Final Report

Project Title: Co-delivery of polyethylenimine cholate–plasmid DNA and aminoglycerol diamine liposome–antisense oligodeoxyribonucleotide in human carcinoma cells

By

Associate Professor Dr. Wanlop Weecharangsan

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Abstract

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Investigator : Associate Professor Dr. Wanlop Weecharangsan

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Project Period : 2 years (3 June 2013 to 2 June 2015)

Abstract:

This study aimed to evaluate the co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/antisense oligodeoxyribonucleotide (AS ODN) complexes in HeLa human cervical carcinoma cells. Dimethyldioctadecyl ammonium bromide (DDAB): dioleoyl phosphatidylethanolamine (DOPE) liposome/plasmid DNA complexes, and DDAB:DOPE liposome/AS ODN complexes, and cholic acid-modified polyethylenimine (PEI-CA)/plasmid DNA and AS ODN complexes were formulated and characterized in terms of agarose gel electrophoretic mobility, particle size and zeta potential. The complexes were evaluated for delivery of pEGFP plasmid DNA and AS ODN in HeLa cells. Cell growth inhibition was evaluated using p53 plasmid DNA and bcl-2 AS ODN, by co-delivery of DDAB:DOPE liposome/p53 plasmid DNA and DDAB:DOPE liposome/bcl-2 AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes. The particle size of DDAB:DOPE liposome/plasmid DNA complexes, and DDAB:DOPE liposome/AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes were nanosized. The AS ODN uptake and green fluorescent protein (GFP) expression upon their co-delivery by DDAB:DOPE liposomes and PEI-CA were both high. Treatment of the cells with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to a greater degree than that with either DDAB:DOPE liposome/p53 plasmid DNA complexes or DDAB:DOPE liposome/bcl-2 AS ODN complexes alone. Treatment of the cells with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes resulted in effective cell growth inhibition that was greater than that of either PEI-

CA/p53 plasmid DNA complexes or PEI-CA/bcl-2 AS ODN complexes alone. The particle size of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage at 4 ^{O}C was in the range of 288.7±5.1 to 472.2±10.0 nm and 149.2±0.5 to 398.4±5.0, respectively. The particle size of PEI-CA/plasmid DNA and AS ODN complexes remained less than 300 nm during storage at 4 ^{O}C for 14 days. These data suggest that co-delivery of plasmid DNA and AS ODN by cationic liposomes and PEI-CA could be an attractive strategy for clinical application in cancer treatment.

Keywords : p53 plasmid DNA, bcl-2 antisense oligodeoxyribonucleotide, co-delivery, cholic acid-modified polyethylenimine, cationic liposome, growth inhibition

Final report content:

1. Abstract

This study aimed to evaluate the co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/antisense oligodeoxyribonucleotide (AS ODN) complexes in HeLa human cervical carcinoma cells. cationic liposome/plasmid DNA complexes, and cationic liposome/AS ODN complexes, and cholic acid-modified polyethylenimine (PEI-CA)/plasmid DNA and AS ODN complexes were formulated and characterized in terms of agarose gel electrophoretic mobility, particle size and zeta potential. The complexes were evaluated for delivery of pEGFP plasmid DNA and AS ODN in HeLa cells. Cell growth inhibition was evaluated using p53 plasmid DNA and bcl-2 AS ODN, by co-delivery of cationic liposome/p53 plasmid DNA and cationic liposome/bcl-2 AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes. The particle size of cationic liposome/plasmid DNA complexes, and cationic liposome/AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes were nanosized. The AS ODN uptake and green fluorescent protein (GFP) expression upon their co-delivery by cationic liposomes and PEI-CA were both high. Treatment of the cells with the co-delivery of cationic liposome/p53 plasmid DNA complexes and cationic liposome/bcl-2 AS ODN complexes inhibited cell growth to a greater degree than that with either cationic liposome/p53 plasmid DNA complexes or cationic liposome/bcl-2 AS ODN complexes alone. Treatment of the cells with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes resulted in effective cell growth inhibition that was greater than that of either PEI-CA/p53 plasmid DNA complexes or PEI-CA/bcl-2 AS ODN complexes alone. The particle size of cationic liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and cationic liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage at 4 ^{O}C was in the range of 288.7±5.1 to 472.2±10.0 nm and 149.2±0.5 to 398.4±5.0, respectively. The particle size of PEI-CA/plasmid DNA and AS ODN complexes remained less than 300 nm during storage at 4 ^{O}C for 14 days. These data suggest that co-delivery of plasmid DNA and AS ODN by cationic liposomes and PEI-CA could be an attractive strategy for clinical application in cancer treatment.

2. Executive summary

Major goal of nucleic acid-based drug therapy is to insert the therapeutic gene or antigene into cancer cells and to have it expressed or blocking. However, most of current nucleic acid-based drug therapy methods are inefficient due to the low efficiency of nucleic acid transfer. Insufficiency of nucleic acid transfer is a main obstacle to the clinical application of nucleic acid-based drug therapy (1, 2). Approaches to improve the efficacy of therapy, delivery systems using cationic polymers and cationic lipids improved the intracellular delivery and therapeutic activity of nucleic acid-based drugs pre-clinically and clinically (3-6). In this current study, PEI-CA and liposome were evaluated for co-delivery of plasmid DNA and antisense oligodeoxyribonucleotide (AS ODN) into human carcinoma cells. The illustration of co-delivery of PEI-CA–p53 gene and cationic liposome–bcl-2 AS ODN in human carcinoma cells in Fig. 1.

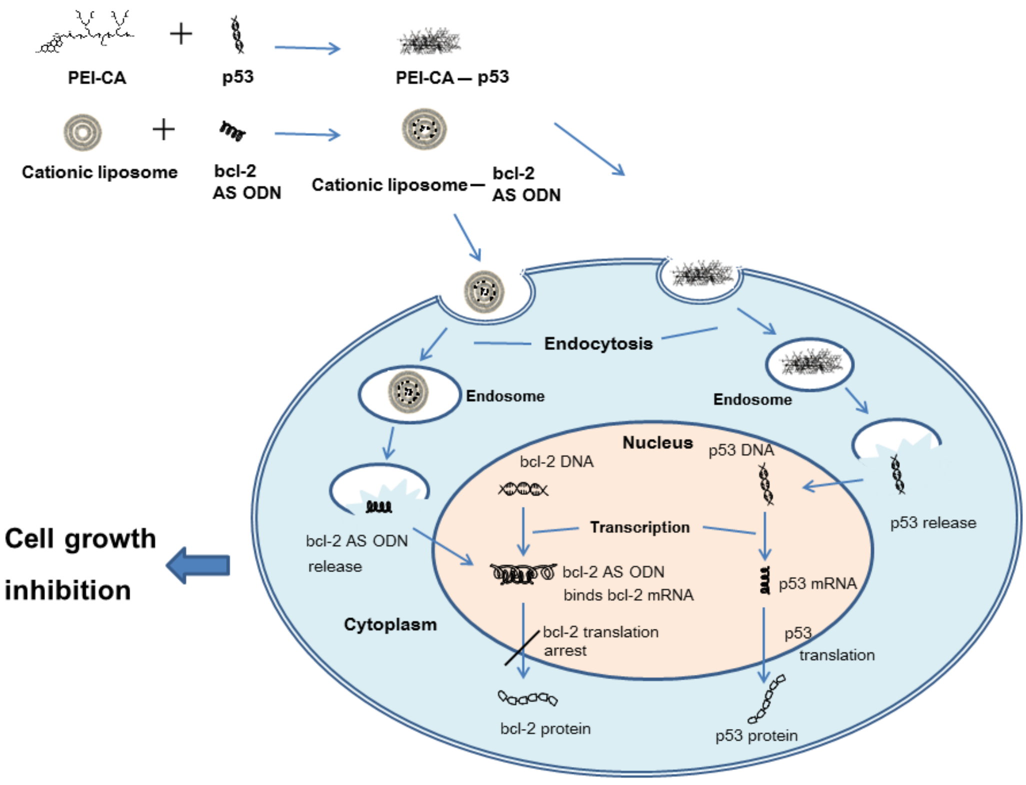

Fig. 1 Illustration of co-delivery of PEI-CA–p53 gene and cationic liposome–bcl-2 AS ODN complexes in human carcinoma cells

Cationic liposome/plasmid DNA complexes, and cationic liposome/AS ODN complexes, and cholic acid-modified polyethylenimine (PEI-CA)/plasmid DNA and AS ODN complexes were formulated and characterized in terms of agarose gel electrophoretic mobility, particle size and zeta potential. The complexes were evaluated for delivery of pEGFP plasmid DNA and AS ODN in HeLa cells. Cell growth inhibition was evaluated using p53 plasmid DNA and bcl-2 AS ODN, by co-delivery of cationic liposome/p53 plasmid DNA and cationic liposome/bcl-2 AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes. The particle size of cationic liposome/plasmid DNA complexes, and cationic liposome/AS ODN complexes, and PEI-CA/plasmid DNA and AS ODN complexes were nanosized. The AS ODN uptake and green fluorescent protein (GFP) expression upon their co-delivery by cationic liposomes and PEI-CA were both high. Treatment of the cells with the co-delivery of cationic liposome/p53 plasmid DNA complexes and cationic liposome/bcl-2 AS ODN complexes inhibited cell growth to a greater degree than that with either cationic liposome/p53 plasmid DNA complexes or cationic liposome/bcl-2 AS ODN complexes alone. Treatment of the cells with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes resulted in effective cell growth inhibition that was greater than that of either PEI-CA/p53 plasmid DNA complexes or PEI-CA/bcl-2 AS ODN complexes alone. The particle size of cationic liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and cationic liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage at 4^{O}C was in the range of 288.7±5.1 to 472.2±10.0 nm and 149.2±0.5 to 398.4±5.0, respectively. The particle size of PEI-CA/plasmid DNA and AS ODN complexes remained less than 300 nm during storage at 4 ^{O}C for 14 days.

3. Objective

To evaluate the co-delivery of PEI-CA–plasmid DNA and cationic liposome–antisense oligodeoxyribonucleotide in human carcinoma cells

4. Research methodology

4.1 Materials

Polyethylenimine (PEI 25KDa, branched), cholic acid (CA), *N,N*-dicyclohexylcarbodiimide (DCC), 4-dimethylamiopyridine (DMAP), *N,N*-dimetylformamide (DMF) and deuterated water (D_2O) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyldioctadecyl ammonium bromide (DDAB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Di-(9Z-octadecenoyl)3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was obtained from Lipoid GMBH (Ludwigshafen, Germany). N-(1-(2,3-dilauroyloxy)propyl)-N'-(3-aminopropyl)carbamide (DLPAP) [1] was provided from Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech (Palo Alto, CA, USA). Plasmid GFP-p53 was a gift from Dr. Tyler Jacks (Addgene plasmid # 12091) [2]. Plasmid Maxi Kit was purchased from Geneaid (Taipei City, Taiwan). AS ODN, a fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'- TACCGTCTGCGACGCCCT-3'), bcl-2 AS ODN, fully phosphorothioated 18-mer oliogonucleotide (Sequence: 5'- TCTCCCAGCGTGCGCCAT-3') and Cy3-labeled bcl-2 AS ODN (Sequence: 5'-Cy3-TCTCCCAGCGTGCGCCAT-3') were purchased from Alpha DNA (Quebec, Canada). Sephadex™LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Six- and 96-well plates were purchased from SPL Life Sciences. (Gyeonggi-do, Korea). MEM media and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Bio Basic Inc. (Ontario, Canada). HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

4.2 Plasmid DNA preparation

pEGFP-C2 plasmid DNA encoding the GFP and p53 plasmid DNA encoding the p53 protein were used. The plasmid DNAs were purified from DH5-α *E. coli* using the Geneaid Plasmid Maxi Kit (Taipei City, Taiwan). DNA concentrations were quantified using a cuvette photometer (Eppendorf Biophotometer plus, Eppendorf AG, Hamburg, Germany). The purity of plasmid DNAs was checked by gel electrophoresis (1.2% agarose gel). The purified plasmid DNAs were redissolved in Tris-EDTA buffer, pH 8.0 and used at a concentration of about 500 μg/ml.

4.3 Antisense oligodeoxyribonucleotides

AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'- TACCGTCTGCGACGCCCT 3'), Cy3-labeled AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'-Cy3-TCTCCCAGCGTGCGCCAT-3'), and bcl-2 AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'-TCTCCCAGCGTGCGCCAT-3'), were purchased from Alpha DNA (Quebec, Canada). The AS ODNs were reconstituted in Tris-EDTA buffer, pH 8.0. The concentrations of AS ODN solutions were determined using a cuvette photometer (Eppendorf Biophotometer plus, Eppendorf AG, Hamburg, Germany). The AS ODN solutions were diluted and used at a concentration of about 500 μg/ml.

4.4 Synthesis of PEI-CA

PEI-CA (PEI: CA; 1: 0.5 molar ratio) was synthesized in the laboratory of Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. The synthesis scheme is depicted in Fig. 2. Briefly, a solution of CA (1.9 mg, 0.0046 mmol), DCC (3.8 mg, 0.0184 mmol) and DMAP (2.2 mg, 0.0184 mmol) in DMF (1 ml) was stirred at room temperature for 15 min. A solution of PEI 25 KDa (230 mg, 0.0092 mmol) in DMF (4 ml) was then added to the activated CA solution under N_2. The reaction was performed at room temperature under stirring for 24 h. The reaction mixture was diluted with 10 mL of methanol (MeOH) and purified by Sephadex™LH-20 (GE

Healthcare Bio-Sciences AB, Uppsala, Sweden) using MeOH as eluting solvent. The collected fractions were dried by rotary evaporation and further dried under vacuum.

