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RESEARCH PAPER

Enhanced Transfection by Antioxidative Polymeric Gene Carrier that Reduces Polyplex-Mediated Cellular Oxidative Stress

Min Sang Lee • Nak Won Kim • Kyuri Lee • Hongtae Kim • Ji Hoon Jeong

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ABSTRACT

Purpose To test the hypothesis in which polyplex-induced oxidative stress may affect overall transfection efficiency, an antioxidative transfection system minimizing cellular oxidative stress was designed for enhanced transfection.

Methods An amphiphilic copolymer (PEI-PLGA) was synthesized and used as a micelle-type gene carrier containing hydrophobic antioxidant, α-tocopherol. Cellular oxidative stress and the change of mitochondrial membrane potential after transfection was measured by using a fluorescent probe (H_2DCFDA) and lipophilic cationic probe (JC-1), respectively. Transfection efficiency was determined by measuring a reporter gene (luciferase) expression level.

Results The initial transfection study with conventional PEI/plasmid DNA polyplex showed significant generation of reactive oxygen species (ROS). The PEI-PLGA copolymer successfully carried out the simultaneous delivery of α-tocopherol and plasmid DNA (PEI-PLGA/Toco/pDNA polyplex) into cells, resulting in a significant reduction in cellular ROS generation after transfection and helped to maintain the mitochondrial membrane potential ($\Delta\Psi$). In addition, the transfection efficiency was dramatically increased using the antioxidative transfection system.

Conclusions This work showed that oxidative stress would be one of the important factors that should be considered in designing non-viral gene carriers and suggested a possible way to reduce the carrier-mediated oxidative stress, which consequently leads to enhanced transfection.

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INTRODUCTION

Recently, the potential toxicity of nanomaterials has attracted considerable attention since materials in nanoscale dimensions often display substantially different properties compared to bulk materials with the same composition and may have unexpected adverse effects on tissues and cells (1). Various inorganic or organic nanoparticles with diameters of a few hundred nanometers have been reported to elicit inflammatory responses, oxidative injury, cellular cytotoxicity, or genotoxicity (2,3). A major pathway responsible for these adverse effects is thought to involve oxidative stress, which is often the result of excess intracellular reactive oxygen species (ROS) (4–6).

Efficient transfection and low cytotoxicity are prerequisite properties for nonviral gene carriers to reach clinical settings (7). Since polycation-based gene delivery systems rely mostly on the formation of nano-sized polyplexes with nucleic acid drugs such as plasmid DNA (pDNA) and siRNA, it is highly likely that the polyplex will induce oxidative stress after cellular uptake. Polyethylenimines (PEIs) are among the most extensively studied cationic polymers for non-viral gene delivery because of their ability to form nano-sized particles with nucleic acid drugs and to mediate consistent transfection in several cell types (8,9). However, like most polycations used in non-viral transfection protocols, the relatively high cytotoxicity of PEI is considered a major drawback (10,11). The interactions of PEI with cellular membranes may reduce membrane fluidity by facilitating the formation of huge clusters on the surface of the membrane, leading to necrotic cell death (12,13). In addition, it was recently reported that PEI and its copolymer can induce oxidative stress responses in

epithelial cells and macrophages (14,15). Grandinetti *et al.* also showed that PEI/DNA polyplexes localized to mitochondria after transfection (16). The interaction of PEI polyplex with mitochondria seems to be related to interference of mitochondrial function and loss of mitochondrial membrane potential. The mitochondrial damages by polyplexes could stimulate the additional production of ROS due to the uncoupling of electron transport machineries, leading to significant increase in cellular oxidative stress (17). In addition, a series of previous studies has shown that a subtoxic level of ROS can act as a key modulator for the expression of several growth factors, cytokines, and transcription factors (18,19). Subtle alterations in the expression of cellular signal transporters induced by ROS may either directly or indirectly affect the reporter gene expression.

In this study, based on the hypothesis that reduction of polyplex nanoparticle-mediated cellular oxidative stress during transfection would increase overall gene transduction efficiency, we designed an antioxidative transfection system that allows the combined delivery of antioxidant and nucleic acid drugs. The antioxidative transfection system yielded a significant reduction in intracellular cellular oxidative stress and mitochondrial membrane perturbation, leading to remarkably enhanced transfection in various cells.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic-*co*-glycolic acid) (RG502H, M_w 14,000, lactide/glycolide ratio = 50/50) was obtained from Boeringer Ingelheim (Ingelheim, Germany). Branched PEI (PEI, M_w 10,000 and 25,000) was purchased from Polysciences (Warrington, PA). Dichlorohexyl carbodiimide (DCC), *N*-hydroxyl succinimide (NHS), α-tocopherol, cholesterol and fluorescamine were from Sigma (St. Louis, MO). Cell culture materials, including Minimum Essential Medium (MEM), Roswell Park Memorial Institute 1640 medium (RPMI1640), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS), were obtained from Invitrogen (Carlsbad, CA).

