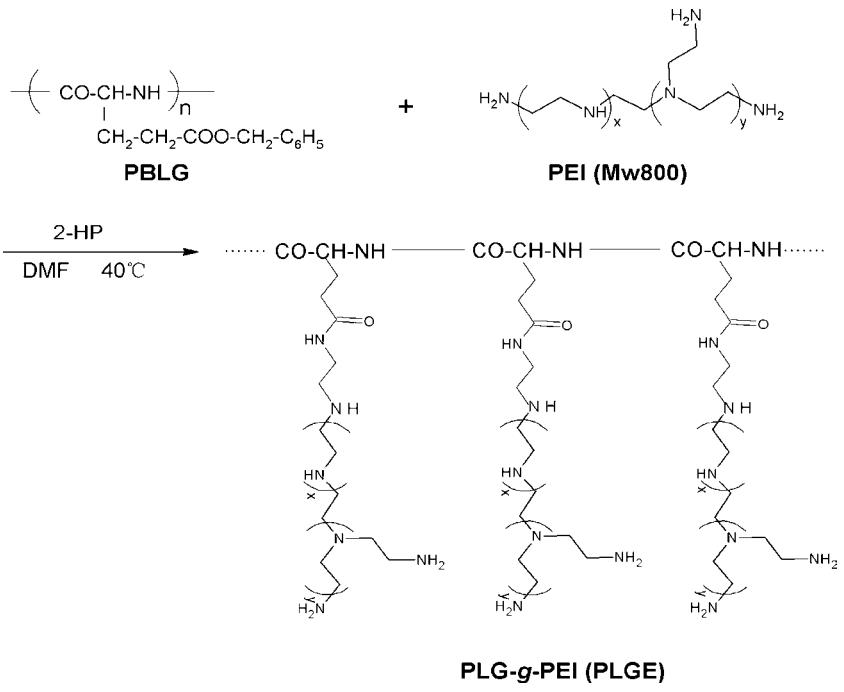
Formation of pDNA/histone/PLGE Terplex

Plasmid pEGFP-C1 was condensed with both PLGE and histone to form terplex, as Scheme 2. In brief, pEGFP-C1 was mixed with histone solution based on weight ratio of histone to pDNA from 0.5 to 1.5 by gentle vortexing. It was further incubated at room temperature for 15 min to allow spontaneous pDNA/histone complex precursor formation. In order to adjust the N/P ratio (the molar ratio of amino groups of PLGE to phosphate groups of pDNA), various amounts of PLGE were added to pDNA /histone complex and were rapidly mixed by pipetting. The resulting mixture was incubated for 30 min at ambient temperature, yielding the pDNA/histone/PLGE terplex.

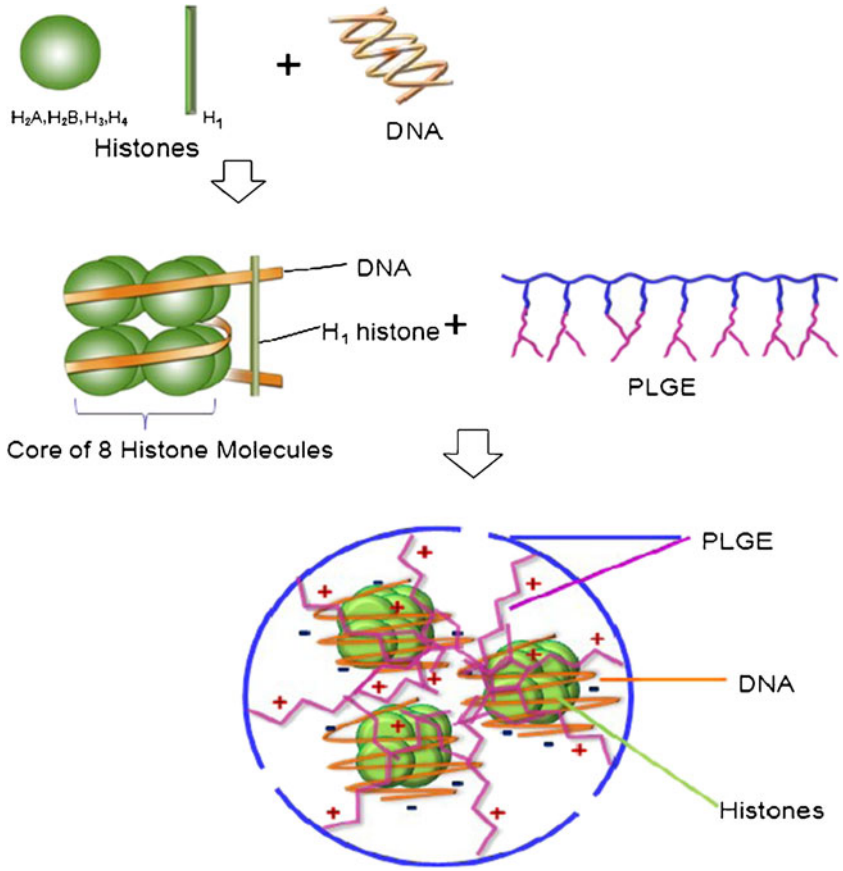
Measurement of the Particle Size and Zeta Potential

The particle size of pDNA/histone/PLGE terplexes was measured by using a photon correlation spectroscopy (PCS) on a Malvern Zetasizer NS90 (Malvern Instruments, UK). The instrument was equipped with a 10-miliWatt helium neon laser producing light at a wavelength of 633 nm. pDNA complexes were dispersed in 5% glucose solution. Measurements were done in cuvettes at 25°C with a fixed scattering angle of 90° through a 400 μm pinhole. Each data point is comprised of at least three independent experiments.

Zeta potential values were also obtained using a Zetasizer NS90 with a He–Ne laser beam. All measurements were done at a wavelength of 633 nm at 25°C with a



Scheme 1 Schematic diagram of PLGE synthesis. Low-molecular-weight PEIs were grafted to poly (L-glutamic acid) to form the comb-shaped PLGE copolymers.



Scheme 2 Formation of pDNA/histone/PLGE terplex.

scattering angle of 90°. Samples were dispersed in 10 mM NaCl solution, and zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results were the mean of five determinations ± standard deviation.

Gel Retardation Assay

To determine whether pDNA was condensed by histone and PLGE, a series of gel retardation assays were performed by electrophoresis. The pDNA/histone/PLGE terplex formulated at the optimal N/P ratio of 15, and varying weight ratios of histone to pDNA were prepared. Each complex sample was loaded into wells of a 1.0% agarose gel prepared in Tris-acetate-EDTA (TAE) buffer containing 0.5 μg/ml ethidium bromide. Samples were subsequently electrophoresed in an electric field of 110 V for 60 min. Bands corresponding to pDNA were visualized on a UV transilluminator (Benchtop UV Transilluminator GDS 8000, USA) and photographed.