Fourier transform infrared (FTIR): ν_{max} 3252, 2928, 2815, 1658, 1614, 1452, 1365, 1348 cm^{-1}; Proton nuclear magnetic resonance (^1H NMR) (400 MHz, D_2O): δ 0.83 (*br s*, 3H, H-18-CA), 1.07 (*br s*, 3H, H-19-CA), 1.22-2.1 (*m*, 30H, methane, ethine and methylene protons of CA), 2.57-3.42 (*m*, -NH-C$\underline{H}_2$-C$\underline{H}_2$-NH-(PEI)), 3.79 (*br s*, 1H, H-3-CA), 3.83 (*br s*, 1H, H-7-CA), 3.96 (*br s*, 1H, H-12-CA).

4.5 Preparation of cationic liposomes

Cationic liposomes were formulated with cationic lipids: DDAB, DOTAP or DLPAP, and neutral lipid DOPE by ethanol dilution as described previously [3] with minor modification. Briefly, the lipids were dissolved in 50 µl of absolute ethanol at a molar ratio of 1:1 and injected into 450 µl of vortexing HEPES buffered solution (20 mM HEPES, pH 7.4) and vortexed for 20 min at room temperature to make a fixed total lipid concentration of 2.5 mg/ml.

4.6 Preparation of PEI-CA/plasmid DNA and cationic liposome/AS ODN complexes

4.6.1 Preparation of PEI-CA/plasmid DNA complexes

PEI-CA was dissolved in 20 mM HEPES, pH 7.4 at the concentration of 0.1 mg/ml. PEI-CA/plasmid DNA complexes were prepared by adding the plasmid DNA solution to PEI-CA solution. The mixture was gently pipetted and vortexed for 3-5 sec to initiate complex formation and incubate for 20 min at room temperature for the complexes to completely form.

4.6.2 Preparation of cationic liposomes and liposome/AS ODN complexes

Cationic liposomes were formulated with cationic cationic lipid, aminoglycerol diamine (AGD) or dimethyldioctadecyl ammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE) by ethanol dilution method. The lipids, at a molar ratio of 1:1, were dissolved in ethanol, and injected into HEPES buffered solution upon vortexing for 20 min at room temperature. Liposome/AS ODN complexes were prepared by mixing liposomes with an equal volume of AS ODN in ultrapure water.

4.7 Agarose gel electrophoresis

Complex formation of PEI-CA/plasmid DNA and liposome/AS ODN was analyzed on 1.2 % agarose gel stained with SYBR green. Electrophoresis was carried out at 100 V for 15 min. The volume of the sample loaded in the gel well was 10 µl of the complexes containing 0.25 µg of plasmid DNA or 0.3125 µg of AS ODN. Plasmid DNA and AS ODN were visualized using an image analyzer.

4.8 Size and zeta potential measurements

The particle size and surface charge of PEI-CA/pEGFP plasmid DNA and AS ODN complexes and DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The complexes were prepared in ultrapure sterile water (50 μl) and diluted with deionized water or bovine serum albumin (BSA) solution to obtain the volume required for each measurement (1 ml). The measurements were performed using the aqueous flow cell in the automatic mode at 25°C.

4.9 Encapsulation efficiency

PEI-CA/pEGFP gene and cationic liposome/AS ODN complexes were prepared in 100 μl of 20 mM HEPES, pH 7.4. Their encapsulation efficiency was determined by centrifugation (11,000 rpm, 10 min). The content of unencapsulated plasmid DNA and AS ODN in the supernatant was determined by measuring optical density at 260 nm on a cuvette photometer (Eppendorf Biophotometer and, Eppendorf AG, Hamburg, Germany). The encapsulation efficiency (%) was calculated as follows. PEI-CA or liposome alone was used as a background control.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Encapsulated nucleic acid}}{\text{Total nucleic acid}} \times 100$$

4.10 Cell culture

HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in MEM (Invitrogen, Grand Island, NY, USA) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% amphotericin B, under a humidified atmosphere of 5% CO_2 in air at 37°C.

4.11 AS ODN uptake

HeLa cells were seeded into 6 well plates at a density of 2.5×10^{5} cells/well in 1.5 ml of growth medium 20 h prior to transfection. The growth medium were replaced with 1 ml of PEI-CA complexed with pEGFP and Cy3-labeled AS ODN, and DDAB:DOPE liposomes complexed with pEGFP and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN. Untreated cells and cells treated with uncomplexed nucleic acids were used as control. After 4 h incubation at 37 $^{\circ}$C, the cells were treated with 0.4% trypan blue for 1 min and rinsed with phosphate bufferred saline (PBS), pH 7.4. Cells were harvested by trypsinization with 0.2% trypsin and rinsed with PBS, pH 7.4. The cells were then fixed with 4% paraformaldehyde.

The fluorescent intensity of Cy3 was measured by flow cytometry on a FACSCanto™II flow cytometer (BD Biosciences, San Jose, CA, USA). In the separated experiments, cells were stained with DAPI (2 μg/ml) and imaged using a fluorescence microscope (IX83-ZDC, Olympus, Tokyo, Japan).

4.12 GFP expression

HeLa cells were seeded into 6 well plates at a density of 2.5×10^{5} cells/well in 1.5 ml of growth medium 20 h prior to transfection. The cells were incubated with 1 ml of PEI-CA/pEGFP plasmid DNA and AS ODN complexes, and DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h. Untreated cells were used as control. The medium were removed and the cells were incubated in growth medium at 37 °C. After 20 h incubation, the cells were rinsed with PBS, pH 7.4, harvested, and fixed with 4% paraformaldehyde. The GFP expression was quantified by flow cytometry on a FACSCanto™II flow cytometer (BD Biosciences, San Jose, CA, USA). In the separated experiments, cells were imaged using a fluorescence microscope (IX83-ZDC, Olympus, Tokyo, Japan).

4.13 P53 protein expression and bcl-2 protein down regulation detection

P53 protein expression and bcl-2 protein down regulation were evaluated by Western blot analysis of p53 and bcl-2 proteins. PEI-CA/p53 and cationic liposome/bcl-2 AS ODN transfection of HeLa cells were performed as described above. After 24 h incubation, the cells were harvested by trypsinizing with 0.25 % trypsin-EDTA and washing with PBS, pH 7.4. The cells were then lysed with 50 µl of lysis buffer, pH 7.4 containing protease Inhibitor cocktail set III and incubated on ice for 10 min. The cell lysate was then centrifuged at 12,000 rpm at 4 °C for 10 min and the supernatant is collected. The total protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Cell lysate containing 20 µg of total protein was separated on a 12.5% SDS-polyacrylamide gel under denaturing conditions at 180 V for 1 h 30 min (Mini-PROTEAN® Tetra Cell; Biorad, Hercules, CA, USA) and then blotted to polyvinylidine difluoride membranes (Hybond™-P; Amersham Bioscience, GE Healthcare, Piscataway, NJ, USA) at 350 mA for 1 h (Mini-PROTEAN® Tetra Cell; Biorad, Hercules, CA, USA) at 4°C. The membranes were blocked for 1 h in 10% powdered nonfat milk in Tris-buffered saline/Tween-20, and then incubated with monoclonal mouse anti-human p53 (Dakocytomation, Glostrup, Denmark) and monoclonal mouse anti-human bcl-2 (Dakocytomation, Glostrup, Denmark) at a dilution of 1:2000 or mouse anti-human β-actin antibody (Invitrogen, Grand Island, NY, USA) at a dilution of 1.4:10,000 overnight at 4°C. Following four washing steps, the membranes were incubated with houseradish peroxidase-linked antimouse IgG (Invitrogen, Grand Island, NY, USA) at a dilution of 1.4:10,000 or at a dilution of 1:14,000 for 1 h at room temperature. The membranes are then developed with Amersham ECL Prime Western Blotting Detection (GE Healthcare Bioscience AB, Upsala, Sweden) and imaged on an image analyzer (ImageQuant™ LAS 4000 mini, GE Healthcare Bioscience AB, Upsala, Sweden). The bcl-2 protein level was quantified using an image analysis software (Image

Analysis Software v7.0, GE Healthcare Bioscience AB) and normalized with the β-actin level from the same sample.

4.14 Growth inhibition

The efficacy of the co-delivery of plasmid DNA and AS ODN by liposomes were evaluated using a therapeutic gene and AS ODN, p53 and bcl-2 AS ODN, in terms of growth inhibition. The evaluation of growth inhibition were performed with three different categories. Firstly, to evaluate the efficacy of the co-delivery of p53 plasmid DNA and bcl-2 AS ODN, the growth inhibition was investigated with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes, and DDAB:DOPE liposome/p53 plasmid DNA complexes, DDAB:DOPE liposome/bcl-2 AS ODN complexes, and the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a concentration of p53 plasmid DNA and bcl-2 AS ODN of 4 and 5 μg/ml, respectively. Secondly, the effect of the lipid-to-DNA and lipid-to-ODN ratios on the growth inhibition was investigated with different lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10. The amount of p53 plasmid DNA and bcl-2 AS ODN was fixed at 4 and 5 μg/ml, respectively. Thirdly, the efficacy of the co-delivery of p53 plasmid DNA and bcl-2 AS ODN by cationic liposomes on growth inhibition were evaluated with liposomes formulated with PEI-CA and PEI, and various cationic lipids including DDAB, DLPAP, and DOTAP, and a lipid-based transfection reagent, Lipofectamine™2000.

For the evaluation of growth inhibition, HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells/well in 100 μl of growth medium and incubated for 20 h at 37°C under 5% CO_2 atmosphere. Prior to treatment, the medium was removed and the cells were rinsed with PBS, pH 7.4. The cells were incubated with 62.5 μl of PEI-CA/nucleic acid complexes or cationic liposome/nucleic acid complexes for 4 h at 37°C under 5% CO_2 atmosphere. Untreated cells and cells transfected with free nucleic acids were used as controls. After transfection, the cells were rinsed with PBS, pH 7.4, and continued to be cultured in 100 μl of fresh growth medium at 37 °C under 5% CO_2 atmosphere for 20 h. After 20 h incubation, the viability of cell was determined by the MTT assay by measurement of UV absorbance at 570 nm using a microplate spectrophotometer (Zenyth 200 rt; Anthos Labtech Instruments GmbH, Salzburg, Austria). Cell growth inhibition (%) was calculated relative to cells treated with the medium as a control.

4.15 Colloidal stability

The colloidal stability of PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes, and DDAB:DOPE liposome/plasmid DNA complexes and DDAB:DOPE liposome/AS ODN complexes was evaluated by monitoring change in the average particle size during storage and incubation with 10% BSA solution at 4°C for 14 days.

4.16 Statistical analysis

All results were demonstrated as the mean ± standard deviation (S.D.). Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by an LSD post hoc test. $p < 0.05$ was defined as statistical significance.

5. Results

5.1 Synthesis of PEI-CA

Conjugation of CA to PEI 25KDa through the amide linkage was shown in Fig. 2. The carboxylic group of CA was activated with DCC, and conjugated to amine groups of PEI. The degree of substitution (DS) was determined by ^{1}H NMR spectroscopic method from the ratio of the peak area corresponding to the terminal methyl group in the CA to that of the methylene in PEI [4, 5]. It was found that the degree of substitution of PEI-CA was about 1.5.

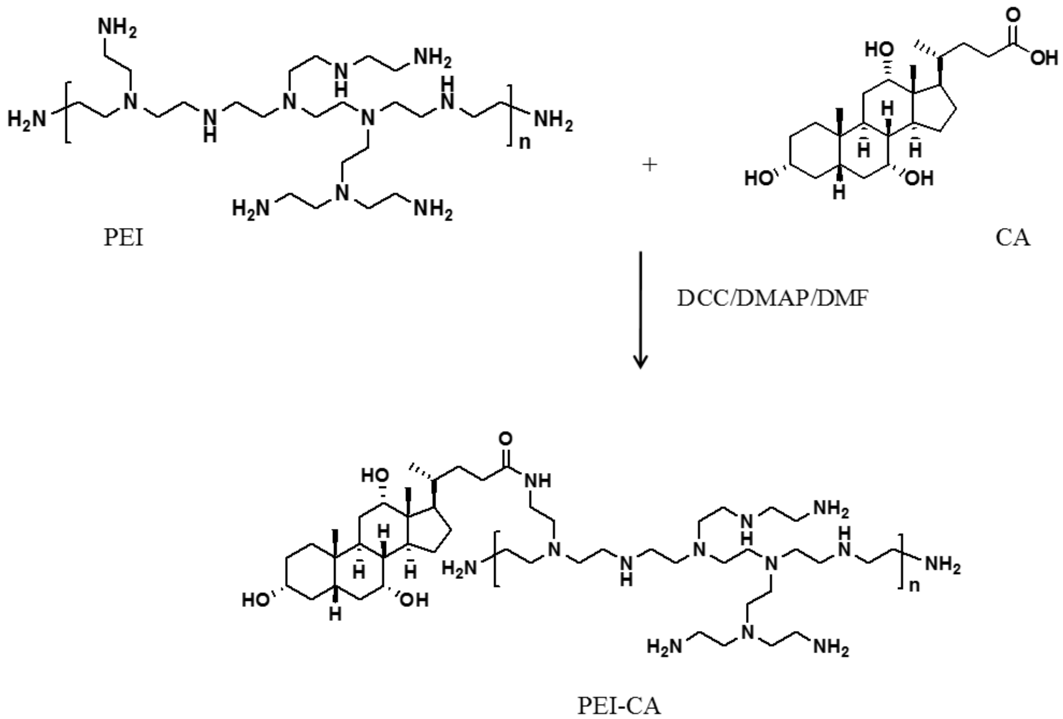

Fig. 2 Synthesis Scheme of PEI-CA.

The conjugation of CA to PEI was characterized by FTIR and ^{1}H NMR spectroscopy. FTIR-spectrum of PEI-CA is shown in Fig. 3 where peaks for the N-H bending, C=O absorption band, C-H stretching, and N-H stretching were observed at 1614, 1658, 2815 to 2928, and 3252 cm^{-1}, respectively. The presence of C=O absorption band at 1658 cm^{-1} indicated that the formation of an amide linkage between PEI and CA was occurred [6]. ^{1}H NMR spectrum of PEI-CA shows a PEI peak at 2.5-3.4 ppm and the terminal methyl groups of CA at 0.81 and 1.07 ppm (Fig. 4). These results confirmed the linkage between PEI and CA.