Synthesis and Characterization of PEI-PLGA Copolymer

PEI-PLGA copolymer was synthesized as previously described (20). Briefly, PLGA (0.11 mmol) dissolved in 20 mL tetrahydrofuran was activated by DCC/NHS chemistry (PLGA/DCC/NHS stoichiometric molar ratio = 1:6:6). The insoluble by-product was removed by filtration and the solvent was removed using a rotary evaporator. The activated PLGA was precipitated in hexane and dried under reduced pressure. The copolymer was synthesized by adding the activated PLGA (0.02 mM) dissolved in 10 ml methylenechloride/DMSO (1:1) co-solvent to bPEI (M_w = 10,000, 0.01 mM) in DMSO (PLGA/bPEI stoichiometric molar ratio = 2:1) with stirring at room temperature. After overnight reaction, dichloromethane (DCM) was removed using a rotary evaporator. The resulting polymer in DMSO was dialyzed against deionized water (MWCO 50,000, Spectrum, Rancho Dominguez, CA). The solution was then filtered to remove insoluble products and freeze-dried. The conjugation and degree of substitution of the PEI-PLGA were determined by using ^{1}H-NMR, FT-IR (amine groups of PEI (N-H stretch, 3400 and 1651 cm^{-1}); carbonyl groups of PLGA (C=O stretch, 1733 – 1626 cm^{-1}); amide linkage of PEI-PLGA (HN-C=O, 1624 cm^{-1})). Gel permeation chromatography (GPC) was performed on Agilent 1100S HPLC equipped with a differential refractometer as a detector and Shodex GPC K-803 column. Tetrahydrofuran (THF) was used as a mobile phase with a flow rate of 1.0 ml/min. Polystyrene (Aldrich, St. Louis, MO) was used as molecular weight standards. Molecular weight and polydispersity index (M_w/M_n, PDI) of the conjugate were determined by using Agilent ChemStation^{TM} software. The number of primary amine group of the conjugate was determined by fluorescamine assay (21). A series of known concentration of ethylenediamine dissolved in 900 μL sodium borate buffer (0.1 M, pH 9.0) was mixed with 100 μL fluorescamine solution (3 mg/mL). The solution was incubated at an ambient temperature for 30 min, after which fluorescence intensity was measured by spectrofluorometery (RF-5301PC, Shimadzu, Japan) at an excitation and an emission wavelength of 390 and 475 nm, respectively, and used to construct a standard curve. Based on the standard curve, the amount of primary amines per mg polymer was determined to calculate the degree of substitution according to a following equation: Degree of substitution (%) = $(1\text{-}FL_{modified}/FL_{unmodified}) \times 100$, where $FL_{modified}$ = fluorescence intensity of PEI-PLGA and $FL_{unmodified}$ = fluorescence intensity of PEI.

Preparation of α-Tocopherol-Loaded PEI-PLGA Micelles

Tocopherol-loaded PEI-PLGA micelles (PEI-PLGA/Toco) were prepared by a film casting method. Briefly, PEI-PLGA copolymer and α-tocopherol were dissolved in DCM and stirred for 30 min at room temperature. After evaporating the solvent using a rotary evaporator, the thin film was dispersed in 10 ml deionized water by sonication. The micelles were filtered to remove unincorporated α-tocopherol and freeze-dried. The loading content and

loading efficiency were 11% and 73.3%, respectively, as determined by HPLC.

In Vitro Release of α-tocopherol

The release of α-tocopherol from PEI-PLGA/Toco micelles was monitored using a dialysis method. The PEI-PLGA/Toco micelles were placed into dialysis membrane (MWCO 10,000, Spectra/Por 7, Rancho Dominguez, CA). The drug release was performed in 100 ml PBS containing 0.1% Tween 20 on an orbital shaker at 37°C. The sample was collected at predetermined time internal and analyzed by HPLC equipped with a UV detector (292 nm, Waters 486 Tunable Absorbance Detector) and Phenomenex Gemini C18 column (150 × 3.0 mm). Fifty percent aqueous acetonitril was used as a mobile phase with a flow rate of 0.5 ml/min.

Transmission Electron Microscopy (TEM)

The micelle solution in deionized water was dropped onto a 300-mesh carbon-coated copper grid and dried at an ambient temperature. The grid was stained with 2% uranyl acetate and observed under a transmission electron microscope (JEM-3010, ZEOL, Tokyo, Japan).

Formation of Polyplexes

Polyplexes were freshly prepared prior to use. To generate polyplexes, predetermined amounts of micelles and pDNA (pCMV-Luc, 1.5 μg) were separately diluted and mixed. All the polyplex formulations were prepared on the basis of polymer/pDNA weight ratio (w/w). The polyplexes were allowed to stabilize by incubating for 20 min at room temperature. The hydrodynamic size and surface charge were determined using a light scattering method (Zeta Plus, Brookhaven Instrument Co., NY) with a He-Ne laser at a wavelength of 632 nm and a 90° detection angle.

Cell Culture and Transfection

Human hepatoma cancer cells (HepG2), human breast cancer cells (MCF-7), and human colorectal cancer cells (HCT116) cells were used in the transfection experiments. HepG2, MCF-7, and HCT-116 cells were cultured in MEM, RPMI1640, and DMEM, respectively, supplemented with 10% FBS. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells (1.5×10^5/well) were seeded in a 12-well plate (SPL Life Sciences, Korea) in growth medium with 10% FBS and incubated for 24 h. For transfection, 1 μg of the plasmid DNA and the desired amount of polymer were diluted separately in PBS and mixed. The mixture was allowed to generate polyplexes for 15 min at room temperature. The cell culture medium was replaced with serum-free medium before the addition of the polyplex formulations. As an additional transfection parameter, H_2O_2 and α-tocopherol were treated in the transfection medium along with desired polyplex formulation. The effect of oxidative stress on polyplex-mediated transfection efficiency was observed by adding varying concentration of H_2O_2 for inducing oxidative stress. For the addition of α-tocopherol, it was first dissolved in DMSO (1.5 mM stock solution), serially diluted in the serum-free medium, and added to the transfection medium along with desired polyplex formulation (final concentration = 15 μM). After 4 h incubation, the transfection medium was replaced with fresh medium containing 10% FBS. The transfection efficiency was determined by measuring the expression of the luciferase reporter gene 24 h after transfection. Luciferase activity was monitored using a commercial assay kit (Promega, Madison, WI) in a GloMax 20/20 luminometer (Promega, Madison, WI). The transfection efficiency was expressed as relative light units per mg of cell protein, the concentration of which was determined using the Micro-BCA assay (Pierce, Rockford, IL).

In Vitro ROS Measurements

Intracellular ROS was measured using a cell-permeable fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate probe (H_2DCFDA, Invitrogen, Carlsbad, CA). Transfection of polyplex formulations was performed described as above. As a positive control, the cells were treated with PEI/DNA polyplex and 100 μM H_2O_2 (final concentration) and incubated for 4 h. For the 24-h incubation group, the medium was replaced with fresh medium containing 10% FBS at 4 h. After incubation for 4 h or 24 h, the cells were trypsinized and washed twice with PBS. The cells were then treated with 50 μM H_2DCFDA for 30 min, washed once with PBS, resuspended, and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). The green fluorescence of DCF was measured using the FL-1 setting and 10,000 events were recorded in each analysis. The cells in an arbitrarily selected gate region ($100 <$ FL-1H < 10000) were considered under oxidative stress. The relative oxidative stress level was expressed as percent ROS increase by relating that of mock-treated cells (0% ROS increase).