Atomic Force Microscopy (AFM)

The morphology of pDNA/histone/PLGE terplex was observed by AFM. All AFM experiments were carried out using a Digital Instruments Nanoscope IIIa (Digital Instruments, Santa Barbara, CA, USA). In a typical experiment, the pDNA/histone/PLGE terplex was prepared according to the aforementioned protocol. A drop of freshly prepared samples (15 μl) was immediately dropped onto the surface of freshly prepared mica. The sample was allowed to dry at room temperature for 24 h, and the images were scanned and saved.

Cell Culture

HeLa cells were cultured in RPMI-1640 medium supplemented with 10% defined fetal bovine serum (FBS), penicillin G (100 kU/l), and kanamycin (0.1 g/l). Cells were cultured in a culture incubator at 37°C and in a 90% humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

Cytotoxicity of PLGE, histone and chloroquine in formulations was evaluated by MTT colorimetric assay. Briefly, HeLa cells were seeded in 96-well plates at a density of 5,000 cells/well. After 24 h, culture medium was replaced with serial dilutions of samples in full medium, and cells were incubated for another 24 h. Then, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide) in 5 mg/mL in phosphate-buffered saline solution was added to each sample. After the samples were incubated for 4 h, the supernatant was aspirated, and the formazan crystals were dissolved in 150 μl DMSO. Absorption was measured photometrically at 570 nm with a background correction using a Bio-Tek ELX800 ELISA reader. Values of eight measurements were normalized to 100% for the control group (exposure to full medium). Cells without addition of MTT were used as a blank for calibration of the spectrophotometer to zero absorbance. The turnover of the substrate relative to control cells was expressed as relative cell viability and was calculated by the $A_{(\text{test})}/A_{(\text{control})} \times 100\%$ formula.

In Vitro Transfection Efficiency Study

HeLa cells were seeded 24 h prior to transfection into 24-well plates at a density of 1.5×10^4 cells per well in 1.0 ml of culture medium. Prior to the experiment, the cells were rinsed twice with warm phosphate-buffered saline (PBS, pH 7.4). Then, 0.2 ml of pDNA/histone/PLGE terplex and 0.8 ml of serum-free or 10% serum-containing medium were added. The final pDNA concentration was 1.0 μg/well. After transfection for 4 h at 37°C in 90% humidified atmosphere and 5%CO_2, the cells were rinsed with warm PBS and supplied with 1.0 mL of culture medium. After 48 h of incubation allowed for enhanced green fluorescent protein expression, the cells were rinsed twice with PBS. The green fluorescence was observed by fluorescence spectroscopy (Olympus BX51 Fluorescence Spectroscopy, Japan). Subsequently, cells were treated with trypsin/EDTA for 2 min, collected by centrifugation, suspended in 0.5 ml PBS and kept on ice until analysis. The percentage of GFP-expressing cells was employed to quantify transfection efficiency via flow cytometry using the Beckman Coulter ESP ELITE System (Beckman Coulter, USA) equipped with an argon laser with an excitation wavelength of 488 nm. The filter setting for emission was 530/30 nm bandpass. Data were acquired in linear mode and visualized in linear mode.

In Vivo Gene Expression Study

In vivo gene delivery of the optimal formulations was evaluated by the level of GFP expression in drosophila melanogaster. The wild-type Canton S flies are widely used as the animal model for drug delivery and pharmacological investigation (22,23). Here, wild-type Canton S flies were maintained in bottles containing an agar, corn meal, corn syrup, water, and dried yeast medium at 25°C and in a 60–70% humidified atmosphere. Two-day-old wild-type Canton S flies with 2–2.5 cm in length were used in these studies. The optimal DNA/histone/PLGE terplex formulation was prepared at the N/P ratio of 15 and histone/pDNA weight ratio of 0.8. PEI 25 K and Lipofectamine 2000 under their corresponding optimal conditions were used as positive controls. Samples containing 0.8 μg of pEGFP-C1 were injected into the thoracic cavity of wild-type Canton S flies using a XenoWorks™ Digital Microinjector (Sutter Instrument, USA). After injection, the flies were placed into vials and fed for 48 h. The GFP expression levels in wild-type Canton S flies were observed by fluorescence spectroscopy (Olympus BX51 Fluorescence Spectroscopy, Japan). The GFP pictures were taken under the fluorescent light with exposure time 4 sec and a gain of 2. As a measure of fluorescence intensity, the gray value for each fly in GFP-expression fields was determined with the Image J software.

Statistical Analysis

All data were expressed as averages with standard deviations. First, ANOVA was used to determine statistical difference among the groups, and then pair-wise comparison was conducted using the student *t*-test. A p-value of <0.01 on a two-tailed test was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of Comb-Shaped PLGE Polymers

It is well known that the transfection efficiency of PEI is highly dependent upon its molecular weight. For example, PEI with the high molecular weight has high transfection efficiency, whereas PEI with low molecular weight shows lower transfection efficiency (24). High-molecular-weight PEI 25 K is considered one of the most efficient non-viral vectors for gene delivery and has been used as a standard reference when compared with other newly designed polymers (25). Unfortunately, the-high molecular-weight PEI has high cytotoxicity that restricts its clinical applications. It is thought that PEI 25 K formed the aggregates with high charge-density on cell surface, resulting in cell necrosis (26). In general, the cytotoxicity of PEI 25 K can be reduced by conjugating poly (ethylene glycol) to the periphery of the branched PEI 25 K. However, the PEGylated PEI 25 K showed a reduction in its transfection efficiency (27). We have recently developed a method to synthesize a novel polycation, poly (L-glutamic acid) grafted low-molecular-weight polyethylenimine copolymer (PLGE). The chemical structure of the PLGE comb-shaped polymers is shown in Scheme 1. The PLGE exhibited significantly lower cytotoxicity compared with PEI 25 K. This comb-shaped copolymer was composed of biodegradable poly (L-glutamic

acid) as backbone and low-toxic PEI 800 as side chains. Many PEI 800 side chains were connected on backbone to form the high-molecular-weight PEI derivates. We made the effort to combine the merits of high transfection efficiency of the high-molecular-weight PEI and low toxicity of low-molecular-weight PEI. In this study, we used the PLGE as the core composition to condense DNA into nano-size particles and protect DNA from the enzyme degradation in the non-viral gene delivery formulation.