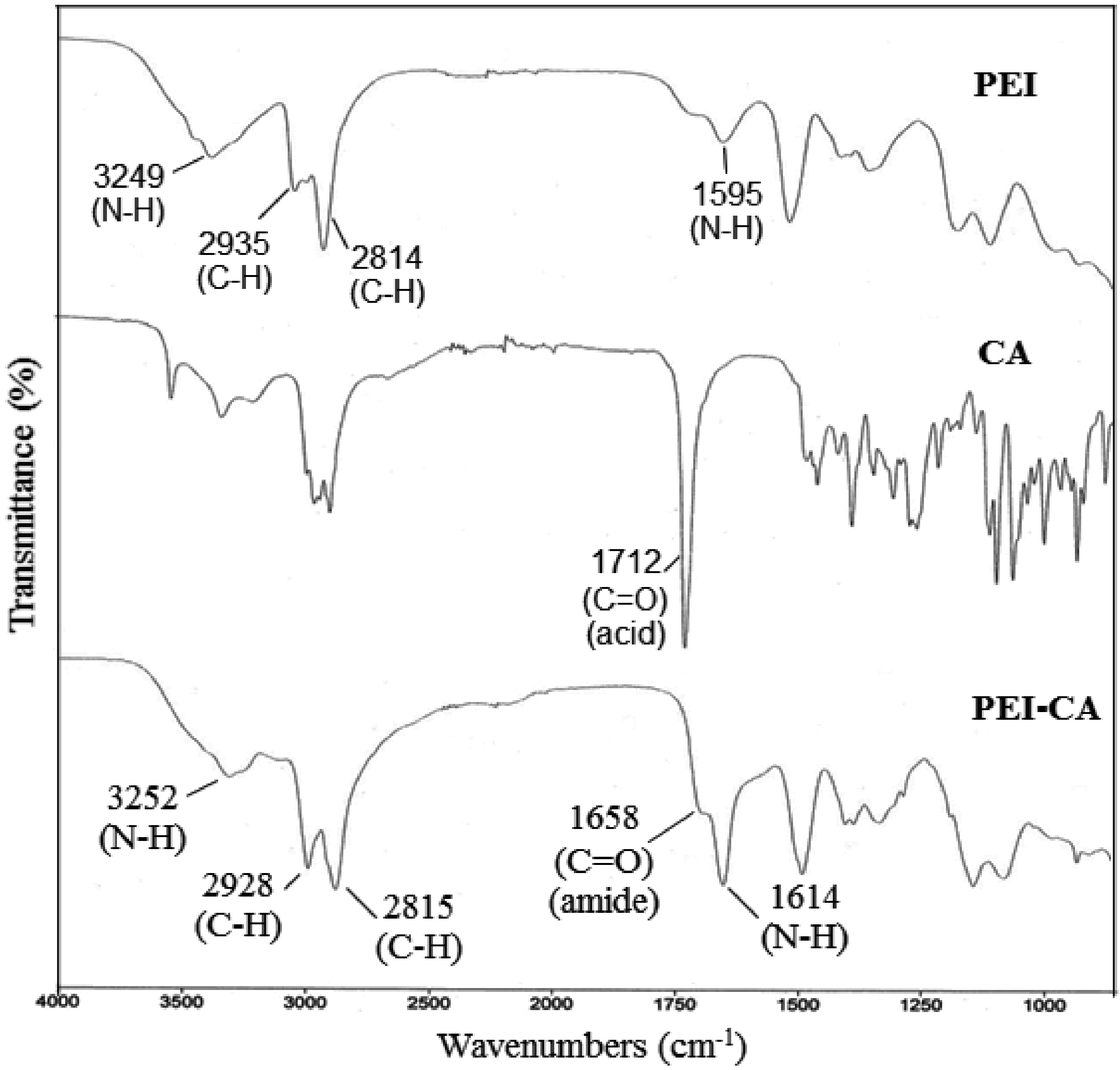

Fig. 3 ^{1}H NMR spectrum of PEI, CA, and PEI-CA

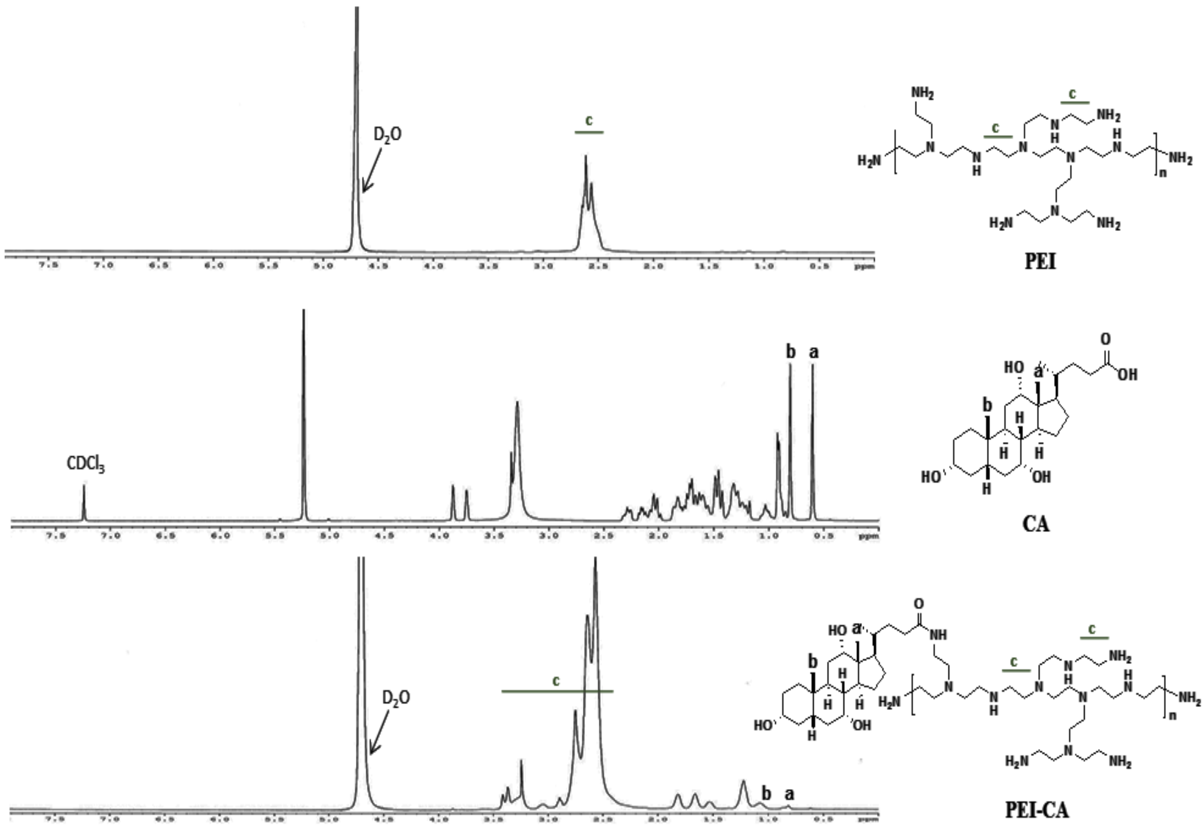

Fig. 4 ^{1}H NMR spectrum of PEI, CA, and PEI-CA.

5.2 Characterization of PEI-CA/pEGFP and AS ODN complexes and DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes

5.2.1 PEI-CA/pEGFP and AS ODN complexes

The formation of complexes between PEI-CA and pEGFP plus AS ODN was assessed through retardation of nucleic acid electrophoretic mobility by agarose gel electrophoresis at polymer-to-nucleic acid (NA) ratios of 0.0625, 0.125, 0.25, 0.5, 1 and 2. The free plasmid and AS ODN lane showed the DNA and ODN bands, whereas the band density decreased when the amount of PEI-CA increased. The nucleic acids were completely retarded at polymer-to-NA ratios as low as 0.5 (Fig. 5). The particle size and zeta potential of PEI-CA/pEGFP and AS ODN complexes were evaluated at varying polymer-to-NA ratio (Fig. 6). The particle size of PEI-CA/pEGFP and AS ODN complexes decreased with increasing polymer-to-NA ratios. The particle size of PEI-CA/pEGFP and AS ODN complexes at polymer-to-NA ratios of 0.2 to 1 was in the range of 87.7±29.9 to 182.8±6.8 nm. The zeta potential of PEI-CA/pEGFP and AS ODN complexes was negative at a polymer-to-NA ratio of 0.2, and increased to positive va

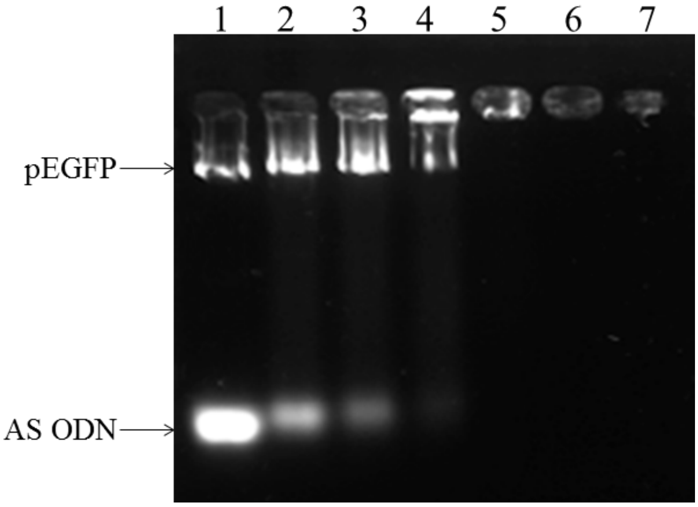

Fig. 5 Gel electrophoresis assay of PEI-CA/pEGFP and AS ODN complexes. Lane 1: 0.25 μg free pEGFP and 0.3125 μg free AS ODN. Lanes 2-8: PEI-CA/pEGFP and AS ODN complexes at polymer-to-NA ratios of 0.0625, 0.125, 0.25, 0.5, 1, and 2, respectively.

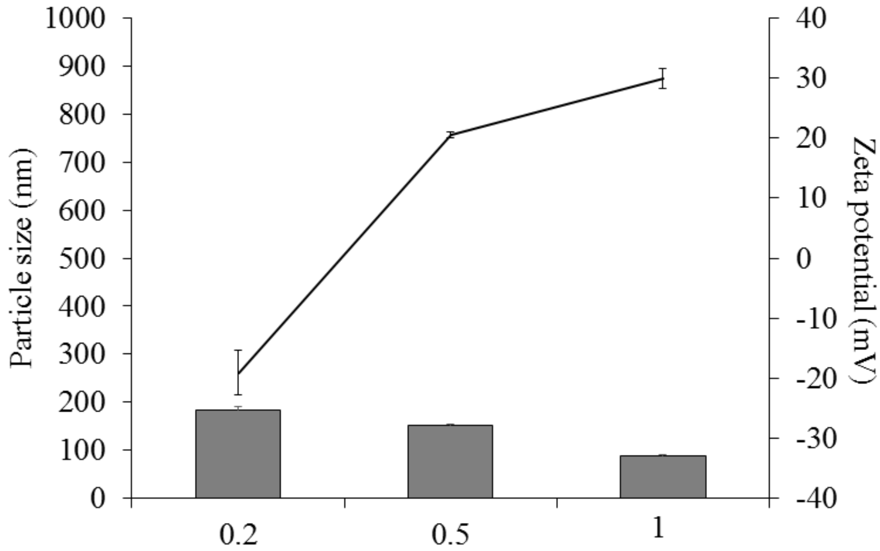

Fig. 6 Particle size (▩) and zeta potential (─) of PEI-CA/pEGFP and AS ODN complexes at varying polymer-to-NA ratios in ultrapure water. Each value represents the mean±S.D. of three measurements.

5.2.2 DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes

The complexation of cationic liposomes to pEGFP plasmid DNA and AS ODN was measured by agarose gel electrophoresis. DDAB:DOPE liposome/pEGFP complexes at different lipid-to-DNA ratios with a range of 1.5, 3, 6, 12.5, 25, 50 and 100, and DDAB:DOPE liposome/AS ODN complexes at lipid-to-ODN ratios of 1.25, 2.5, 5, 10, 20, 40 and 80, with increasing the amount of added cationic liposomes to a fixed amount of pEGFP and AS ODN were investigated. As shown in (Fig. 7), when DDAB:DOPE liposomes were mixed with pEGFP and AS ODN, the amount of free pEGFP and AS ODN decreased proportionally with increasing amounts of DDAB:DOPE liposomes.

pEGFP and AS ODN showed different nucleic acid condensation patterns. In the case of pEGFP, the complete complexes of DDAB:DOPE liposomes and pEGFP were formed at lipid-to-DNA ratios above 6. On the other hand, the complete complexes of liposomes and AS ODN were formed at lipid-to-ODN ratios above 20. In all experiments, DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes was chosen at lipid-to-DNA and lipid-to-ODN ratios below complete complexes due to the high cytotoxicity of high amount of cationic lipids.