JC-1 Assay for Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\Psi$) was assessed in live HepG2 cells using a lipophilic cationic probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Biotium, Hayward, CA). In healthy cells having intact mitochondrial membrane potential, JC-1 preferentially localizes in mitochondrial matrix and forms aggregate

emitting red fluorescence (600 nm) in a concentration-dependent manner. Lose of mitochondrial membrane potential due to apoptosis for instance makes JC-1 remain in the cytoplasm and exist as a monomer emitting green fluorescence (535 nm). The cells were treated with the desired polyplex formulation and incubated as described above. After incubation for 4 h or 24 h, the cells were trypsinized, washed twice with PBS, and stained with JC-1 as previously described (22). Red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) was measured using a fluorescence microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). The ratio of red fluorescence intensity to green fluorescence intensity was determined. The relative mitochondrial membrane potential ($\Delta\Psi$) was expressed as percent of the ratio by relating the ratio of mock-treated cells (= 100%).

RESULTS AND DISCUSSION

Cationic Polyplex-Mediated Oxidative Stress

To observe cationic polyplex-mediated cellular oxidative stress, the cellular oxidative stress level after PEI25k/pDNA polyplex transfection was measured by flow cytometry using a cell-permeable probe, H_2DCFDA, which is converted to a non-fluorescent deacetylated intermediate by cellular esterases. The intermediate can be readily oxidized by ROS to form fluorescent 2',7'-dichlorodihydrofluorescene (DCF). Figure 1a shows that transfection with PEI/pDNA polyplexes (1 μg pDNA, weight ratio = 1) induced the production of ROS in HepG2 cells, and the generation of cellular ROS increased as incubation time increased. After 24 h, ROS production induced by the PEI polyplexes was higher that induced by pretreatment with 0.1 mM hydrogen peroxide (H_2O_2) (Fig. 1a). The intracellular ROS production after the transfection of the PEI polyplexes was also visualized by DCF under fluorescence microscope (Fig. 1b). Cationic nanoparticles are known to induce the generation of cellular ROS and depletion of reduced glutathione, leading to oxidative stress-mediated toxic responses (4). It appears that cationic nanoparticle-mediated cellular ROS production and cytotoxicity are closely related to mitochondrial damage, rather than inflammatory responses such as the up-regulation of inflammatory cytokines (4,23). It was recently reported that

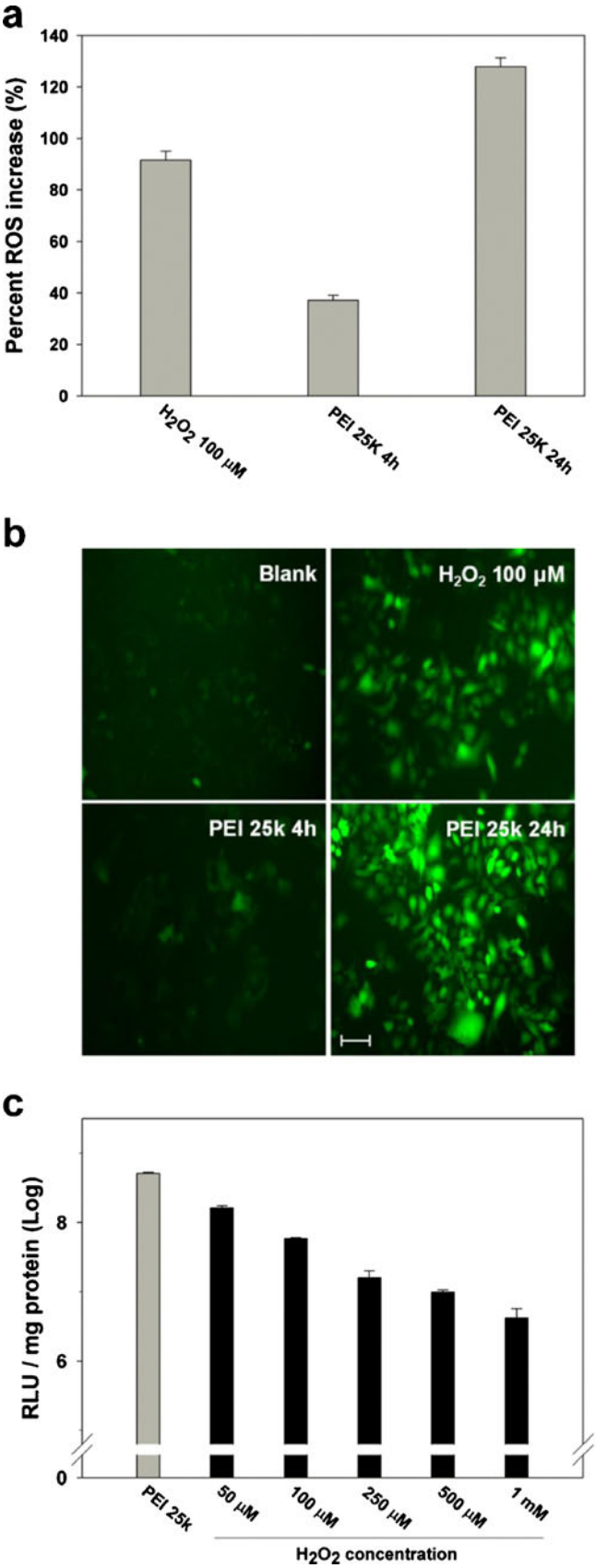

Fig. 1 (**a**) Effect of PEI 25 K/pDNA polyplex (PEI/pDNA weight ratio, w/w = 1) on cellular ROS production in HepG2 cells. The ROS level was determined by measuring fluorescent DCF production using flow cytometry and is expressed as the percent ROS increase. As a positive control, cells were treated with 100 μM hydrogen peroxide (H_2O_2). Data represent the mean ± SD of triplicate experiments. (**b**) Fluorescence microscope images of PEI 25 K/pDNA polyplexes 4 h and 24 h after transfection. The cells under oxidative stress were shown in green fluorescence from DCF (bar = 40 μm). (**c**) Effects of oxidative stress induced by H_2O_2 on transfection efficiency of PEI 25 K/pDNA polyplex in HepG2 cells. Data represent the mean ±SD of triplicate experiments.

transfected PEI/pDNA polyplexes were localized and accumulated in mitochondria in a time-dependent manner (16). The interactions of PEI polyplexes with mitochondria would result in the production of additional ROS and further increase in cellular oxidative stress level by perturbing mitochondrial functions. To observe the influence of increased cellular oxidative stress on transfection, PEI/pDNA polyplexes were transfected to HepG2 cells in the presence of varying concentration of H_2O_2. Transfection efficiency decreased as the H_2O_2 concentration increased (Fig. 1c), indicating that the alteration of cellular oxidative stress level affects polyplex-mediated transfection.