Formation of pEGFP-C1/histone/PLGE Terplex

In polymeric gene delivery system, the vectors transfer DNA into cells and dissociate it in the cytoplasm, but the

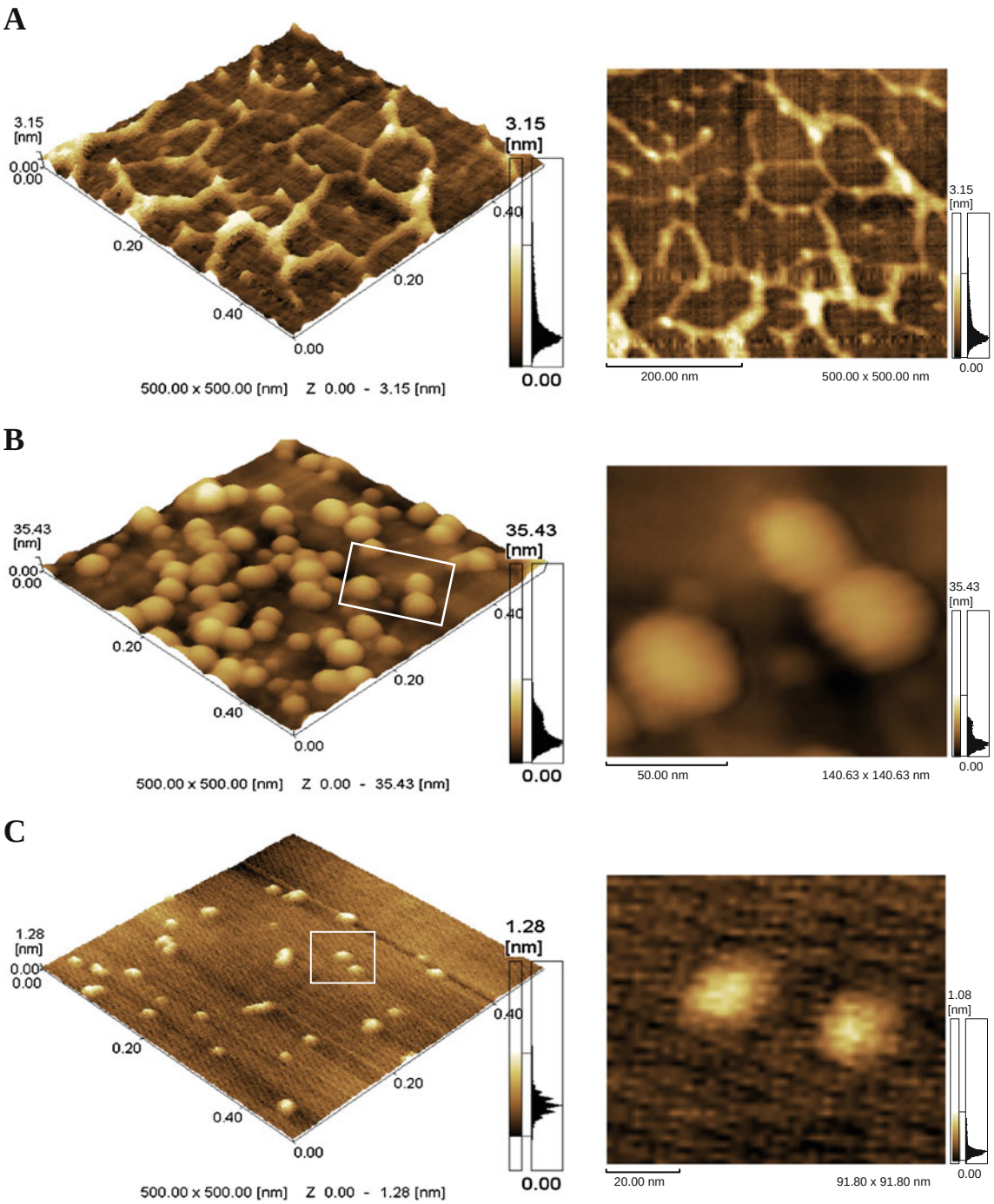


Fig. 1 AFM images (**A**) The image for pEGFP-C1; (**B**) The image for pEGFP-C1/ histone complex at weight ratios of 1.0; (**C**) The image for pEGFP-C1/ histone/PLGE terplex at the N/P ratio of 15 and the weight ratio of histone to pDNA of 1.0.

entry into nucleus is dependent on DNA itself. Therefore, the rate-limiting step for intracellular transport of DNA polyplexes is thought to be the nuclear transport (15). In this study, the histones containing NLSs were added to the gene delivery formulation to aid the delivery of DNA into the cell nucleus. The plasmid pEGFP-C1, histone and PLGE polycation in the formulation were a formed ternary complex via electrostatic interactions, as depicted in Scheme 2. First, pEGFP-C1 was interacted with histones to form the binary complex. The histone, as a basic protein, has a general high affinity for DNA. The structure of histone is usually formed octamers that consist of pairs of each of the four core-histones (H2A, H2B, H3, and H4) and one linker histone (H1) (28). The models of DNA/histone complex have been suggested such that the histones were arranged differently either relative to the base sequence of DNA or relative to the sugar-phosphate skeleton: H3 and H4 in the large gutter, H2A and H2B in the small gutter, and H1 as a cross-linkage between DNA molecules (29). Then, the comb-shaped PLGE polycations were added in the solution containing DNA/histone complex. The grafting chains of low-molecular-weight PEIs in PLGE could combine with DNA/histone complex to form the pEGFP-C1/histone/PLGE terplex.

In order to gain further insight into the structure of this terplex, AFM imaging of pEGFP-C1, pEGFP-C1/histone complex and pEGFP-C1/Histone/PLGE terplex was performed. The image of bare pEGFP-C1 (4.8 kbp) showed the irregularly branched DNA constructs (Fig. 1a). However, pEGFP-C1 and histone at the weight ratio of 1.0 formed a complex with a uniform spherical structure and an approximate diameter of 50 nm, as shown in Fig. 1b. Based on this pEGFP-C1/histone complex, PLGE polycation further condensed the binary complex at the N/P of 15 into highly compact structures. The particle size was about 20 nm (Fig. 1c). These AFM images confirmed the successful formation of the desired pEGFP-C1/histone/PLGE terplex as schematically illustrated in Scheme 2.

We also used the agarose gel electrophoresis to assay the formation of pEGFP-C1/histone/PLGE terplex, as shown