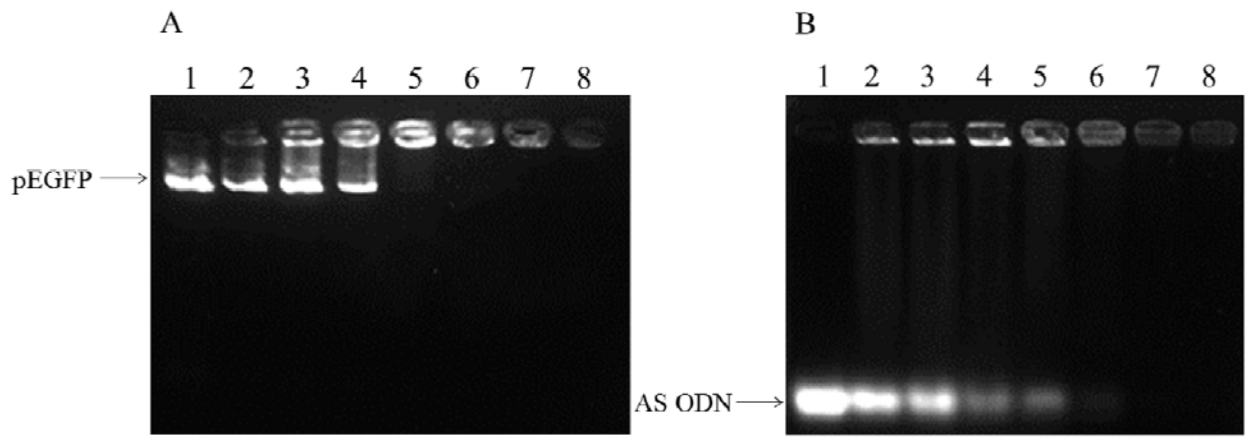

Fig. 7 Gel electrophoresis analysis of **(A)** DDAB:DOPE liposome/pEGFP complexes on 1.2% agarose gel. Lane 1: free pEGFP (0.25 μg). Lanes 2-8: DDAB:DOPE/pEGFP complexes at lipid-to-DNA ratios of 1.5, 3, 6, 12.5, 25, 50 and 100, respectively, and **(B)** DDAB:DOPE liposome/AS ODN complexes on 1.2% agarose gel. Lane 1: free AS ODN (0.3125 μg). Lanes 2-8: DDAB:DOPE liposome/AS ODN complexes at lipid-to-ODN ratios of 1.25, 2.5, 5, 10, 20, 40 and 80, respectively.

The particle size and zeta potential of DDAB:DOPE liposomes and DDAB:DOPE liposome/nucleic acid complexes were determined with a Zetasizer Nano ZS. The particle size of DDAB:DOPE liposomes were 166.3±3.3 nm with polydispersity index of 0.533. As cationic liposomes, they had a positive charge. The zeta potential of DDAB:DOPE liposomes were +25.9±1.4 mV. This demonstrates that the liposomes with nanosized and positive zeta potential could be suitable for cellular entry of plasmid DNA and AS ODN, and thus they were employed for complex formation with nucleic acids.

The particle size and zeta potential of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were plotted versus lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10, respectively (Fig. 8). The particle size of DDAB:DOPE liposome/pEGFP complexes decreased with increasing lipid-to-DNA ratio from 3 to 12.5. For DDAB:DOPE liposome/AS ODN complexes, the particle size was relatively constant in the range of the lipid-to-ODN ratios of 2.5 to 10. The particle size of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were in the range of 197±3.0 to 372±2.4 nm for DDAB:DOPE liposome/pEGFP complexes and of 180±2.0 to 203±7.7 nm for DDAB:DOPE liposome/AS ODN complexes. Zeta potential of DDAB:DOPE liposome/pEGFP complexes increased with increasing lipid-to-DNA ratio and lipid-to-ODN ratio in both DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes. An initial negative value of the zeta potential was observed at the low lipid-to-DNA ratios of 3 and 6, and lipid-to-ODN ratios of 2.5 and 5. At the lipid-to-DNA ratio of 6 and lipid-to-ODN ratio of 5, the zeta potential reached a positive value of +2.3±2.2 mV for DDAB:DOPE liposome/pEGFP complexes and of 6.8±0.4 mV for DDAB:DOPE liposome/AS ODN complexes.

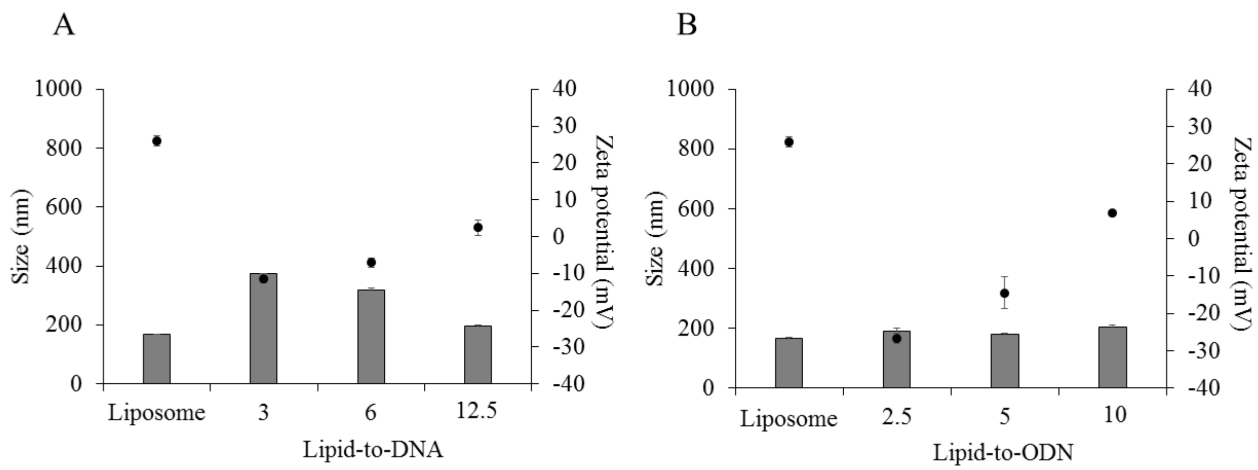

Fig. 8 Particle size ( ) and zeta potential (●) of (A) DDAB:DOPE liposome/pEGFP complexes at varying lipid-to-DNA ratios, and (B) DDAB:DOPE liposome/AS ODN complexes at varying lipid-to-ODN ratios in deionized water. Each value represents the mean±S.D. of three measurements.

Polydispersity index of PEI-CA/plasmid DNA and PEI/plasmid DNA complexes at polymer-to-DNA ratio of 0.5, of PEI-CA/ODN and PEI/ODN complexes at polymer-to-ODN ratios of 0.4, of DDAB:DOPE liposome/plasmid DNA and DLPAP:DOPE liposome/plasmid DNA complexes at lipid-to-DNA ratio of 12.5, and of DDAB:DOPE liposome/ODN and DLPAP:DOPE liposome/ODN complexes at lipid-to-ODN ratio of 10 is shown in Table 1.

Table 1 Polydispersity index of PEI-CA/plasmid DNA, PEI/plasmid DNA, DDAB:DOPE liposome/ODN and DLPAP:DOPE liposome/ODN complexes

Polyplex/Lipoplex	Polydispersity index
Polyplex	
PEI-CA/DNA	0.223
PEI/DNA	0.252
PEI-CA/ODN	0.213
PEI/ODN	0.199
Lipoplex	
DDAB:DOPE/DNA	0.346
DLPAP:DOPE/DNA	0.140
DDAB:DOPE/ODN	0.230
DLPAP:DOPE/ODN	0.234

Entrapment efficiency of PEI-CA/plasmid DNA and PEI/plasmid DNA complexes at polymer-to-DNA ratio of 0.5, of PEI-CA/ODN and PEI/ODN complexes at polymer-to-ODN ratios of 0.4, of DDAB:DOPE liposome/plasmid DNA and DLPAP:DOPE liposome/plasmid DNA complexes at lipid-to-DNA ratio of 12.5, and of DDAB:DOPE liposome/ODN and DLPAP:DOPE liposome/ODN complexes at lipid-to-ODN ratio of 10 is shown in Table 2.

Table 2 Entrapment efficiency of PEI-CA/plasmid DNA, PEI/plasmid DNA, DDAB:DOPE liposome/ODN and DLPAP:DOPE liposome/ODN complexes

Polyplex/Lipoplex	Entrapment efficiency (%)
Polyplex	
PEI-CA/DNA	41.7
PEI/DNA	46.1
PEI-CA/ODN	27.7
PEI/ODN	28.0
Lipoplex	
DDAB:DOPE/DNA	71.8
DLPAP:DOPE/DNA	76.6
DDAB:DOPE/ODN	48.0
DLPAP:DOPE/ODN	84.8

5.3 Transfection efficiency

FACS analysis showed that the Cy3-labeled AS ODN-positive cells increased after co-delivery of nucleic acids using PEI-CA (Fig. 9). Compared with naked nucleic acids, PEI-CA/pEGFP and Cy3-labeled AS ODN complexes produced a 15.1-fold increase in fluorescence intensity. Fluorescence microscopy revealed that the fluorescent of Cy3-labeled AS ODN was concentrated in the cytoplasm and some Cy3-labeled AS ODN was located in the nucleus of the cells at 4 h after the addition of PEI-CA/pEGFP and Cy3-labeled AS ODN complexes to the cells, indicating that PEI-CA/pEGFP and Cy3-labeled AS ODN complexes entered the cells and AS ODN delivered into the cytoplasm, and thus transferred into the nucleus (Fig. 10A).Transfection efficiency of PEI-CA/pEGFP and AS ODN was evaluated in HeLa cells using pEGFP encoding for green fluorescent protein as a reporter gene. Fig. 9 shows the transfection efficiency of PEI-CA/pEGFP and AS ODN at a polymer-to-NA ratio of 0.2 at 24 h post transfection. The transfection efficiency of PEI-CA/pEGFP and AS ODN was 14.8-fold higher than that of uncomplexed nucleic acids. The GFP was observed in the cells (Fig. 10B).

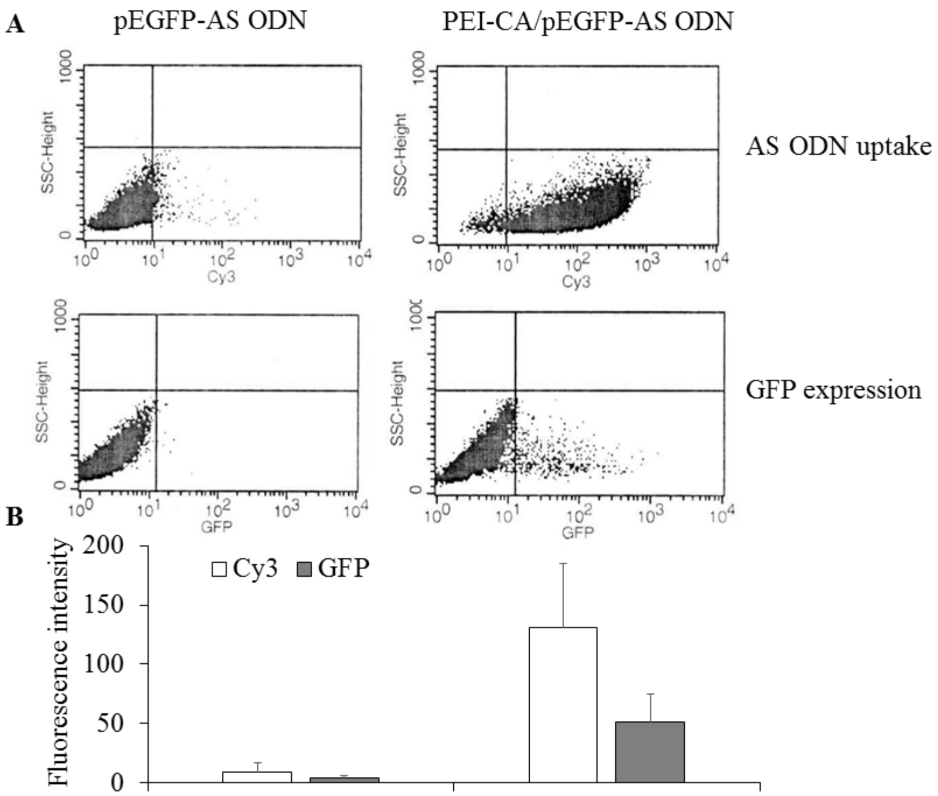

Fig. 9 AS ODN uptake and GFP expression of PEI-CA/pEGFP and AS ODN complexes at a polymer-to-NA ratio of 0.2 in HeLa cells by flow cytometric analysis (A). Fluorescence intensity (B).

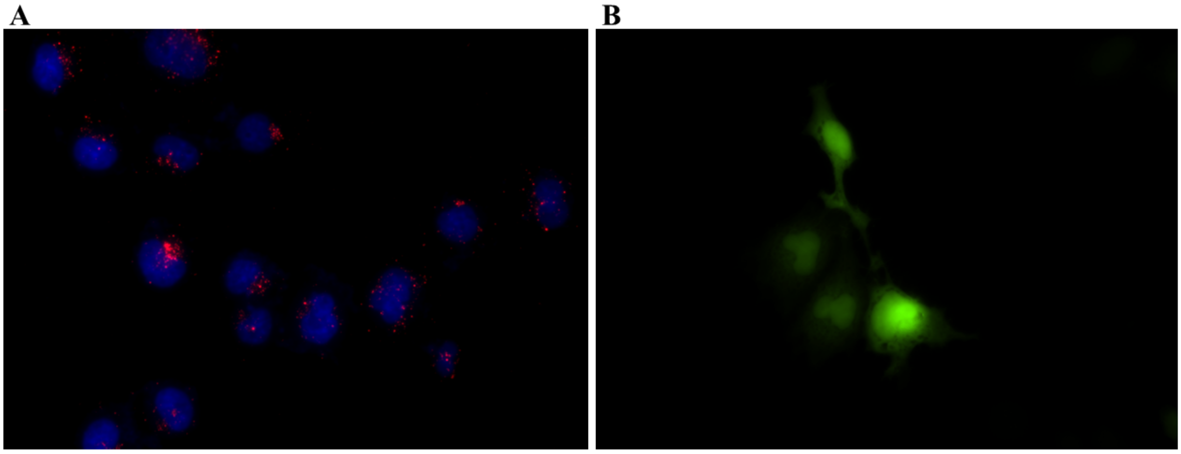

Fig. 10 Fluorescent images of PEI-CA/pEGFP and AS ODN complexes at a polymer-to-NA ratio of 0.2 in HeLa cells. (A) AS ODN uptake. (B) GFP expression.