Effect of Antioxidative Transfection on Cellular Oxidative Stress

We hypothesized that concurrent delivery of an antioxidant with polyplexes may improve the polyplex-mediated oxidative stress during gene delivery. The concept of the antioxidative transfection system was illustrated in Fig. 2. In our previous study we synthesized a conjugate of PEI and poly(lactic-co-glycol acid) (PEI-PLGA) that showed a desirable level of transfection efficiency with low cytotoxicity (20). The synthesis of the conjugate was confirmed by ^{1}H-NMR and FT-IR analysis (Fig. 3a and b). The molecular weight of PEI-PLGA was 26,000 (M_w) and 13,600 (M_n), as determined by GPC analysis (see Supplementary Material, Fig. S1). Therefore, considering the nominal M_w of PLGA and PEI are 14,000 and 10,000, respectively, ca. one PLGA molecule was conjugated to PEI backbone, which is in agreement with the degree of modification determined by fluorescamine assays based on the number of primary amine group of PEI (DS = 0.75).

Since the conjugate spontaneously forms cationic micelles in an aqueous medium, we selected a water-insoluble antioxidant, α-tocopherol, which can be loaded in the hydrophobic core of the micelles. The loading efficiency was 73.3% when the initial target loading content was 15%, as determined by HPLC analysis. Both PEI-PLGA and α-tocopherol-loaded micelles (PEI-PLGA/Toco) did not elicit any cytotoxicity in HepG2 cells even at high concentration (500 μg/ml), whereas PEI 10 kDa caused significant cell death at relatively lower

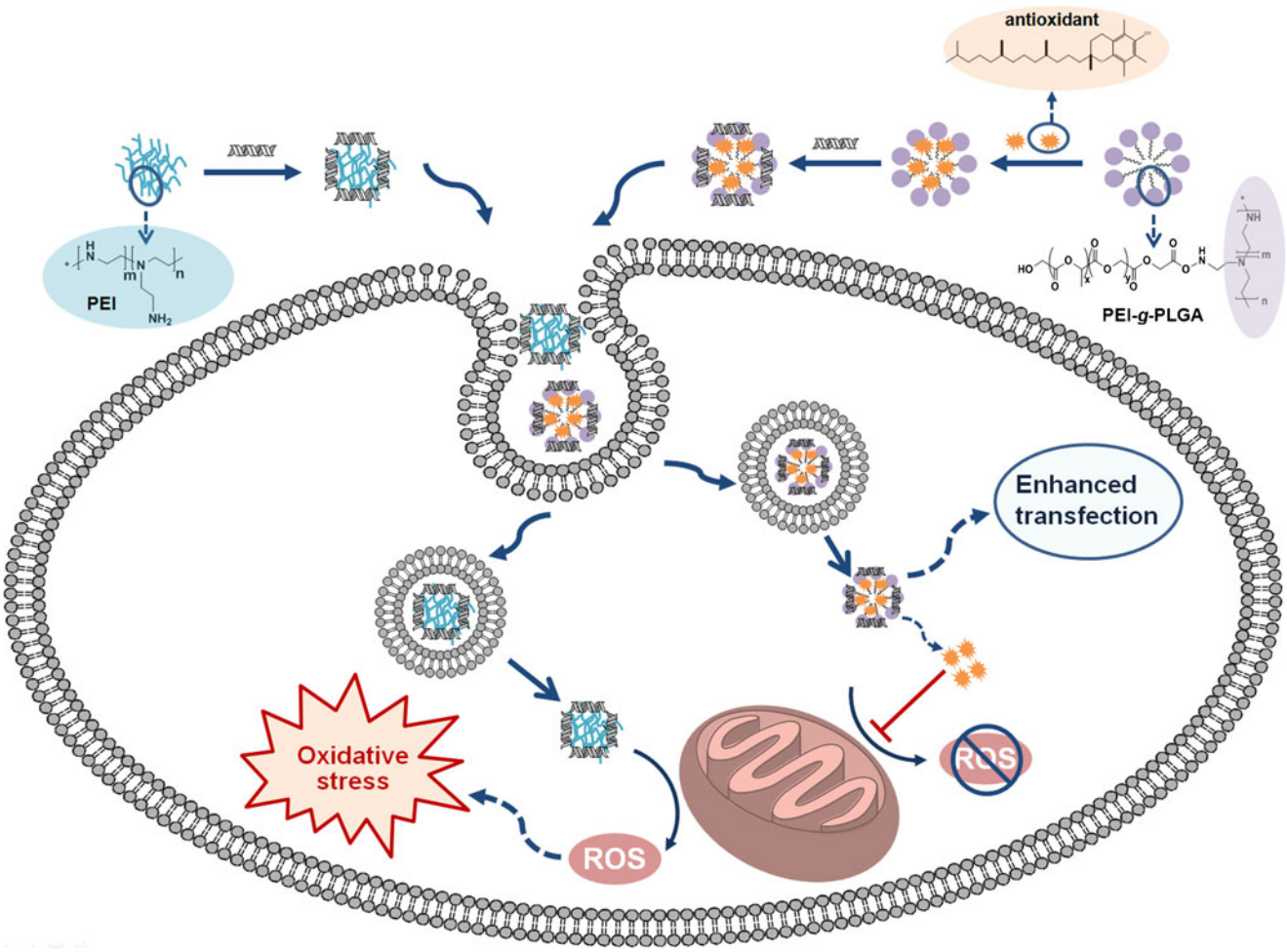

Fig. 2 Schematic illustration of enhanced gene transfection by reducing polyplex-mediated cellular oxidative stress using antioxidative polymeric gene carrier.