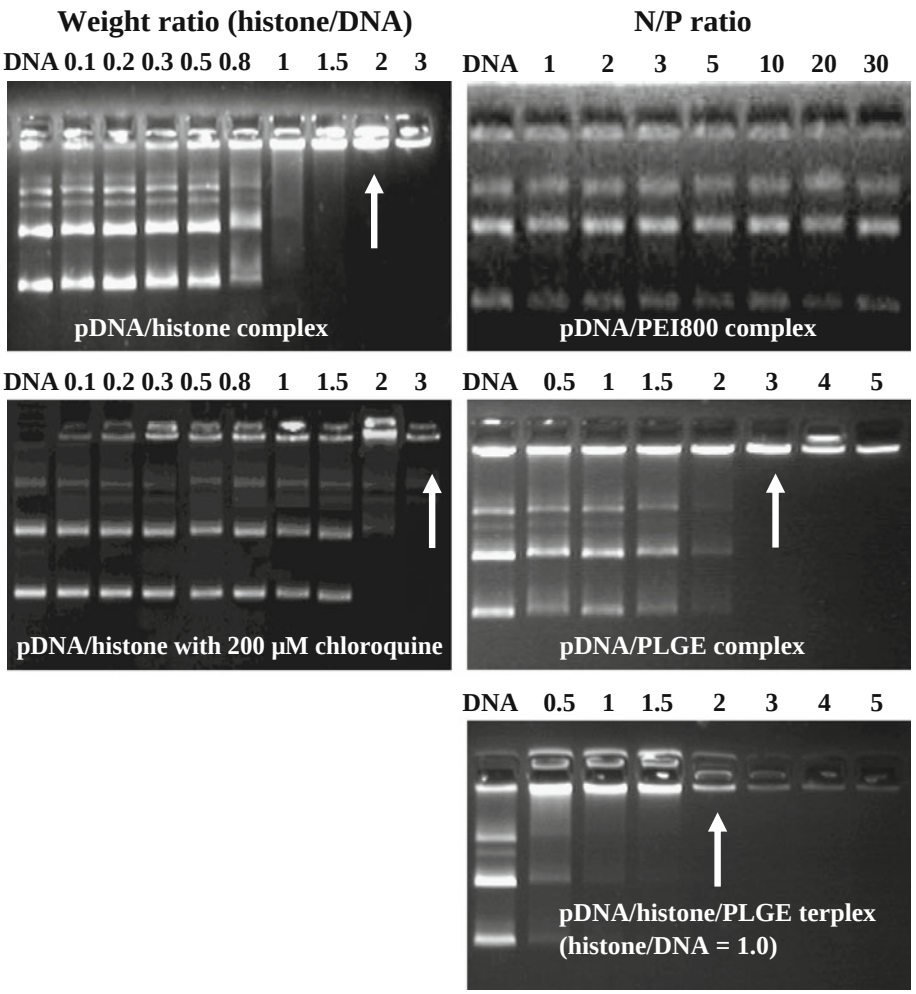


Fig. 2 Agarose gel electrophoresis to assay the formation of pEGFP-C1/histone/PLGE terplex.

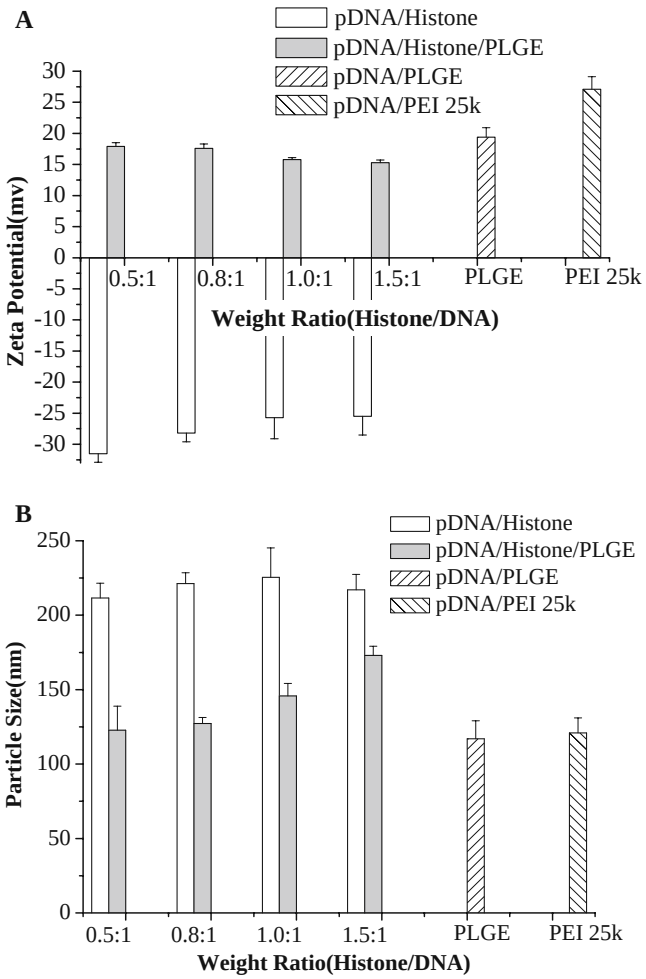


Fig. 3 Zeta potential (**A**) and particle size (**B**) of pDNA/histone/PLGE terplex at the N/P ratio of 15 and varying weight ratios of histone to pDNA in 5% glucose solution.

in Fig. 2. The electrophoretic migration of pEGFP-C1/histone complex in agarose gel was completely retarded above the histone/DNA weight ratio of 2.0. After the pDNA/histone complex incubated with 200 μM chloroquine for 15 min, the migration of pDNA was retarded completely at the histone/DNA weight ratio increased to 3.0. That suggested chloroquine weakened some interactions between pEGFP-C1 and histone. For PLGE alone, pEGFP-C1 was completely retarded at the N/P ratio of 3.0 and higher N/P ratio. This retarded N/P ratio was much lower than that of PEI 800, which could not retard pEGFP-C1 at the N/P of 30. Furthermore, pEGFP-C1/histone/PLGE terplex with the histone/pDNA weight ratio of 1.0 could completely retard pDNA at the N/P ratio of 2.0. The retarded N/P ratio of histone/PLGE was close to PEI 25 K (N/*P*=1.6) (30). The results indicated that histone/PLGE as the combined vector had the lower retarded N/P ratio than that of PLGE alone. This suggested that histone could assist PLGE to condense DNA.

Particle Size and Zeta Potential of pEGFP-C1/Histone/PLGE Terplex

The interaction between DNA, nuclear proteins and polycations is thought to be the electrostatic interaction (31). To further interact with PLGE polycations, the negatively charged pEGFP-C1/histone complex was prepared first. Without PLGE condensation, pEGFP-C1/histone complex at the weight ratio of histone to pDNA from 0.5 to 1.5 in 5%

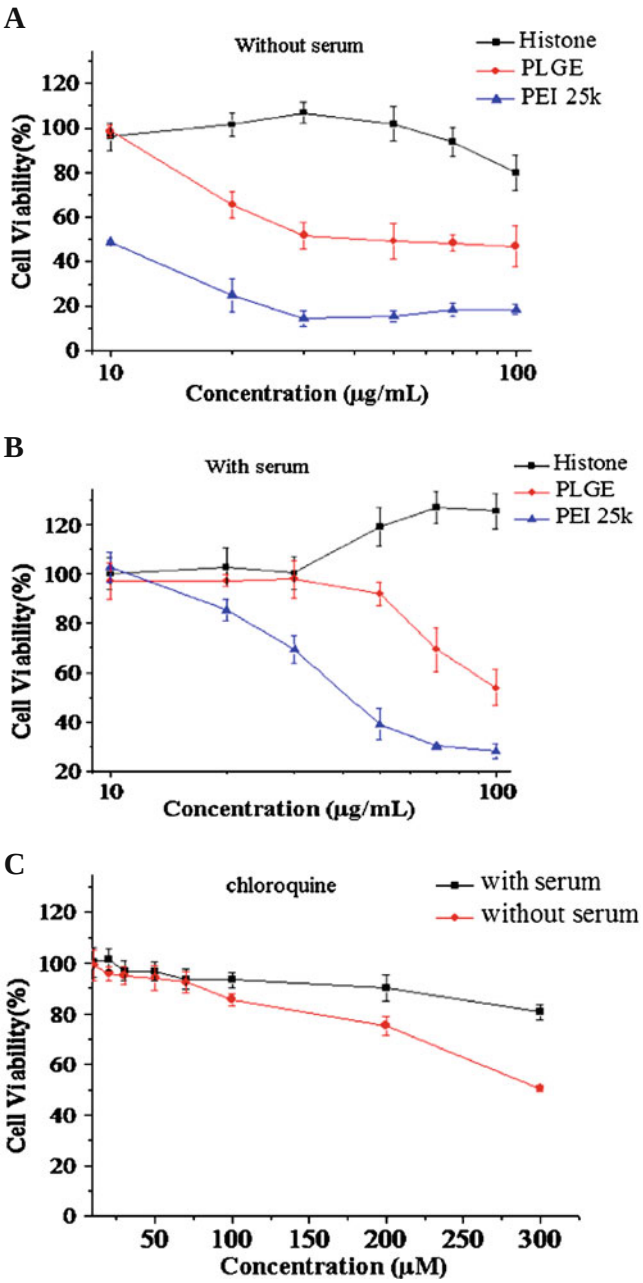


Fig. 4 Cytotoxicity of (**A**) histone, PLGE, PEI25K in serum-free medium, (**B**) histone, PLGE, PEI25K in serum-containing medium, and (**C**) Cytotoxicity of chloroquine on HeLa cells.