The AS ODN uptake of the co-delivery of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were assessed in HeLa cells with the pEGFP and AS ODN concentrations of 4 and 5 μg/ml using Cy3-labeled AS ODN (Fig. 11A, Fig. 12B). DDAB:DOPE liposomes were complexed with pEGFP and AS ODN mixed with Cy3 labeled AS ODN at lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10, respectively. The untreated cells yielded Cy3 positive cells of 1.71±1.2% fluorescence intensity. The fluorescence intensity of the cells treated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/Cy3-labeled AS ODN complexes examined by flow cytometry was significantly increased from 6.8±0.9 to 10.8±2.7% fluorescence intensity with increasing lipid-to-DNA ratio from 3 to 6 and lipid-to-ODN ratio from 2.5 to 5 ($p < 0.05$) and decreased to 6.2±1.5% fluorescence intensity at a lipid-to-DNA ratio of 12.5 and lipid-to-ODN ratio of 10 (Fig. 10A). The fluorescent of Cy3-labeled AS ODN was observed mainly in the cytoplasm of the cells at 4 h after the addition DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/ Cy3-labeled AS ODN complexes to the cells, indicating that DDAB:DOPE liposome/AS ODN complexes entered the cells and AS ODN delivered into the cytoplasm. (Fig. 12B).

Improving nucleic acid transfection efficiency is an ultimate goal for the development of nucleic acid delivery system. To investigate cationic liposomes mediated gene delivery, in vitro gene transfection was performed in HeLa cells using pEGFP-C2 plasmid DNA encoding green fluorescence protein.

The GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5, 5 and 10, used for AS ODN uptake study, were employed to assess the GFP expression of pEGFP. The GFP expression of all lipid-to-DNA and lipid-to-ODN ratios was higher than that of untreated cells. The GFP expression significantly increased with increasing lipid-to-DNA ratio from 3 to 6 and lipid-to-ODN ratio from 2.5 to 5 with the fluorescence intensity from 10.3±3.0 to 20.2±5.8% ($p < 0.05$) and decreased after a lipid-to-DNA ratio of 6 and lipid-to-ODN ratio of 5 to 11.3±2.7% fluorescence intensity (Fig. 11B). Fig. (12C) shows the GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes in HeLa cells.

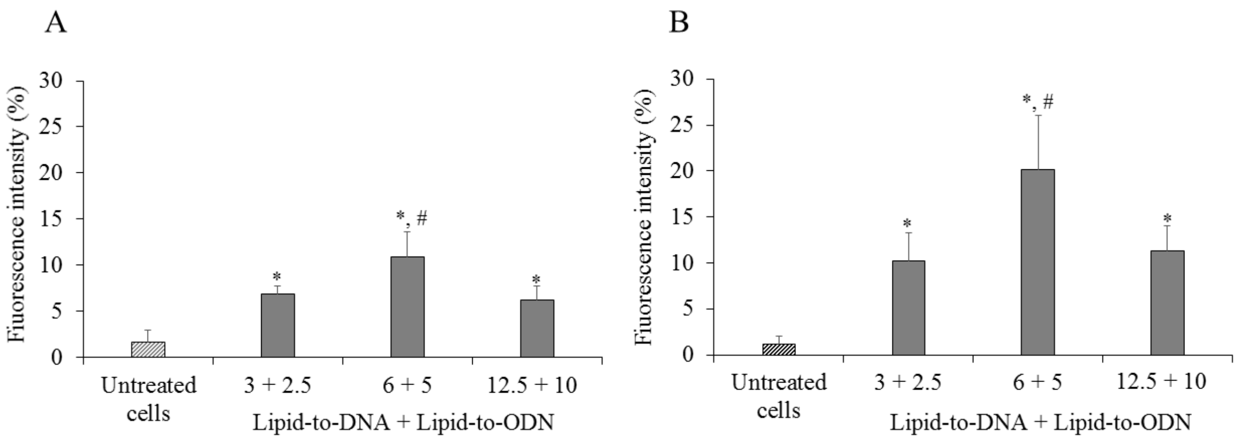

Fig. 11 A) AS ODN uptake, and B) GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at varying lipid-to-DNA ratios and DDAB:DOPE liposome/AS ODN complexes at varying lipid-to-ODN ratios in HeLa cells. In AS ODN uptake experiment, cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN for 4 h at 37 °C. In GFP expression experiment, cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h at 37 °C and in growth medium for 20 h at 37 °C. The fluorescent intensity were measured on a FACSCanto™II flow cytometer. *$p < 0.05$ when compared with untreated cells; $^{\#}p < 0.05$ when compared with cells treated with the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5, and cells treated with the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 12 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 10. Each value represents the mean±S.D. of three measurements.

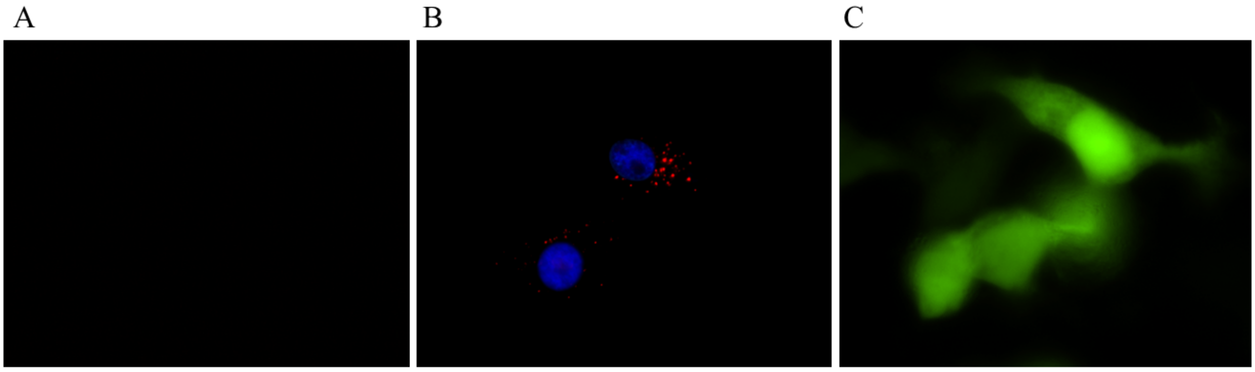

Fig. 12 Fluorescent images of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 6 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN of 5 in HeLa cells. A) Untreated cells. B) AS ODN uptake. Cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN for 4 h at 37 °C. C) GFP expression. Cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h at 37 °C and in growth medium for 20 h at 37 °C.

5.4 Growth inhibition

Growth inhibition of HeLa cells by PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes was evaluated by MTT assay. p53 plasmid DNA and bcl-2 AS ODN co-delivered using PEI-CA exhibited greater growth inhibition level than p53 plasmid DNA or bcl-2 AS ODN delivered using PEI-CA alone (Fig. 13).

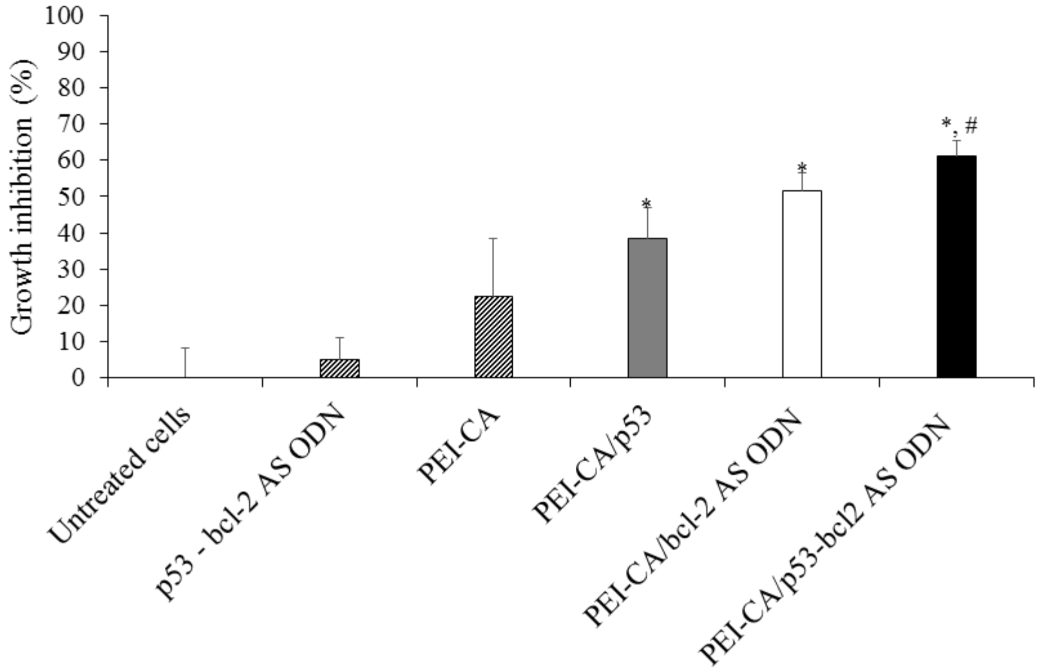

Fig. 13 Growth inhibition by PEI-CA/p53 plasmid DNA complexes, PEI-CA/bcl-2 AS ODN complexes, and PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes, at a polymer-to-NA ratio of 0.5 in HeLa cells. **p* < 0.05 when compared with cells treated with p53 plasmid DNA and bcl-2 AS ODN, and cells treated with PEI-CA; #*p* < 0.05 compared with cells treated with PEI-CA/p53 plasmid DNA. Each value represents the mean±S.D. of three wells.

To determine whether treatment of the cells with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes inhibits cell growth, the cells were transfected with varying concentrations of p53 plasmid DNA and bcl-2 AS ODN for 4 h at a polymer-to-NA ratio of 0.5. Increasing the concentration of p53 plasmid DNA of 1, 2 and 4 µg/ml, and bcl-2 AS ODN of 1.25, 2.5 and 5 µg/ml in polyplexes increased growth inhibition level on HeLa cells to 10.7±11.8, 53.4±5.7 and 61.0±4.6% that of the control, respectively (Fig. 14). Significant growth inhibition was observed from the complexes at p53 plasmid DNA concentrations of 2 and 4 µg/ml, and bcl-2 AS ODN concentrations of 2.5 and 5 µg/ml ($p<0.05$). PEI-CA alone at the concentrations as those used for complexes of p53 plasmid DNA of 1 and 2 µg/ml and bcl-2 AS ODN of 1.25 and 2.5 µg/ml did not induce significant cytotoxicity as compared with cells treated with p53 plasmid DNA and bcl-2 AS ODN (p>0.05). However, a high concentration of PEI-CA alone, at dose equivalence of complexes of p53 plasmid DNA of 4 μg/ml and bcl-2 AS ODN of 5 μg/ml did induce cytotoxicity (77.5±16.1% cell viability) (p<0.05).

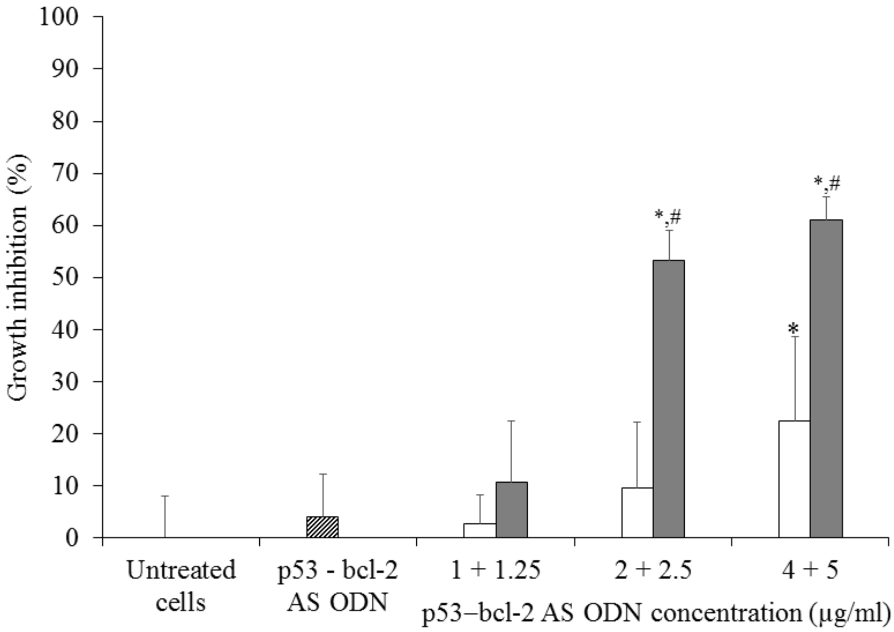

Fig. 14 Effect of p53 plasmid DNA and bcl-2 AS ODN concentrations of PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes at a polymer-to-NA ratio of 0.5 on HeLa cell growth. Dash bar: p53 plasmid DNA and bcl-2 AS ODN; white bar: PEI-CA; grey bar: PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes. *p < 0.05 compared with untreated cells, and cells treated with p53-bcl-2 AS ODN; #p < 0.05 compared with cells treated with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes at the p53 plasmid DNA and bcl-2 AS ODN concentrations of 1 and 1.25 μg/ml. Each value represents the mean±S.D. of three wells.

PEI-CA/p53 plasmid DNA and bcl-2 AS ODN inhibited cell growth as effectively as PEI/p53 plasmid DNA and bcl-2 AS ODN (Fig. 15). However, the cytotoxicity of PEI-CA was less. This indicated that PEI-CA could be an alternative delivery carrier for plasmid DNA and AS ODN.