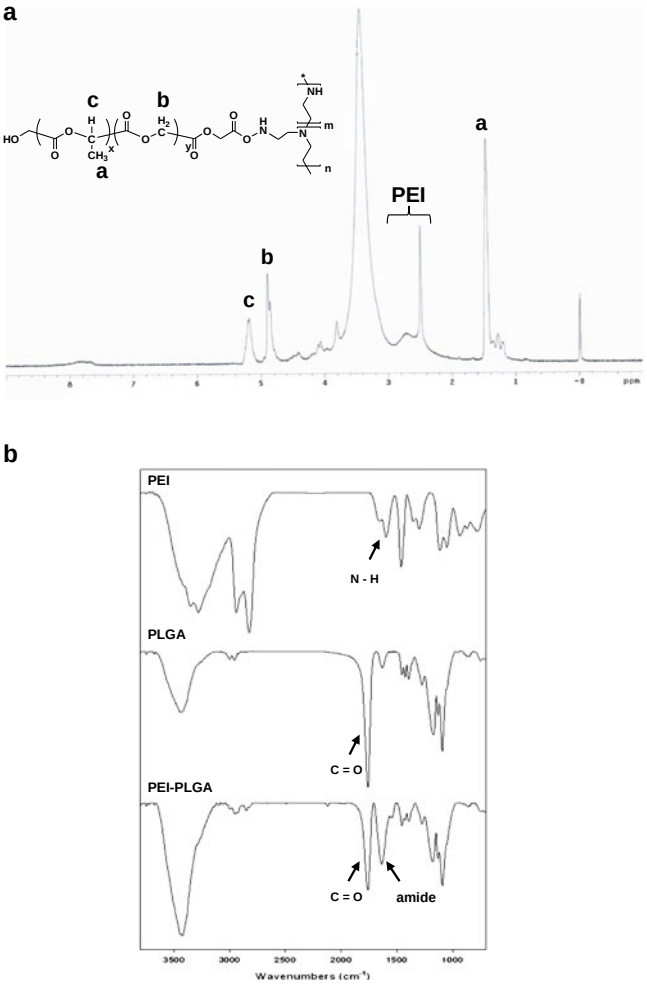

Fig. 3 ^{1}H-NMR (**a**) and FT-IR spectrum (**b**) of PEI-PLGA copolymer.

concentration (IC_{50} = 100 μg/ml) (see Supplementary Material, Fig. S2). PEI-PLGA and PEI-PLGA/Toco exhibited similar spherical morphologies with a diameter of approximately 160 nm (Table I). The hydrodynamic diameter and ζ-potential of PEI-PLGA were 181.4 ± 4.5 nm and 51.1 ± 2.6 mV, respectively, while PEI-PLGA/Toco exhibited smaller size (166.2 ± 1.1 nm) and similar surface potential (49.1 ± 1.0 mV). The release of α-tocopherol from PEI-PLGA/Toco micelles was shown in Fig. 4. The release of α-tocopherol was carried out in the presence of non-ionic detergent, Tween 20 (Sigma, St. Louis, MO), for the enhanced dissolution of the hydrophobic antioxidant.

The condensation of pDNA and the formation of polyplexes by the micelles were confirmed by observing the band migration shift in an electrophoretic field (data not shown). The change in hydrodynamic diameters and surface charges of the polyplexes according to the change in the polymer/DNA weight ratio (w/w) were measured by a light scattering method (Fig. 5). The final formulation used in the subsequent experiments contained positively charged micelles with a diameter of approximately 150 nm. The size, zeta-potential, and morphology (TEM image) of pDNA/PEI-PLGA/Toco polyplexes were shown in Table I.

The antioxidant effect of PEI-PLGA/Toco/pDNA polyplexes on ROS production was observed by measuring DCF fluorescence after transfection. For transfection, PEI 25 kDa (PEI 25 K/pDNA, weight ratio w/w = 1), PEI 10 kDa (PEI 10 K/pDNA, w/w = 1), and PEI-PLGA (PEI-PLGA/pDNA, w/w = 10) were selected as it was determined for optimal transfection without eliciting significant cellular cytotoxicity (20) and used without further optimization for other parameters. For the formation of PEI-PLGA/Toco/pDNA polyplexes, only the weight of the polymer (PEI-PLGA) without α-tocopherol was considered for determining the weight ratio (PEI-PLGA/Toco/pDNA, w/w = 10, as a weight of PEI-PLGA). Polyplexes formed from PEI 25 kDa, PEI 10 kDa, and PEI-PLGA stimulated the production of ROS in HepG2 cells after 4 h and 24 h of transfection. In contrast, the polyplexes generated from PEI-PLGA/Toco effectively reduced the generation of cellular ROS after both 4 h and 24 h incubation (Fig. 6). It should be noticed that the amount of ROS generated by the control polyplexes increased ca. 150%, compared to mock-treated cells (0%) after 24 h incubation, but PEI-PLGA/Toco exhibited only slight increase (39%) in the ROS production (Fig. 6b). In contrast, the same amount of cholesterol-loaded micelles (PEI-PLGA/Chol) did not influence the cellular ROS level after transfection, indicating the specific ability of α-tocopherol to reduce polyplex-induced ROS production. Cholesterol was used as a control due to its hydrophobicity and lack of antioxidant effect. Both PEI-PLGA/Toco/pDNA and PEI-PLGA/Chol/pDNA polyplexes exhibited similar size, surface charge, and morphological shape (Table I), suggesting their physico-chemical characteristics would not significantly affect the transfection result. The result suggests primary antioxidant function of α-tocopherol is to scavenge peroxyl radicals (24). It is noteworthy that the addition of an equivalent amount of α-tocopherol (15 μM) to the cell culture medium resulted in only a small reduction in ROS. Using the micelles, the simultaneous delivery of α-tocopherol, a potent ROS scavenger, may effectively compensate for the adverse action of the polyplex, a ROS stimulator, because both the preventer and stimulator can exist at a high local concentration in the same intracellular space and time.