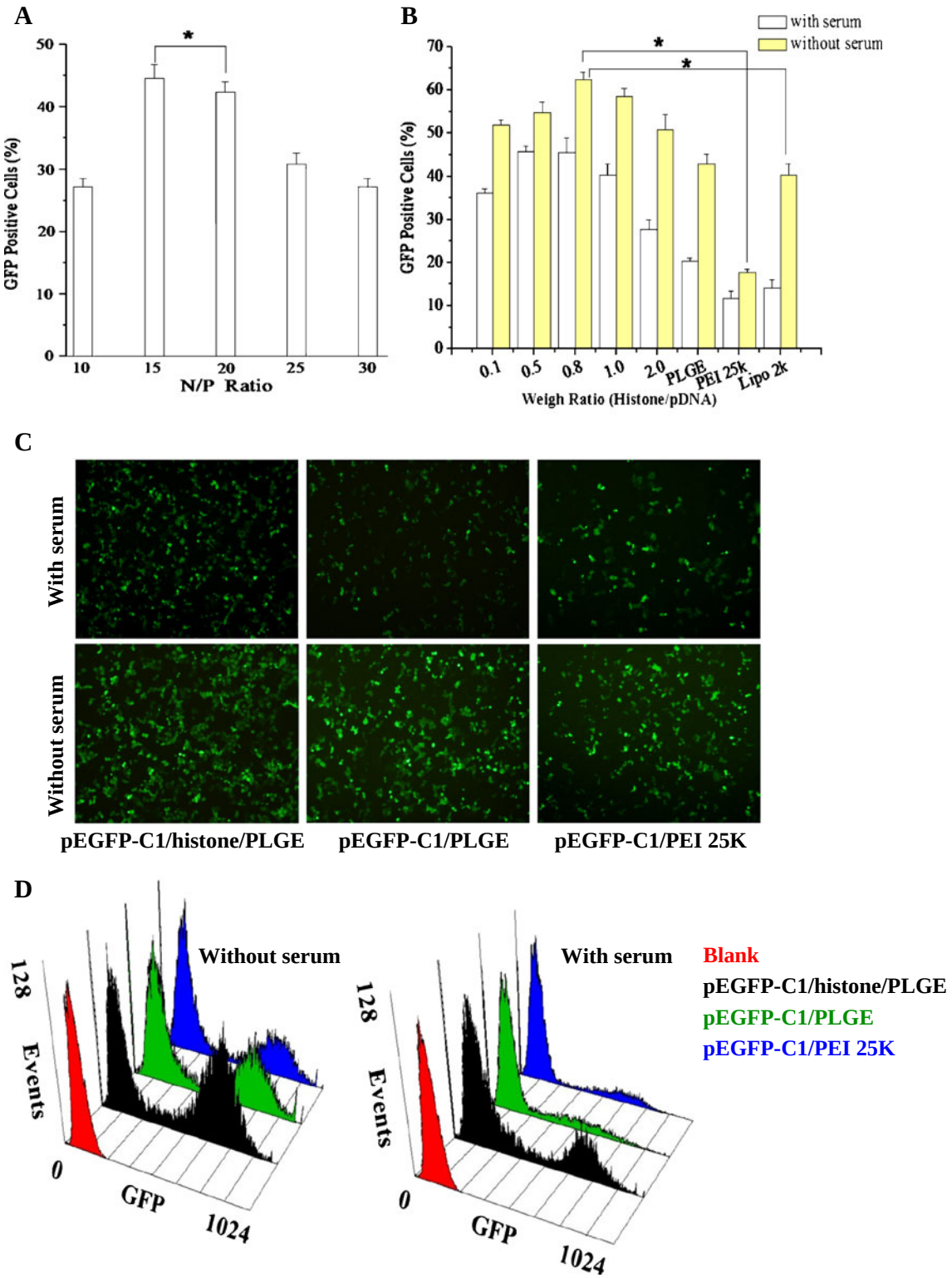


Fig. 5 (**A**) PLGE was optimized to its best transfection efficiency based on the N/P ratio. Significant differences were marked with an asterisk, $*p < 0.01$. (**B**) effect of histone on the PLGE polycations mediated gene delivery. HeLa cells were transfected with pEGFP-C1/histone/PLGE terplex at the N/P ratio of 15 and varying weight ratios of histone/pDNA. The percentage of cells transfected was assessed 48 h post-transfection. Significant differences were marked with an asterisk, $*p < 0.01$. (**C**) HeLa cells were transfected with pEGFP-C1/histone/PLGE terplex. The cells were analysed for GFP expression using a fluorescent microscope. (**D**) FACS analysis showing profile of the optimal transfection efficiencies on HeLa cells.

glucose isotonic solution showed the negatively charged surface with the zeta potential range between −35 mV and−25 mV, as shown in Fig. 3a. These findings indicated that phosphate anions in pEGFP-C1 were not sufficiently masked by histones. At the N/P ratio of 15, all the samples of the pEGFP-C1/histone/PLGE terplex had the positive zeta potential values, demonstrating that the PLGE polycation interacted with pEGFP-C1/histone complex. It is worth mentioning that the absolute zeta potential values of both the pEGFP-C1/histone and pEGFP-C1/histone/PLGE complex slightly reduced with increasing amount of histone. This may be because histone has an amphoteric character and contains the positively charged residues of lysine and arginine and negatively charged aspartic acid and glutamic acid, which could interact with both pDNA and PLGE. Figure 3b shows that the size of pEGFP-C1/histone/PLGE terplex with varying amount of histone at the N/P ratio of 15 was about 120–180 nm. All the measured sizes of terplex were smaller than that of pEGFP-C1/histone complex. These results were consistent with the size trend observed by AFM.