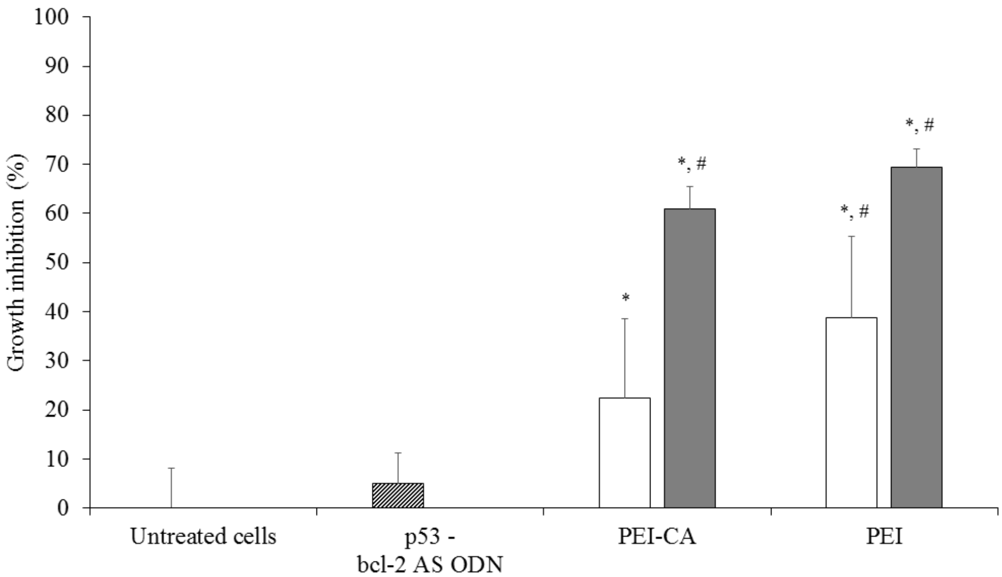

Fig. 15 Growth inhibition of HeLa cells by PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes and PEI/p53 plasmid DNA and bcl-2 AS ODN complexes at a polymer-to-NA ratio of 0.5 in HeLa cells. White bar: polymer; grey bar: polymer/p53 plasmid DNA and bcl-2 AS ODN complexes. **p* < 0.05 compared with untreated cells; #*p* < 0.05 compared with untreated cells, and cells treated with p53 plasmid DNA and bcl2 AS ODN. Each value represents the mean±S.D. of three wells.

The growth inhibition on HeLa cells following 24 h incubation after co-transfection with DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 2.5, and after transfection with DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 6, DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 5, free p53 plasmid DNA and bcl-2 AS ODN at a concentration of 4 and 5 µg/ml, respectively, and liposome alone is shown in (Fig. 16). Free p53 plasmid DNA and bcl-2 AS ODN revealed negligible cell growth inhibition. DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 47.7±8.6% (p < 0.05) and 37.4±2.4% (p < 0.05) of the control, respectively. The p53 plasmid DNA and bcl-2 AS ODN co-delivered using DDAB:DOPE liposomes exerted growth inhibition level of 63.1±7.6% (p < 0.05), which was greater than either p53 plasmid DNA or bcl-2 AS ODN delivered using DDAB:DOPE liposomes. DDAB:DOPE liposomes alone had low toxicity to cells.

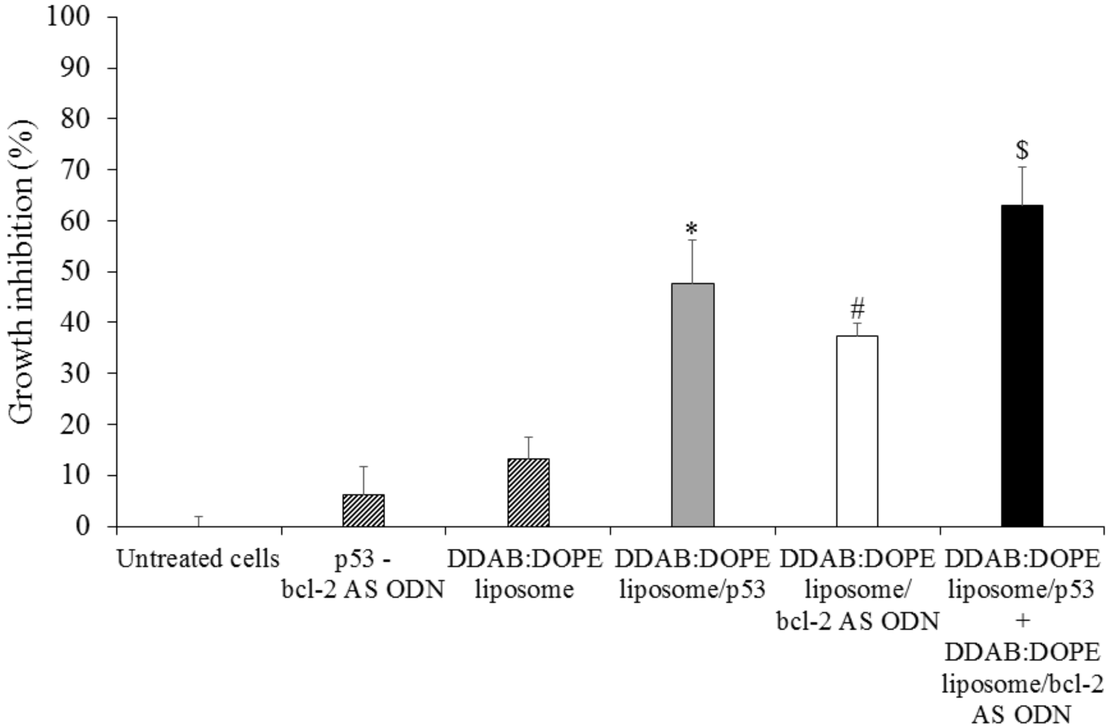

Fig. 16 Effect of the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 2.5, DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 6, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 5 on HeLa cell growth. Cells were incubated with the complexes at the p53 plasmid DNA and bcl-2 AS ODN concentrations of 4 and 5 µg/ml for 4 h and in growth medium for 20 h. $^{*}p < 0.05$ when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, cells treated with DDAB:DOPE liposomes, and cells treated with DDAB:DOPE liposome/bcl-2 AS ODN; $^{\#}p < 0.05$ when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, and cells treated with DDAB:DOPE liposomes; $^{\$}p < 0.05$ when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, cells treated with DDAB:DOPE liposomes, cells treated with DDAB:DOPE liposome/p53 plasmid DNA complexes, and cells treated with DDAB:DOPE liposome/bcl-2 AS ODN complexes. Each value represents the mean±S.D. of three wells.

To study the effect of the lipid-DNA and lipid-ODN ratios of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes on the cell growth inhibition, different amounts of total lipids with a fixed p53 plasmid DNA concentration of 4 µg/ml and bcl-2 AS ODN concentration of 5 µg/ml were evaluated in HeLa cells. Fig. 17 shows the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at lipid-to-ODN ratios of 2.5, 5 and 10, on HeLa cell growth inhibition. The co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at lipid-to-ODN ratios of 2.5, 5 and 10, inhibited cell growth to 63.1±7.6 to 75.2±3.5% ($p<0.05$) that of the control. Free p53 plasmid DNA and bcl-2 AS ODN exhibited a low growth inhibition (6.2±5.5%). The cytotoxicity of DDAB:DOPE liposome alone increased with an increasing the amount of liposomes. The cytotoxicity of DDAB:DOPE liposome alone at the amount corresponding to DDAB:DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratio of 3, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at lipid-to-ODN ratio of 2.5 was low with 13.2±4.3% growth inhibition.

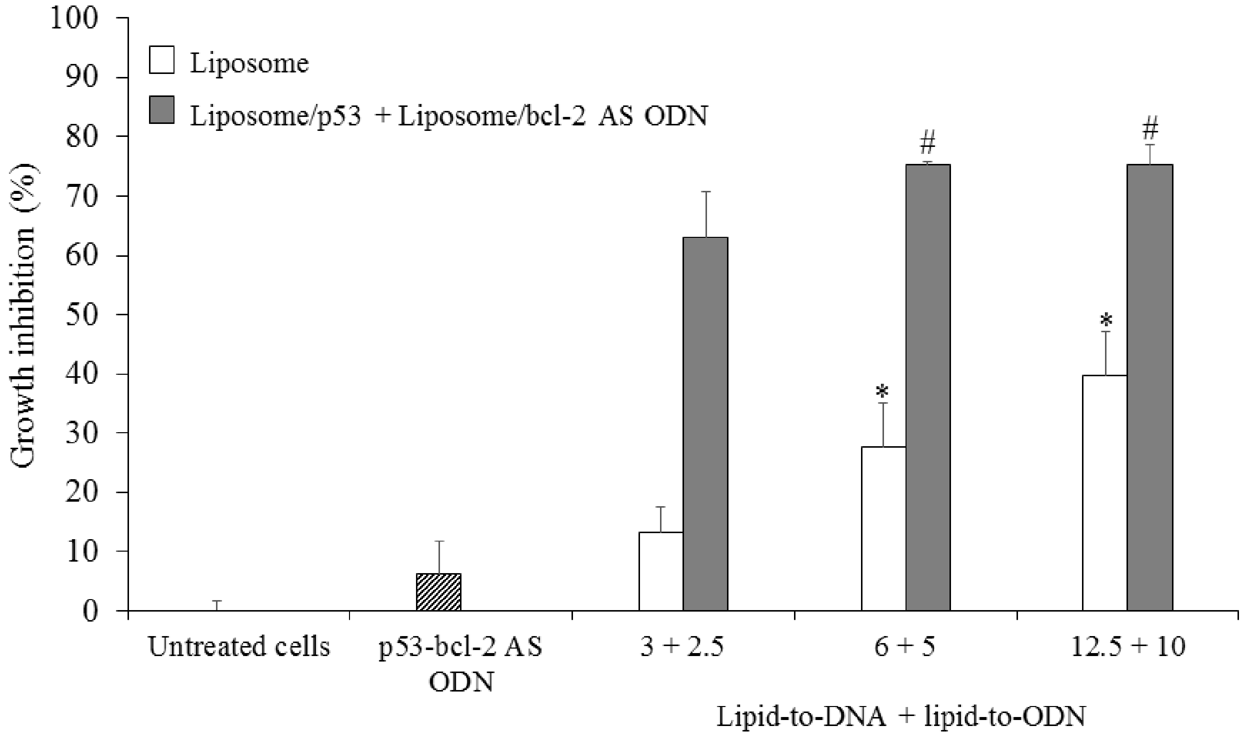

Fig. 17 Effect of lipid-to-DNA and lipid-to-ODN ratios of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes at the p53 plasmid DNA and bcl-2 AS ODN concentrations of 4 and 5 μg/ml on HeLa cell growth. Cells were incubated with DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes for 4 h and in growth medium for 20 h. $^{*}p < 0.05$ when compared with cells treated with p53 plasmid DNA and bcl-2 AS ODN; $^{\#}p < 0.05$ when compared with cells treated with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5. Each value represents the mean±S.D. of three wells.

Fig. 18 shows the growth inhibition level of p53 plasmid DNA and bcl-2 AS ODN using cationic-based liposomes formulated with various cationic lipids including DDAB, DLPAP and DOTAP at a lipid-to-DNA ratio of 3 and lipid-to-ODN ratio of 2.5:1, and a lipid-based transfection reagent, Lipofectamine™2000. The co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 63.1±7.6%, whereas the co-delivery of DLPAP:DOPE liposome/p53 plasmid DNA complexes and DLPAP:DOPE liposome/bcl-2 AS ODN complexes, and the co-delivery of DOTAP:DOPE liposome/p53 plasmid DNA complexes and DOTAP:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 76.7±1.3% and 71.3±8.8% respectively. The cytotoxicity of DDAB:DOPE liposomes, DLPAP:DOPE liposomes and DOTAP:DOPE liposomes was 13.2±4.3, 36.5±4.3 and 20.5±4.6%, respectively. The co-delivery of Lipofectamine™2000 (1.84 μl/ml)/p53 plasmid DNA complexes and Lipofectamine™2000 (1.84 μl/ml)/bcl-2 AS ODN complexes inhibited cell growth to 78.7±2.6%. Lipofectamine™2000 alone inhibited cell growth to 18.7±8.3%.

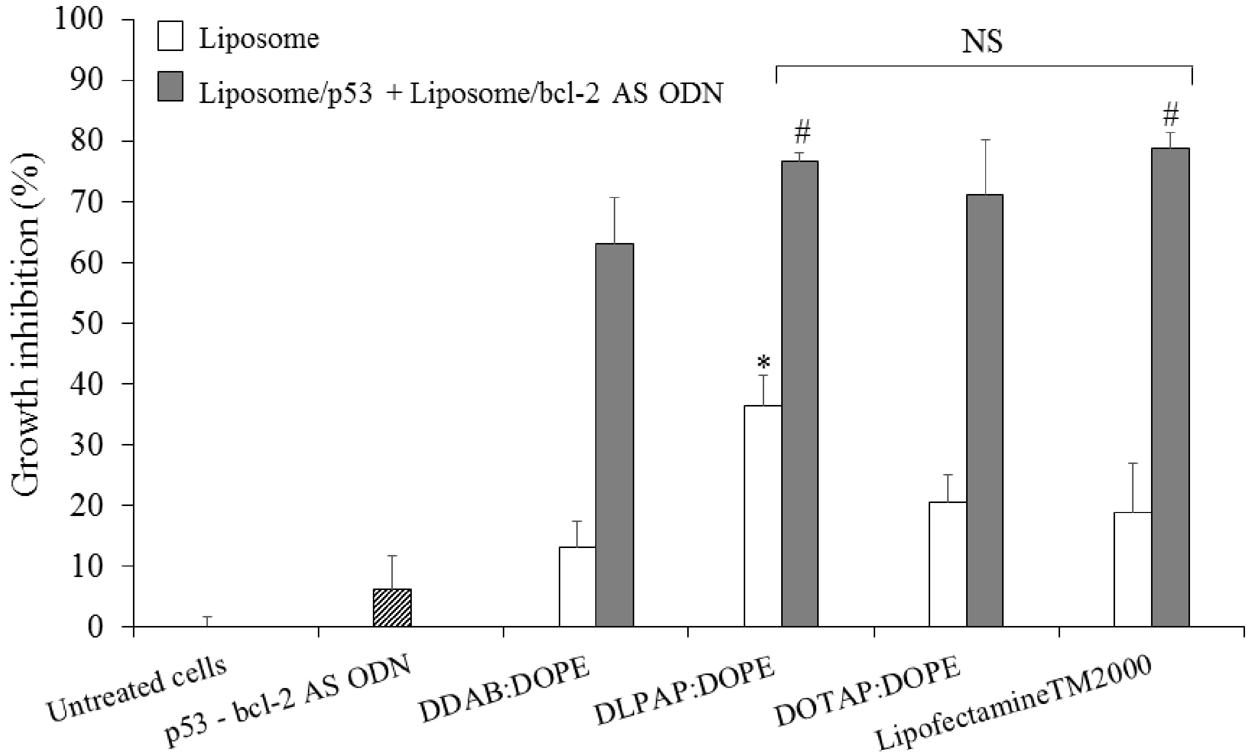

Fig. 18 Growth inhibition of the co-delivery of cationic liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and cationic liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5 in HeLa cells by DDAB:DOPE liposomes, DLPAP:DOPE liposomes, DOTAP:DOPE liposomes, and a lipid-based transfection reagent, Lipofectamine^{TM}2000. $^{*}p$ < 0.05 when compared with cells treated with DDAB:DOPE liposomes, DOTAP:DOPE liposomes, and Lipofectamine^{TM}2000; $^{\#}p$ < 0.05 when compared with cells treated with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes; NS, not significant. Each value represents the mean±S.D. of three wells.