Effect of Antioxidative Transfection on the Mitochondrial Membrane

Mitochondria are one of the main targets of oxidative damage, which is involved in mitochondrial dysfunction and many aspects of cell death in several diseases (25,26). Mitochondrial membrane potential ($\Delta\Psi$) is considered important because it provides the essential driving force for oxidative phosphorylation in the mitochondrial respiratory chain. A reduction in the membrane potential contributes to

Table I Characterization of PEI-PLGA Micelles, α-tocopherol-loaded PEI-PLGA Micelles (PEI-PLGA/Toco), Cholesterol-loaded PEI-PLGA Micelles (PEI-PLGA/Toco), PEI-PLGA/Toco/pDNA Polyplexes, and PEI-PLGA/Chol/pDNA Polyplexes. [a] determined by light scattering method

	PEI-PLGA	PEI-PLGA/Toco	PEI-PLGA/Chol	PEI-PLGA/Toco/pDNA polyplex (w/w=20)	PEI-PLGA/Chol/pDNA polyplex (w/w=20)
Size[a]	181.4±4.5 nm	166.2±1.1 nm	154.1±1.6 nm	156.±1.4 nm	177.2±1.9 nm
Zeta-potential[a]	51.1±2.6 mV	49.1±1.0 mV	48.1±0.8 mV	48.4±0.5 mV	50.4±1.5 mV
TEM images	Bar=200 nm	Bar=200 nm	Bar=100 nm	500 nm	500 nm

further ROS production, mitochondrial uncoupling, and reduced generation of ATP (25,26). It was recently reported that PEI/pDNA polyplexes interact with and accumulated in mitochondria (16), which may affect the integrity and function of the mitochondrial membrane. The effect of PEI-PLGA/Toco was further evaluated by observing the change in mitochondrial membrane potential after delivery of the polyplexes. Polyplexes formed from PEI 25 kDa, PEI 10 kDa, and PEI-PLGA reduced the membrane potential (Fig. 7). The PEI-PLGA/Toco/pDNA polyplex exerted a significant protective effect on the membrane potential, while no significant effect was observed after co-delivery of cholesterol, a control hydrophobe with no antioxidant effect (PEI-PLGA/Chol/pDNA). This finding is consistent with observations of a previous study in which, in addition to its direct effect on oxidative injury, α-tocopherol acted as an efficient inducer of Phase II enzymes that could prevent ROS-mediated damage of cellular biomolecules and effectively protect the mitochondria from the reduction in membrane potential (27).

Transfection Efficiency of the Antioxidative Transfection System

To determine the effect of the polyplex-induced oxidative stress on trans-gene expression in the cells, transfection experiments were carried out in various cells including HepG2, MCF-7, and HCT-116 cells. PEI-PLGA/Toco/pDNA demonstrated a remarkable increase in transfection efficiency, which was two to three orders of

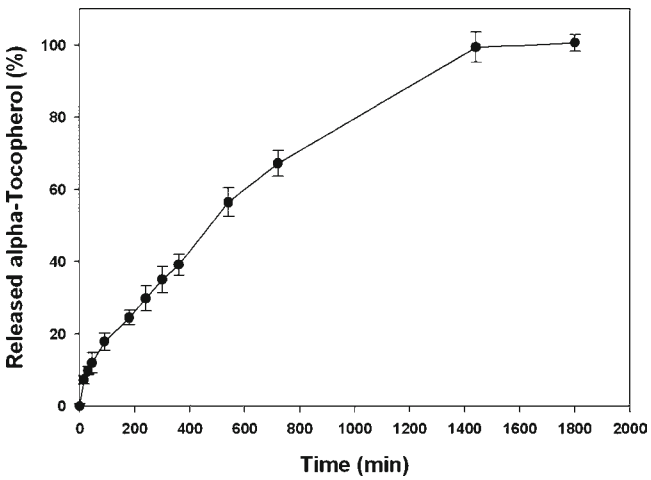

Fig. 4 *In vitro* release profile of α-tocopherol from PEI-g-PLGA/Toco micelle in PBS containing 0.1% Tween 20 at 37°C. Each point data represents the mean ±SD of triplicate.

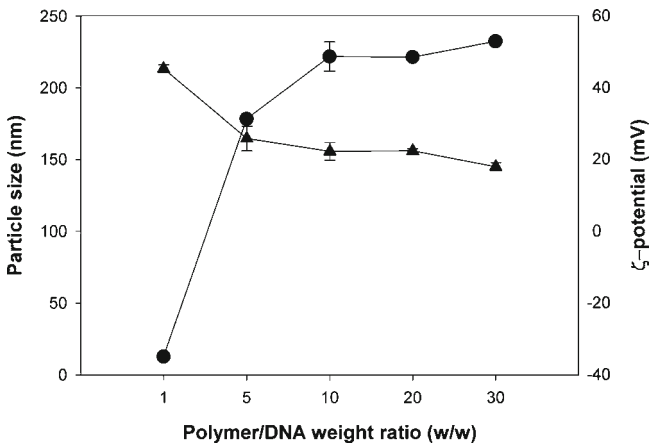

Fig. 5 Hydrodynamic diameter (▲) and surface zeta-potential (●) of PEI-PLGA/Toco/pDNA polyplexes at various polymer/pDNA weight ratios. It should be noted that only polymer (PEI-PLGA) weight was considered for the weight ratios. Data represent the mean ± SD of triplicate experiments.

magnitude higher than that of PEI-PLGA/pDNA, depending on the cell type (Fig. 8). However, co-delivery of cholesterol (PEI-PLGA/Chol/pDNA, w/w = 10) by the same micellar carrier did not result in a significant increase in transfection, suggesting the enhancement in transfection does not come from the stabilization of the micellar core by the hydrophobic small molecules. The addition of 15 μM α-tocopherol to the transfection medium only slightly increased the transfection efficiencies of PEI 25 kDa, PEI 10 kDa, and PEI-PLGA. Taken together with the results shown in Fig. 6, these findings suggest that the elevated level of ROS would affect the reporter gene expression and that effective removal of local ROS by α-tocopherol leads to the observed dramatic increase of trans-gene expression in the cells. The effective protection of mitochondrial membrane from the potential oxidative damage would additionally contribute to the increased transfection efficiency possibly by maintaining the mitochondrial