Cytotoxicity of the Components in Formulation

The cytotoxicity of PEI is known to be concentration-dependent, structure-dependent as well as molecular weight-dependent (32). The branched and high molecular weight of PEI 25 K shows high cytotoxicity, which is the major problem in its application *in vivo*. The components in the gene delivery formulation presented in this study were evaluated to conclude their safety profile for the possible further applications. The colorimetric MTT assay was carried out. The effect of histone, PLGE and chloroquine on HeLa cell viability was determined as functions of concentration or incubation medium, as shown in Fig. 4. A commercial PEI 25 K was used as a positive control to compare the relative toxicity of PLGE. Figure 4a showed that PLGE maintained the cell viability >90% at the concentration of 10 μg/ml. However, the cell viability was less than 50% when the cells were treated with PEI 25 K at the same concentration. All the samples of PLGE showed significantly higher cell viability as compared with PEI 25 K in serum-free or serum-containing medium. The enhanced cell viability of PLGE was undoubtedly the result of the low-toxic composition in the comb-shaped PLGE polymers, which was formed by the low-molecular-weight PEI molecules grafted to the biodegradable PLG backbone. The histone did not show any toxicity at the concentration of 70 μg/mL in serum-free medium and even at a high concentration of 100 μg/mL in serum-containing medium, respectively. These results demonstrated that both PLGE and histone exhibited a significantly lower cytotoxicity in comparison with PEI 25 K. The chloroquine inhibited HeLa proliferation also by concentration-dependent manner. The optimal concentration of chloroquine used in transfection experiments was 200 μM, showing slight toxicity with the cell viability of 90% in serum-containing medium and more toxicity with the cell viability of 75% in serum-free medium.

The Multiple Formulations Optimized for Their Transfection Efficiency

The relatively low efficiency of gene transfer is a major drawback of polycations as non-viral vectors. Poor release of the DNA polyplex from the endosome into the cytoplasm and, subsequently, inefficient transfer into the nucleus are thought to contribute to the low gene transfer efficiency of polycations (33, 34). In this study, we prepared the gene delivery formulation mainly composed of PLGE polycation, histone and chloroquine. In the formulation, low cytotoxic

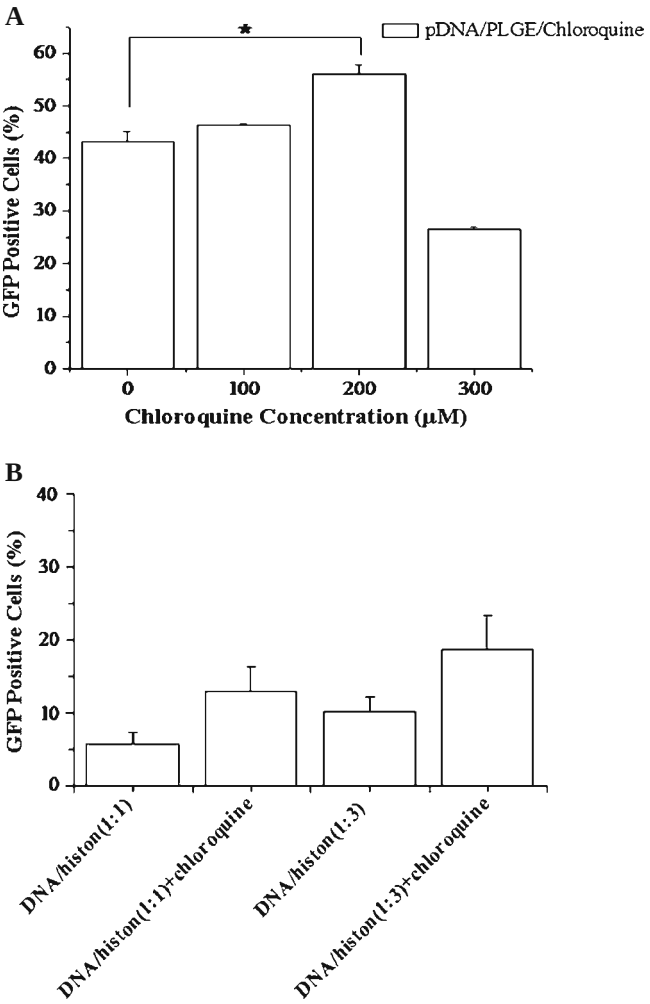


Fig. 6 Effect of chloroquine on the PLGE polycations and histone-mediated gene delivery. (**A**) HeLa cells were transfected with pEGFP-C1/PLGE complex in the presence of varying concentration of chloroquine. The percentage of cells transfected was assessed 48 h post-transfection (*$p < 0.01$). (**B**) HeLa cells were transfected with pEGFP-C1/ histone complex in the presence of 200 μM chloroquine in serum-free medium.