5.5 Colloidal stability

Fig. 19 shows the 14 day-colloidal stability of PEI-CA/plasmid DNA and AS ODN complexes. The particle size of PEI-CA/plasmid DNA and AS ODN complexes remained less than 300 nm during storage at 4 ^{O}C for 14 days.

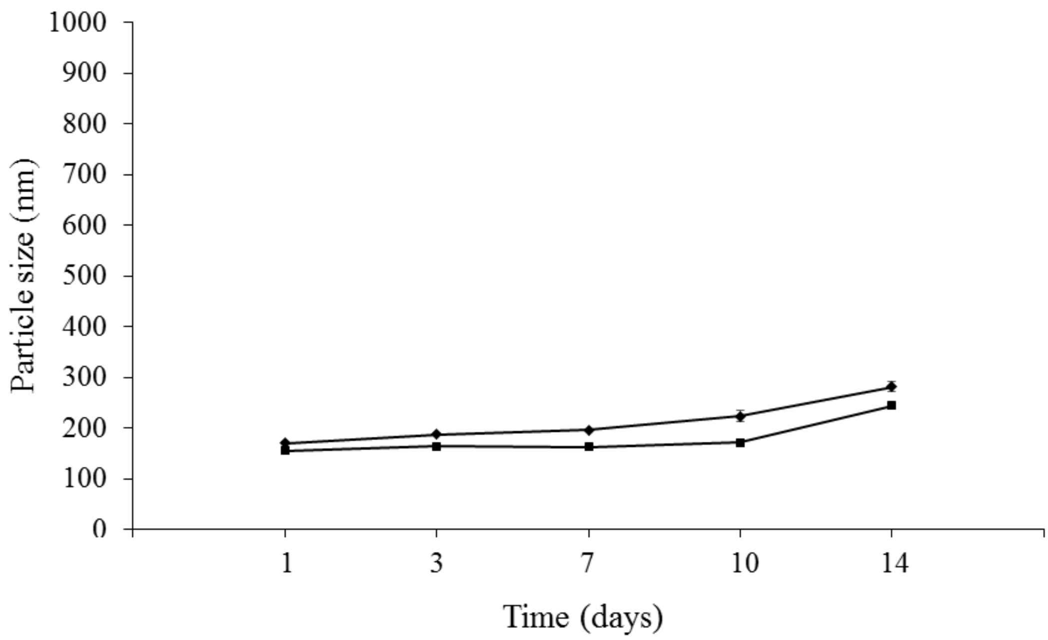

Fig. 19 Particle size of PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes at a polymer-to-NA ratio of 0.5 in 10% BSA solution in 14 days. Polyplexes were kept at 4 ^{O}C. PEI-CA (◆) and PEI (■). Each value represents the mean±S.D. of three measurements.

The colloidal stability of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes was monitored during storage at 4°C in deionized water and in 10% BSA solution. The particle size of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage at 4°C was in the range of 288.7±5.1 to 472.2±10.0 nm and 149.2±0.5 to 398.4±5.0, respectively (Fig. 20).

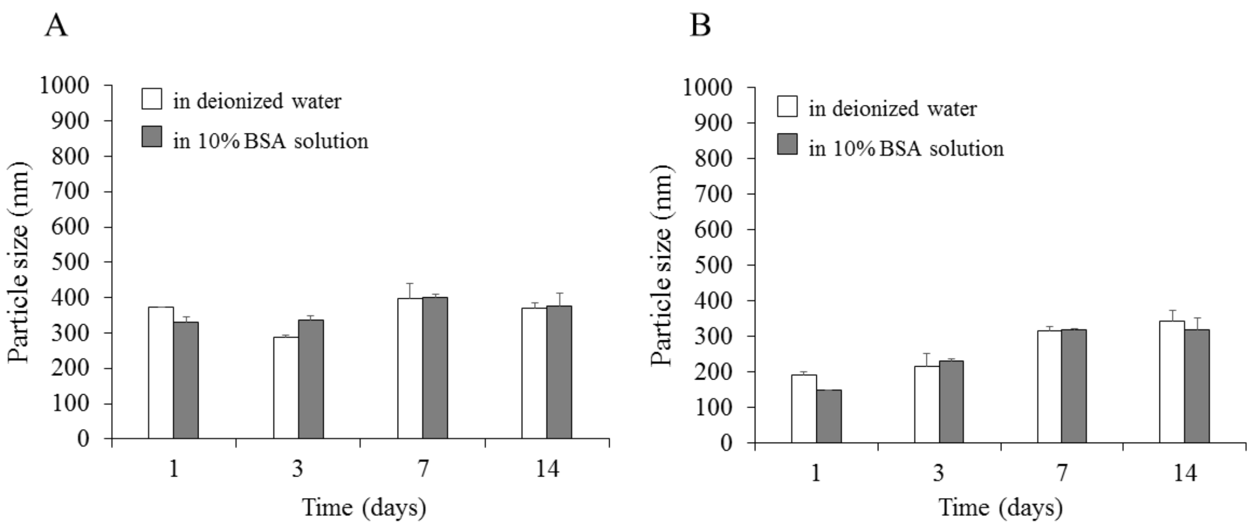

Fig. 20 Particle size of A) DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3, and B) DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5 in 14 days. Liposome/nucleic acid complexes were kept at 4 $^{\circ}$C. Each value represents the mean±S.D. of three measurements.

6. Discussion and Conclusion

The ability to form complexes with the nucleic acid is a required characteristic of a nucleic acid delivery system. The capability of polymer to condense nucleic acid was evaluated by agarose gel electrophoresis, and particle size and zeta potential measurements. PEI-CA efficiently condensed plasmid DNA and AS ODN to nanosized complexes. Polymer gene carriers have been reported to efficiently condense plasmid DNA and siRNA into nanosized complexes [7, 8].

PEI-CA/pEGFP and AS ODN complexes facilitated efficient AS ODN uptake and GFP gene expression. Polyplexes with particle size of less than 200 nm can readily enter cells [9]. PEI/DNA complexes have been demonstrated to enter cells via sulfated proteoglycans [10]. Kim *et al.* showed that deoxycholic acid-modified PEI1.8KDa had higher siRNA uptake than PEI 25KDa and PEI 1.8KDa [11].

The study showed that the growth inhibition activity was more substantial in HeLa cells treated with PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes than those treated with PEI-CA/p53 plasmid DNA complexes or PEI-CA/bcl-2 AS ODN complexes alone. This finding was in agreement with a previous report that co-delivery of p53 plasmid DNA and bcl-2 AS ODN by cationic liposomes significantly inhibited cell growth and at a higher rate than either p53 or bcl-2 AS ODN alone [12].

Delivery of p53 plasmid DNA and bcl-2 AS ODN has been reported in cancer growth inhibition. B16-F16 tumor metastases in lung of melanoma lung metastasis mouse model were inhibited by aerosol delivery of PEI/p53 DNA complexes [13]. PLGA nanoparticles containing p53 plasmid DNA increased tumor apoptosis, inhibited tumor growth and promoted survival time in MDA-MB-435 carcinoma mouse model [14]. Tumor volume in Swiss albino mice was decreased by chitosan nanoparticles loaded with bcl-2 siRNA [15].

Co-delivery strategy of nucleic acid by polymer-based delivery systems has been shown to be a promising approach that improves the biological or therapeutic activity. Chen *et al.* reported that co-transfection of CCR7 and gp100 plasmid DNA using spermine-dextran exhibited greater tumor inhibition than that with gp100 plasmid DNA alone [16].

The growth inhibition by the co-delivery of p53 plasmid DNA and bcl-2 AS ODN by PEI-CA was influenced by the concentrations of p53 plasmid DNA and bcl-2 AS ODN. The growth inhibition of HeLa cells after co-delivery with PEI-CA increased with increased p53 plasmid DNA concentration from 1 to 4 μg/ml, and bcl-2 AS ODN concentration from 1.25 to 5 μg/ml. Weecharangsan *et al.* showed that growth inhibition increased with increasing concentration of bcl-2 AS ODN in KB human oral carcinoma cells transfected with human serum albumin-coated liposome-bcl-2 AS ODN complexes [17].

Recently, conjugation of CA to PEI 25KDa has been reported to decrease cytotoxicity of PEI [18, 19]. Deoxycholic acid-modified PEI1.8KDa had lower cytotoxicity than PEI1.8KDa in human coronary artery smooth muscle cells [11].

PEI-CA was able to condense plasmid DNA and AS ODN, and shown to produce nanosized complexes less than 200 nm with positively charge in a single formulation. PEI-CA had a remarkably low toxicity and efficient transfection performance. Co-delivery of PEI-CA/p53 plasmid DNA and bcl-2 AS ODN complexes synergistically inhibited human carcinoma cell growth higher than either p53 plasmid DNA or bcl-2 AS ODN used alone. This study indicated that PEI-CA as a valuable carrier for plasmid DNA and AS ODN, which has potential application in cancer gene therapy.

The co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/AS ODN complexes were efficient in AS ODN delivery and gene expression, and effectively inhibited cell growth.

pEGFP plasmid DNA can be formed complexes with DDAB:DOPE liposomes better than AS ODN. This was because of the different size or MW of plasmid DNA and AS ODN. AS ODN has smaller size compared to plasmid DNA thus requires higher amount of the liposomes to form complete complexes. Previous studies showed that cationic polymer nanoparticles condensed plasmid DNA more efficiently than siRNA with a lower polymer-nucleic acid ratio [20, 21]

Particle size of cationic liposome/nucleic acid complexes is a crucial factor to control cellular uptake, and appropriate particle size of cationic liposome/nucleic acid complexes could enter cells [22, 23]. The particle size of DDAB:DOPE liposomes was 166.3±3.3 nm. The zeta potential of DDAB:DOPE liposomes was positive. Plasmid DNA and AS ODN were considered as large molecules. Mixing with pEGFP and AS ODN led to formation of nanosized DDAB:DOPE liposome/pEGFP complexes and liposome/AS ODN complexes, and decreased surface charge. This finding is in agreement with previous studies [24, 25]. The zeta potential of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were negative and positive values depending on the lipid-to-DNA and lipid-to-ODN ratios. The lipid-to-nucleic acid ratio has been shown to determine the size and zeta potential of cationic liposome/nucleic acid complexes [25, 26]

Successful gene or antisense therapy depends on the delivery of nucleic acid molecules into cells. It has been reported that cationic liposomes are efficient for nucleic acid delivery [27, 28]. The AS ODN uptake and GFP expression in HeLa cells were efficient by DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes at lipid-to-DNA ratios of 3, 6 and 12.5 and lipid-to-ODN ratios of 2.5, 5 and 10. The study shows that AS ODN uptake and GFP expression by the co-delivery of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes in HeLa cells was lipid-to-DNA and lipid-to-ODN ratios dependent. Several reports showed that the cellular delivery and transfection efficiency of liposomes/nucleic acid complexes depended on the lipid-to-nucleic acid ratio [27-29]. The efficient AS ODN uptake and GFP expression could be due to the cellular uptake by cationic lipid, DDAB, and the endosomal rupture property of DOPE [24, 30, 31].

Inhibition of tumors by p53 gene transfer in tumor xenografted mice treated with poly(lactide-*co*-glycolide)–p53 nanoparticles has been shown to be greater than with p53 alone [32]. Down regulation of bcl-2 protein has been shown to inhibit tumor growth [33]. The study showed that DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth, and extensively by the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes. Uncomplexed p53 plasmid DNA and bcl-2 AS ODN, and DDAB:DOPE liposomes slightly caused cytotoxicity to HeLa cells. Misra *et al.* [34] demonstrated that liposome/p53 plasmid DNA complexes exhibited cytotoxicity in H1299 and HEK293T cells. Weecharangsan *et al*. [24, 35] showed that the growth of KB human oral carcinoma cells were inhibited by disulfide-linked bcl-2 ODN liposomes and human serum albumin-coated liposome-bcl-2 AS ODN complexes. Ren *et al.* [36] showed that the growth of UM-UC-3 bladder cancer cells and UM-UC-3 bladder xenograft tumor was inhibited by Lipofectamine/bcl-2 AS ODN complexes. Rui *et al.* [37] showed that co-transfection of p53 and p16 gene using liposomes inhibited K562 human erythroleukemic cell proliferation stronger than either by p53 or p16 gene. He *et al*. [38] demonstrated that co-delivery of E7 antisense RNA and IL-12 gene using DC-Chol liposomes significantly improved C3 mouse tumor growth inhibition.

Increasing the lipid-to-DNA of 3, 6 and 12.5 of DDAB:DOPE liposome/p53 plasmid DNA complexes and the lipid-to-ODN of 2.5, 5 and 10 of DDAB:DOPE liposome/bcl-2 AS ODN complexes significantly increased HeLa cell growth inhibition. The growth inhibition of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes reached a high level of growth inhibition at the lipid-to-DNA above 3 and lipid-to-ODN above 2.5. The cytotoxicity of DDAB:DOPE liposomes increased with an increasing the amount of the liposomes could be due to high positive zeta potential of cationic liposomes [39].