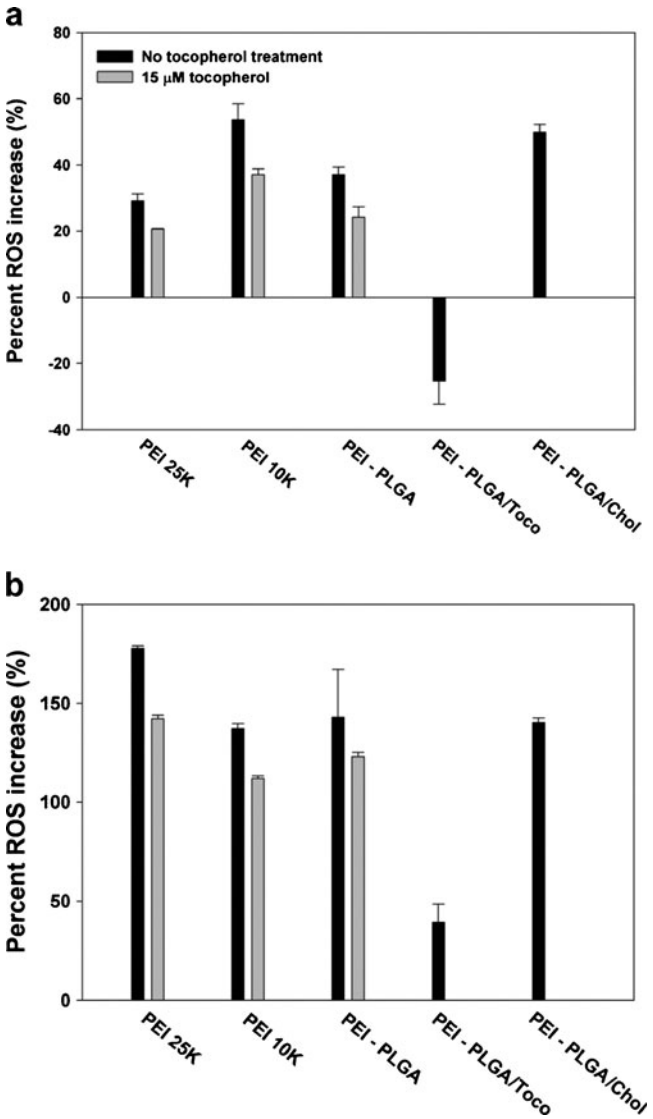

Fig. 6 Effect of the antioxidative gene carrier (PEI-PLGA/Toco/pDNA polyplex, polymer/pDNA weight ratio, w/w = 10) on cellular ROS production in HepG 2 cells after transfection. The ROS level was determined by measuring fluorescent DCF production using flow cytometry 4 h (**a**) and 24 h (**b**) after transfection and is expressed as the percent ROS increase (mock-treated cells = 0%). PEI 25 K/pDNA polyplex (w/w = 1), PEI 10 K/pDNA polyplex (w/w = 1) and PEI-PLGA/Chol/pDNA polyplex (w/w = 10) were used as controls. α-tochopherol (15 μM) was additionally treated to desired controls to observe antioxidant effect. Data represent the mean ± SD of triplicate experiments.

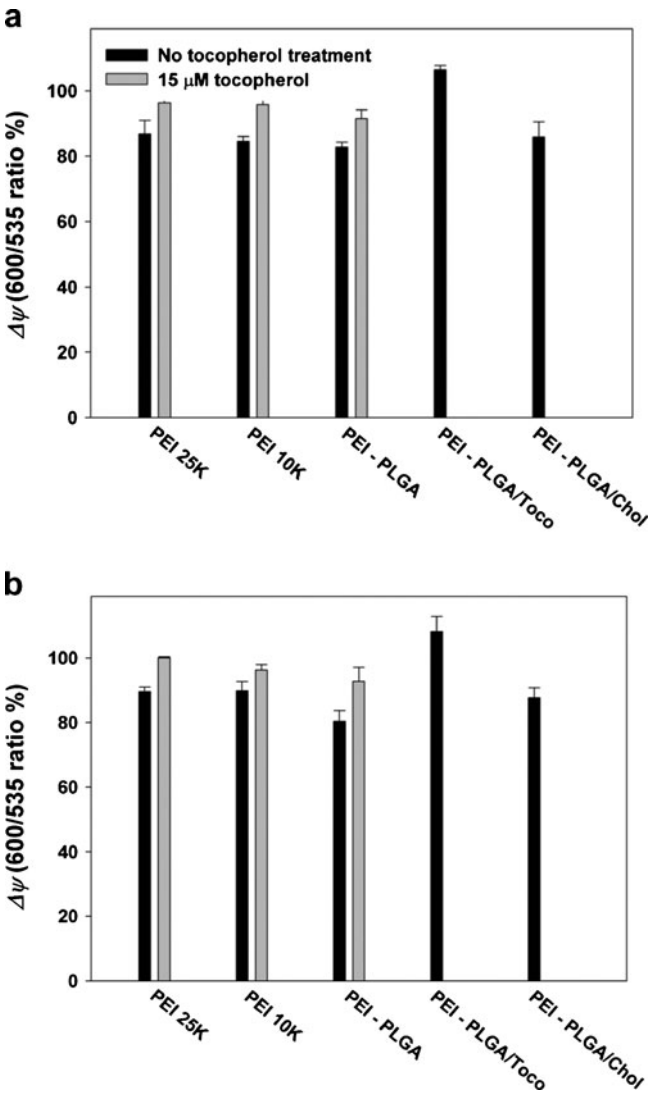

Fig. 7 Effects of the antioxidative polyplex (PEI-PLGA/Toco/pDNA polyplex, polymer/pDNA weight ratio, w/w = 10) on mitochondrial membrane potential ($\Delta\Psi$) in HepG 2 cells. Mitochondrial membrane potential was assayed by JC-1 staining. PEI 25 K/pDNA (w/w = 1), PEI 10 K/pDNA (w/w = 1) and PEI-PLGA/Chol/pDNA (w/w = 10) polyplexes were used as controls. α-tochopherol (15 μM) was additionally treated to desired controls to observe antioxidant effect. Data are presented as the percent red (E_x = 550 nm, E_m = 600 nm)/green (E_x = 485 nm, E_m = 535 nm) fluorescence ratio (mock-treated cells = 100%). Data for 4 h (**a**) and 24 h (**b**) post-transfection are given as the mean ± SD of triplicate experiments.