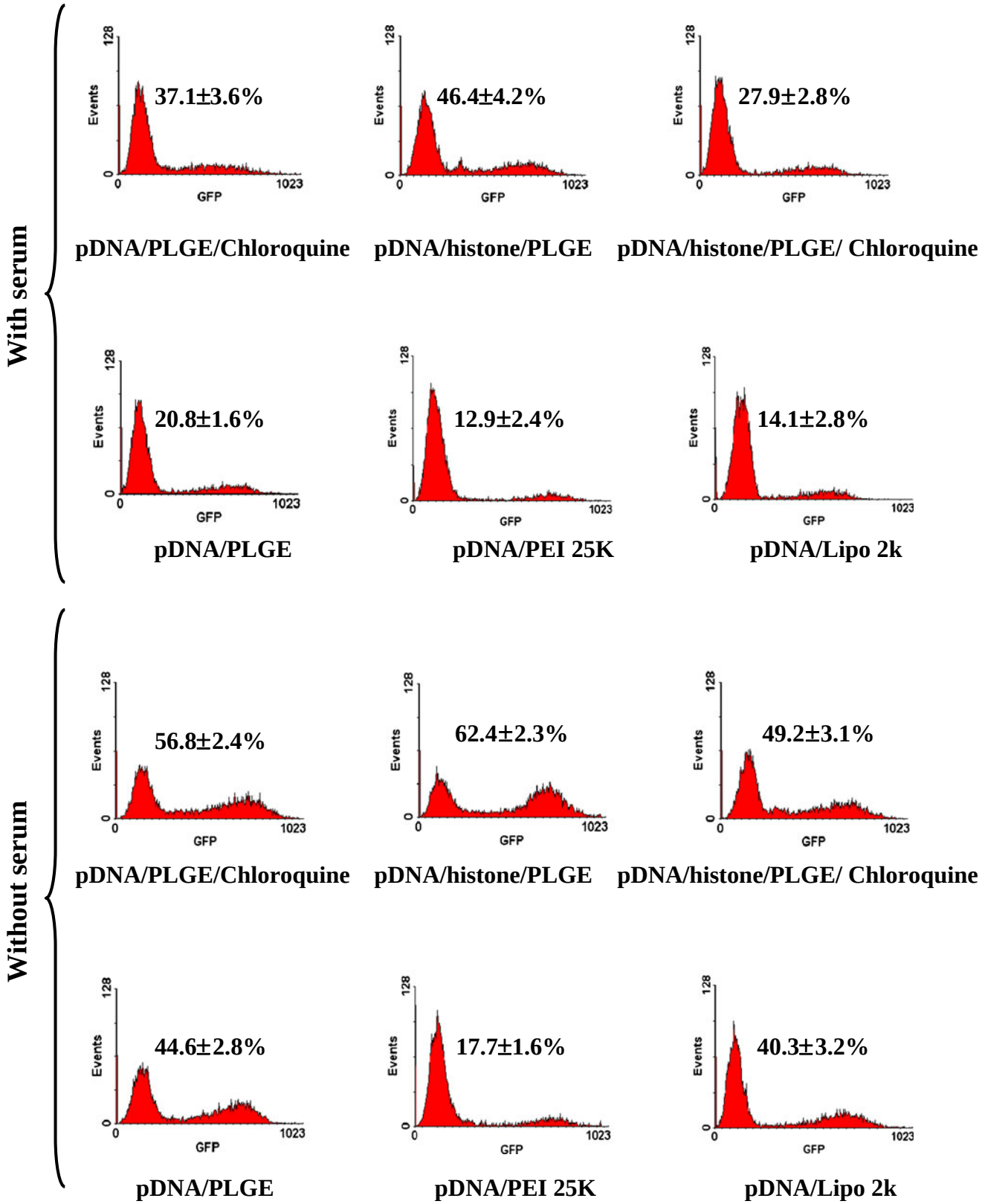
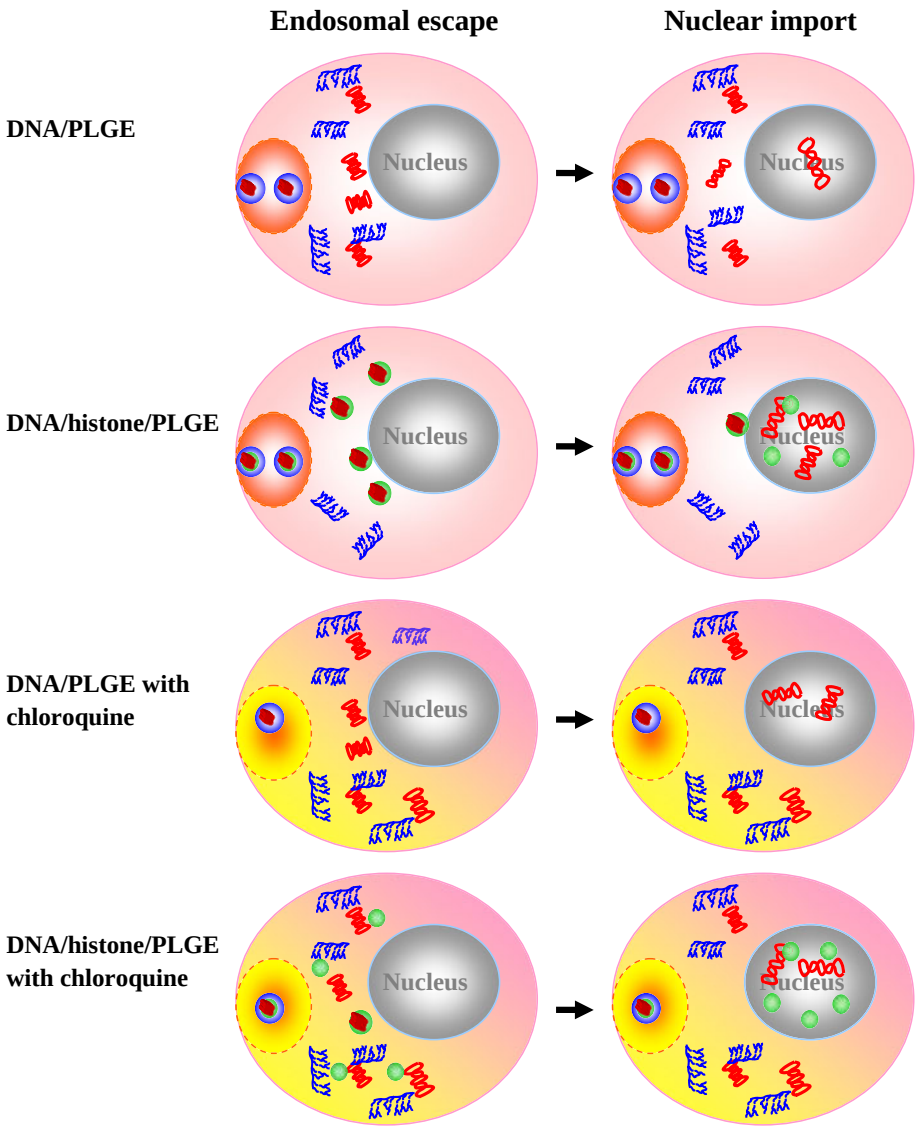


Fig. 7 Effect of histone and chloroquine on the PLGE polycations-mediated gene delivery. FACS analysis showed the profiles of HeLa cells transfected with these formulations under their optimal conditions.

PLGE polycation is the main component to condense and transfer exogenous gene, the chloroquine is the endosomolytic agent, and histone containing NLSs was expected to enhance the endosomal escape and nuclear import, respectively. To investigate whether this formulation could increase the efficiency of nonvirus-mediated gene transfer, the formulation of pDNA/histone/PLGE terplex and chloroquine was assessed for *in vitro* transfection efficiency by green fluorescent protein (GFP) assay using pEGFP-C1. The percentage of cells transfected with the pEGFP-C1 as determined by FACS analysis and GFP expression levels were observed by fluorescent microscope. The optimal condition of PLGE for pEGFP-C1 delivery on HeLa cells was at the N/P ratio of 15, as shown in Fig. 5a. Figure 5b showed the GFP of HeLa cells transfected with the pDNA/histone/PLGE terplex at their optimal N/P ratio and various weight ratios of histone to pDNA. All the samples of pDNA/histone/PLGE terplex exhibited higher transfection efficiency than that of pDNA/PLGE complex without histone. It indicated that histone could enhance the gene transfer of polycations. The most effective pDNA/histone/PLGE terplex was at the weight ratio of histone to pDNA of 0.8 and at the N/P ratio of 15. The optimal pDNA/histone/PLGE terplex was approximately three or four times more effective than the commercial PEI 25 K in serum-free and serum-containing medium, respectively. The optimal pDNA/histone/PLGE terplex also showed the statistically significant difference in transfection efficiency compared to the well-established transfection reagent Lipofectamine 2000. This improvement of transfection over PLGE and PEI 25 K could also be seen by comparing the fluorescent micrographs and FACS histogram of GFP-expressing cells produced using the histone/PLGE, PLGE and PEI 25 K, as shown in Fig. 5c and d. The optimal



Scheme 3 Schematic diagram of the intracellular gene delivery by these formulations. *Blue lines*, PLGE; *green balls*, histone; *red coils*, DNA; *yellow color*, chloroquine.

pDNA/histone/PLGE terplex showed a much higher level of GFP fluorescence than PEI 25 K, especially in serum-containing medium.

Figure 6a showed the GFP of HeLa cells transfected with the pDNA/PLGE complex at the optimal N/P ratio of 15 in the presence of increasing concentrations of chloroquine (100–300 μM). The results suggested that in the presence of chloroquine (<300 μM), pDNA/PLGE complex significantly increased the percentage of GFP-positive cells, apparently because of chloroquine achieving more effective endosomal escape (35). The percentage of GFP-positive cells was maximal when the transfection was performed in the presence of 200 μM of chloroquine. It was noted that the abnormal cell morphology was observed after the cells were treated with a high concentration of chloroquine at 300 μM. It was consistent with the results of MTT assay.