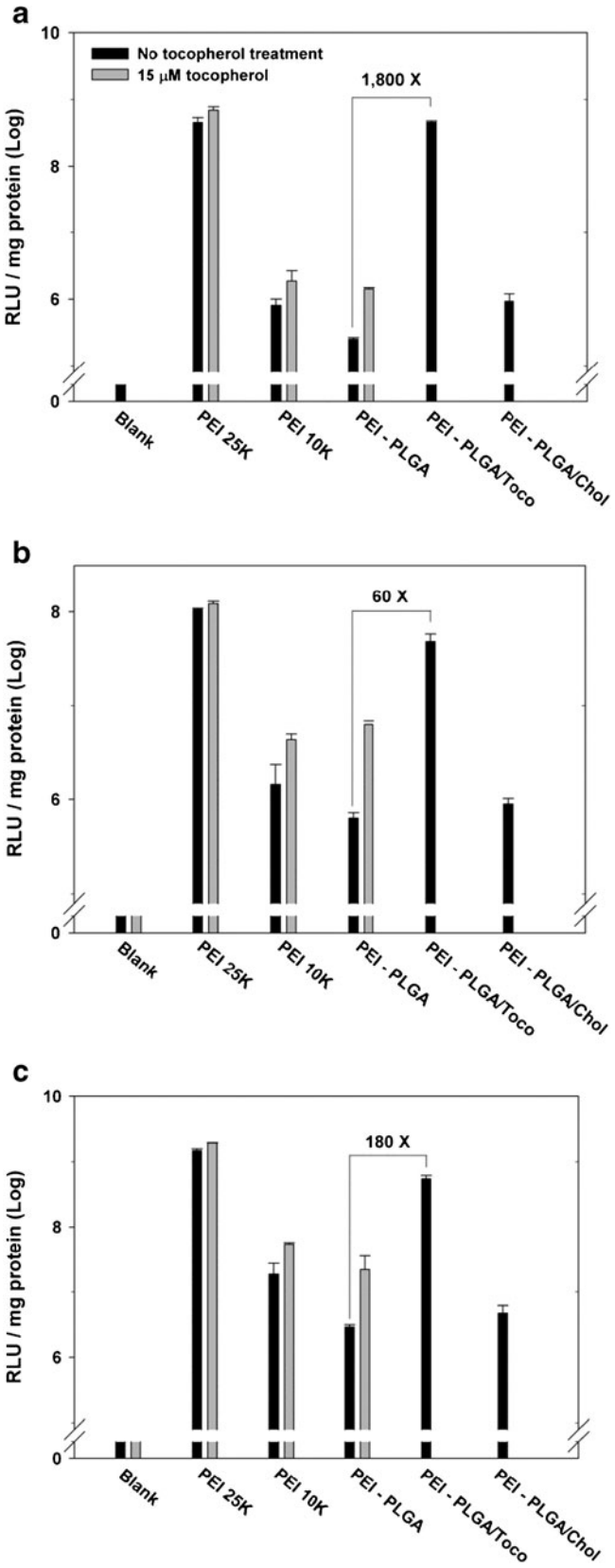

Fig. 8 Transfection of PEI-PLGA/Toco/pDNA polyplex (polymer/pDNA weight ratio, w/w = 10) in (**a**) HepG2, (**b**) MCF-7, and (**c**) HCT-116 cells. PEI 25K/pDNA (w/w = 1), PEI 10K/pDNA (w/w = 1) and PEI-PLGA/Chol/pDNA (w/w = 10) polyplexes were used as controls. α-tochopherol (15 μM) was additionally treated to desired controls to observe antioxidant effect. Data represent the mean ±SD of triplicate experiments.

electron-transport machinery that plays crucial role in cellular ATP production. Transfection with polyplexes (PEI-PLGA/Retinol/pDNA) formed from PEI-PLGA micelles containing retinol, another antioxidant, also showed significant increase in transfection efficiency in HepG2 cells (see Supplementary Material, Fig. S3). This result also supports our initial hypothesis in which the reduction of polyplex-mediated cellular oxidative stress by the co-delivery of antioxidant can significantly enhance transfection efficiency. Moreover, a polymer-peptide conjugate (L-carnosine-PEI) was reported to show improved biocompatibility while maintaining transfection efficiency *via* upregulation of a series of genes that reduce cellular oxidative stress (28). It should be noted that all transfection experiments were carried out at subtoxic polymer concentrations, suggesting that the polyplex-mediated oxidative stress was at a tolerable level for the cells. Since ROS is known to modulate the expression of several regulatory proteins such as transcription factors and cytokines and to interact with intracellular biomolecules including membrane lipid and nucleic acids even at subtoxic concentrations (18,19,29), the changes may affect the trans-gene expression.

CONCLUSIONS

In this study, we revisited the significance of cellular stress during transfection for polycation-based non-viral gene delivery. Our primary concern was transfection-induced cellular ROS because its role in nanoparticle-mediated oxidative stress and toxicity has been strongly emphasized. We demonstrated that polyplexes can induce cellular ROS production even at subtoxic concentrations. The effective removal of ROS by simultaneous delivery of an antioxidant, α-tocopherol, and pDNA using cationic PEI-PLGA micelles resulted in a remarkable increase in transfection efficiency. These results suggest that the importance of cellular stresses in gene transfection should be considered when designing polycation-based non-viral gene delivery systems, especially for clinical applications where a substantial amount of polyplex may be administered. This study provides a simple, but highly efficient example of an antioxidative transfection system. The effect of polyplexes on *in vivo* ROS production and the gene transfection efficacy of the antioxidative transfection system should be further investigated in animal models for successful translation of the system to clinical settings in the future.

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