Figure 6b showed that the transfection efficiency of either pDNA/histone complex or pDNA/histone complex in the presence of 200 μM chloroquine was less than 20%, which was much lower than that of pDNA/PLGE complex. It implied that the PLGE was a key component in the formulations. This result also indicated that the histone in the presence of chloroquine-mediated gene transfer achieved higher transfection efficiency than histone alone. It was consistent with reported results and explained that the chloroquine could help pDNA/histone complex disrupting the endosomal membrane (36,37).

We observed that the use of either histone or chloroquine could improve gene transfer efficiency of PLGE polycations, so the transfection efficiency of PLGE combined with both histone and chloroquine under their optimal condition of the N/P ratio of 15, the weight ratio of histone to pDNA of 0.8, and the chloroquine concentration of 200 μM was further investigated, as shown in Fig. 7. Although the formulation of pDNA/histone/PLGE terplex in the presence of chloroquine yielded better GFP expression and significantly increased transfection efficiency compared with PLGE and PEI 25 K, they could not further enhance the gene transfer activities compared with either of the pDNA/histone/PLGE terplex without chloroquine or the pDNA/PLGE complex in the presence of chloroquine. The chloroquine in cytosol might weaken some interactions bewteen DNA and histone based on the electrophoresis result, thus reducing nuclear translocation of DNA. A schematic sketch of these formulation strategies in this study is depicted in Scheme 3.

In Vivo GFP Expression Mediated by the pDNA/histone/PLGE Formulation

In vivo gene delivery of the optimal formulation, pDNA/histone/PLGE terplex at the N/P ratio of 15 and the histone/ DNA weight ratio of 0.8 was evaluated by the level of GFP expression in drosophila, as shown in Fig. 8. In parallel, commercial transfection reagents, Lipofectamine 2000 and PEI 25 K, under their corresponding optimal conditions, were compared to the pDNA/histone/PLGE formulation. GFP expression was observed throughout the whole body after the flies were injected with pEGFP-C1 and then cultured for 48 h. In fluorescent images, except for negative control physiological saline, all samples observed GFP expression around the thoracic cavity; therefore, we conclude that these formulations yield truly tissue-general expression in flies. Note that GFP expression in flies mediated by the pDNA/histone/PLGE formulation had obviously higher levels than that of Lipofectamine 2000 or PEI 25 K (Fig. 8a). As a

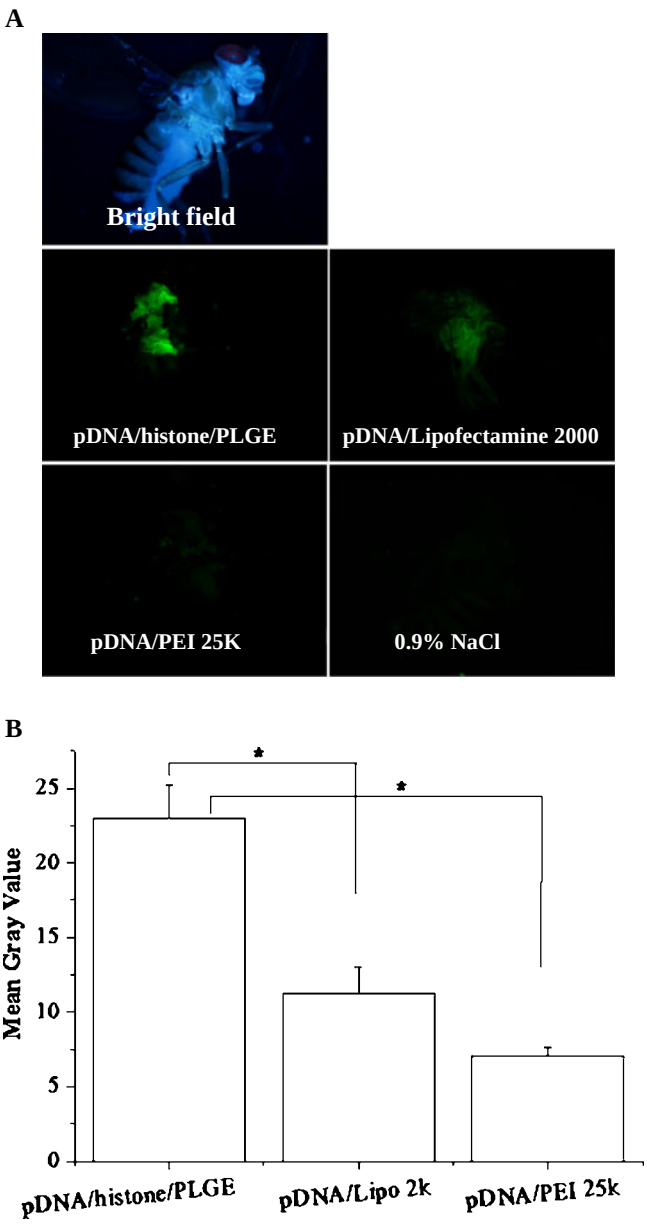


Fig. 8 (**A**) The images of GFP expression in drosophila melanogaster. (**B**) The mean gray value of the GFP expression levels in drosophila ($n = 3$), ($p < 0.01$).

measure of fluorescence intensity, the gray value for each fly in the GFP expression field was determined with the Image J software. The group treated with the pDNA/histone/PLGE formulation expressing the GFP domain showed statistically significant difference in mean gray values yielding a p value less than 0.01, as shown in Fig. 8b. This result demonstrated that the efficiency of *in vivo* gene delivery to flies by the histone/PLGE formulation was superior to Lipofectamine 2000 or PEI 25 K, which was consistent with our *in vitro* transfection findings.

CONCLUSIONS

To improve the efficiency of polycation-based gene vectors, chloroquine as the endosomolytic agent and histone-containing NLSs were included in the formulations based on biodegradable PLGE polycations. They were expected to enhance the endosomal escape and nuclear import that resulted in further improvement of tranfection efficiency of PLGE polycations. The pDNA, histone and PLGE polycations were successfully formed into nano-size particles (<200 nm in diameter). The transfection results suggested that either histone or chloroquine contributed to enhancing the transfection activity of PLGE polycations. Compared with PEI 25 K, the formulation of pDNA/histone/PLGE terplex in the presence of chloroquine showed significant increase in GFP expression and transfection efficiency. However, they could not further enhance the gene transfer activities compared with either of the pDNA/histone/PLGE terplex or the pDNA/PLGE complex in the presence of chloroquine. The optimal formulation was the DNA/histone/PLGE terplex at the N/P ratio of 15 and the histone/DNA weight ratio of 0.8. The efficiency of *in vivo* gene delivery to drosophila melanogaster by optimal formulation was also superior to Lipofectamine 2000 or PEI 25 K. This pDNA/histone/PLGE formulation provided a versatile approach to enhancing the efficiency of polycation-based gene vectors.

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