The study revealed that HeLa cell growth was significantly suppressed by the co-delivery of liposome/p53 plasmid DNA complexes and liposome/bcl-2 AS ODN complexes. Various cationic lipids including DDAB, DLPAP and DOTAP, and a lipid-based transfection reagent, Lipofectamine^{TM}2000, could be used for the co-delivery of p53 plasmid DNA and bcl-2 AS ODN. DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage in the presence and absence of BSA, exhibited particles less than 450 nm for DDAB:DOPE liposome/pEGFP complexes, and less than 400 nm for DDAB:DOPE liposome/AS ODN complexes.

The study reveals that cationic liposomes effectively delivered both plasmid DNA and AS ODN. Co-delivery of p53 plasmid DNA and bcl-2 AS ODN by cationic liposomes significantly provided cell growth inhibition higher than either agent used alone. DDAB:DOPE liposomes at a low lipid-to-DNA and lipid-to-ODN ratios had low cytotoxicity, and their corresponding complexes with plasmid DNA and AS ODN yielded nanosized particles. These data suggest that co-delivery of plasmid DNA and AS ODN by cationic liposomes could be an attractive strategy for clinical application in cancer treatment.

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Co-delivery of Plasmid DNA and Antisense Oligodeoxyribonucleotide into Human Carcinoma Cells by Cationic Liposomes

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Abstract: This study aimed to evaluate the co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/antisense oligodeoxyribonucleotide (AS ODN) complexes in HeLa human cervical carcinoma cells. Dimethyldioctadecyl ammonium bromide (DDAB): dioleoyl phosphatidylethanolamine (DOPE) liposome/plasmid DNA complexes, and DDAB:DOPE liposome/AS ODN complexes were formulated and characterized in terms of agarose gel electrophoretic mobility, particle size and zeta potential. The complexes were evaluated for delivery of pEGFP plasmid DNA and AS ODN in HeLa cells. Cell growth inhibition was evaluated using p53 plasmid DNA and bcl-2 AS ODN, by co-delivery of DDAB:DOPE liposome/p53 plasmid DNA and DDAB:DOPE liposome/bcl-2 AS ODN complexes. The particle size of DDAB:DOPE liposome/plasmid DNA complexes, and DDAB:DOPE liposome/AS ODN complexes were 180.6±2.0 to 372.3±2.4 nm, and zeta potentials were -26.7±1.2 to +6.8±0.4 mV, respectively. The AS ODN uptake and green fluorescent protein (GFP) expression upon their co-delivery by DDAB:DOPE liposomes were both high. Treatment of the cells with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/ bcl-2 AS ODN complexes inhibited cell growth to a greater degree than that with either DDAB:DOPE liposome/p53 plasmid DNA complexes or DDAB:DOPE liposome/bcl-2 AS ODN complexes alone. These data suggest that co-delivery of cationic liposome/p53 plasmid DNA and cationic liposome/bcl-2 AS ODN complexes is an effective strategy to achieve enhanced therapeutic activities.

Keywords: Antisense oligodeoxyribonucleotide, bcl-2, co-delivery, growth inhibition, liposome, plasmid DNA, p53.

INTRODUCTION

Common approaches of nucleic acid-based drug therapy are to introduce a therapeutic gene or antisense agent. However, these are generally ineffective due to low efficiency of nucleic acid delivery. Insufficiency of nucleic acid transfer is a major obstacle to the clinical application of nucleic acid-based drug therapy [1, 2]. In order to improve the efficacy of therapy, delivery systems including those based on cationic polymers or cationic lipids have been developed [3, 4].

Liposomes can be formulated by varying lipid ingredients. Cationic liposomes have been used to deliver p53 to suppress tumor growth and restore the apoptotic pathway in human ovarian cancer cells [5]. Intramuscular injection of cationic liposome/GNE gene complexes in patient with hereditary inclusion body myopathy improved locoregional skeletal muscle function [6].

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Synergistic effect by co-delivery of nucleic acid-based drugs has been shown to enhance therapeutic activity. Co-delivery of BMP-2 and SOX-9 genes enhanced normal chondrogenic property of differentiated chondrocytes [7]. Co-transfection of p16 and p53 genes inhibited human erythroleukemic cell proliferation [8].

Cancer is the result of mutation of DNA accumulated during human life. The loss of function of tumor suppressor gene and the activation of oncogene have crucial role in the pathogenesis of cancer. The deficit or mutation of p53 tumor suppressor gene and overexpression of bcl-2 gene have been shown abnormality in human cancer [9, 10].

In this study, we investigated the co-delivery of plasmid DNA and AS ODN in human carcinoma cells by cationic liposomes. DDAB:DOPE liposomes were formulated and evaluated for co-delivery of plasmid DNA and AS ODN in HeLa human cervical carcinoma cells. Cationic liposome/ plasmid DNA and AS ODN complexes were characterized in terms of particle size and zeta potential. The complexes were evaluated for delivery of plasmid DNA and AS ODN in HeLa cells. Cell growth inhibition in HeLa cells was also evaluated using therapeutic nucleic acids, p53 plasmid DNA

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and bcl-2 AS ODN, by the co-delivery of cationic liposome/p53 plasmid DNA and cationic liposome/bcl-2 AS ODN complexes.

MATERIALS AND METHODS

Materials

Dimethyldioctadecyl ammonium bromide (DDAB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Di-(9Z-octadecenoyl)3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was obtained from Lipoid GMBH (Ludwigshafen, Germany). N-(1-(2,3-dilauroyloxy)propyl)-N'-(3-aminopropyl) carbamide (DLPAP) [11] was provided from Dr. Boon-ek Yingyongnarongkul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech (Palo Alto, CA, USA). Plasmid GFP-p53 was a gift from Dr. Tyler Jacks (Addgene plasmid # 12091) [12]. Plasmid Maxi Kit was purchased from Geneaid (Taipei City, Taiwan). Six- and 96-well plates were purchased from SPL Life Sciences. (Gyeonggi-do, Korea). MEM media and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Bio Basic Inc. (Ontario, Canada).

Plasmid DNA Preparation

pEGFP-C2 plasmid DNA encoding the GFP and p53 plasmid DNA encoding the p53 protein were used. The plasmid DNAs were purified from DH5-α *E. coli* using the Geneaid Plasmid Maxi Kit (Taipei City, Taiwan). DNA concentrations were quantified using a cuvette photometer (Eppendorf Biophotometer plus, Eppendorf AG, Hamburg, Germany). The purity of plasmid DNAs was checked by gel electrophoresis (1.2% agarose gel). The purified plasmid DNAs were redissolved in Tris-EDTA buffer, pH 8.0 and used at a concentration of about 500 μg/ml.

Antisense Oligodeoxyribonucleotides

AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'- TACCGTCTGCGACGCCCT 3'), Cy3-labeled AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'-Cy3-TCTCCCAGCGTGCGCCAT-3'), and bcl-2 AS ODN, fully phosphorothioated 18-mer oliogonucleotide (sequence: 5'- TCTCCCAGCGTGCGCCAT-3'), were purchased from Alpha DNA (Quebec, Canada). The AS ODNs were reconstituted in Tris-EDTA buffer, pH 8.0. The concentrations of AS ODN solutions were determined using a cuvette photometer (Eppendorf Biophotometer plus, Eppendorf AG, Hamburg, Germany). The AS ODN solutions were diluted and used at a concentration of about 500 μg/ml.

Preparation of Cationic Liposomes and Cationic Liposome/Nucleic Acid Complexes

Cationic liposomes were formulated with cationic lipids: DDAB, DOTAP or DLPAP, and neutral lipid DOPE by ethanol dilution as described previously [13] with minor modification. Briefly, the lipids were dissolved in 50 μl of absolute ethanol at a molar ratio of 1:1 and injected into 450 μl of vortexing HEPES buffered solution (20 mM HEPES, pH 7.4) and vortexed for 20 min at room temperature to make a fixed total lipid concentration of 2.5 mg/ml. Cationic liposome/plasmid DNA complexes and cationic liposome/ AS ODN complexes were prepared by mixing an appropriate amount of cationic liposome stock solution with an equal volume of diluted solution of plasmid DNA or AS ODN at lipid-to-DNA ratios of 1.5, 3, 6, 12.5, 25, 50, and 100, and at lipid-to-ODN ratios of 1.25, 2.5, 5, 10, 20, 40 and 80, and vortex mixing for 3-5 sec to initiate complex formation, followed by incubation at room temperature for 20 min. The concentrations of plasmid DNA and AS ODN used were 4 and 5 μg/ml, respectively.

Agarose Gel Electrophoresis

Complex formation of DDAB:DOPE liposomes and pEGFP plasmid DNA and DDAB:DOPE liposomes and AS ODN was analyzed on 1.2 % agarose gel stained with SYBR green (0.74%) in tris-boric acid EDTA buffer. Electrophoresis was carried out at 100 V for 15 min. The volume of the sample loaded in the gel well was 10 μl of the complexes containing 0.25 μg of plasmid DNA or 0.3125 μg of AS ODN. Plasmid DNA and AS ODN were visualized with fluorescence mode using an image analyzer (ImageQuant™ LAS 4000 mini, GE Healthcare Bioscience AB, Upsala, Sweden).

Size and Zeta Potential Measurements

The particle size and zeta potential of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) in the volume weighing mode. The complexes were prepared in deionized water (50 μl) and diluted with deionized water or bovine serum albumin (BSA) solution to obtain the volume required for each measurement (1 ml).

Cell Culture

HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in MEM (Invitrogen, Grand Island, NY, USA) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% amphotericin B, under a humidified atmosphere of 5% CO_2 in air at 37°C.

AS ODN Uptake

HeLa cells were seeded into 6 well plates at a density of 2.5×10^5 cells/well in 1.5 ml of growth medium 20 h prior to transfection. The growth medium were replaced with 1 ml of DDAB:DOPE liposomes complexed with pEGFP and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN. Untreated cells were used as control. After 4 h incubation at 37°C, the cells were treated with 0.4% trypan blue for 1 min and rinsed with phosphate bufferred saline (PBS), pH 7.4. Cells were harvested by trypsinization with 0.2% trypsin and rinsed with PBS, pH 7.4. The cells were then fixed with 4% paraformaldehyde. The fluorescent intensity of Cy3 was measured by flow cytometry on a FACSCanto™II flow cytometer (BD Biosciences, San Jose, CA, USA). In the separated experiments, cells were stained with DAPI (2 μg/ml) and imaged using a fluorescence microscope (IX83-ZDC, Olympus, Tokyo, Japan).

GFP Expression

HeLa cells were seeded into 6 well plates at a density of 2.5×10^5 cells/well in 1.5 ml of growth medium 20 h prior to transfection. The cells were incubated with 1 ml of DDAB: DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h. Untreated cells were used as control. The medium were removed and the cells were incubated in growth medium at 37°C. After 20 h incubation, the cells were rinsed with PBS, pH 7.4, harvested, and fixed with 4% paraformaldehyde. The GFP expression was quantified by flow cytometry on a FACSCanto™II flow cytometer (BD Biosciences, San Jose, CA, USA). In the separated experiments, cells were imaged using a fluorescence microscope (IX83-ZDC, Olympus, Tokyo, Japan).

Growth Inhibition

The efficacy of the co-delivery of plasmid DNA and AS ODN by liposomes were evaluated using a therapeutic gene and AS ODN, p53 and bcl-2 AS ODN, in terms of growth inhibition. The evaluation of growth inhibition were performed with three different categories. Firstly, to evaluate the efficacy of the co-delivery of p53 plasmid DNA and bcl-2 AS ODN, the growth inhibition was investigated with DDAB:DOPE liposome/p53 plasmid DNA complexes, DDAB:DOPE liposome/bcl-2 AS ODN complexes, and the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a concentration of p53 plasmid DNA and bcl-2 AS ODN of 4 and 5 μg/ml, respectively. Secondly, the effect of the lipid-to-DNA and lipid-to-ODN ratios on the growth inhibition was investigated with different lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10. The amount of p53 plasmid DNA and bcl-2 AS ODN was fixed at 4 and 5 μg/ml, respectively. Thirdly, the efficacy of the co-delivery of p53 plasmid DNA and bcl-2 AS ODN by cationic liposomes on growth inhibition were evaluated with liposomes formulated with various cationic lipids including DDAB, DLPAP, and DOTAP, and a lipid-based transfection reagent, Lipofectamine™2000.

For the evaluation of growth inhibition, HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells/well in 100 μl of growth medium and incubated for 20 h at 37°C under 5% CO_2 atmosphere. Prior to treatment, the medium was removed and the cells were rinsed with PBS, pH 7.4. The cells were incubated with 62.5 μl of cationic liposome/nucleic acid complexes for 4 h at 37°C under 5% CO_2 atmosphere. Untreated cells and cells transfected with free nucleic acids were used as controls. After transfection, the cells were rinsed with PBS, pH 7.4, and continued to be cultured in 100 μl of fresh growth medium at 37°C under 5% CO_2 atmosphere for 20 h. After 20 h incubation, the viability of cell was determined by the MTT assay by measurement of UV absorbance at 570 nm using a microplate spectrophotometer (Zenyth 200 rt; Anthos Labtech Instruments GmbH, Salzburg, Austria). Cell growth inhibition (%) was calculated relative to cells treated with the medium as a control.

Colloidal Stability

The colloidal stability of DDAB:DOPE liposome/plasmid DNA complexes and DDAB:DOPE liposome/AS ODN complexes was evaluated by monitoring change in the average particle size during storage and incubation with 10% BSA solution at 4°C for 14 days.

Statistical Analysis

All results were demonstrated as the mean ± standard deviation (S.D.). Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by an LSD post hoc test. $p < 0.05$ was defined as statistical significance.

RESULTS

Agarose Gel Electrophoresis

The complexation of cationic liposomes to pEGFP plasmid DNA and AS ODN was measured by agarose gel electrophoresis. DDAB:DOPE liposome/pEGFP complexes at different lipid-to-DNA ratios with a range of 1.5, 3, 6, 12.5, 25, 50 and 100, and DDAB:DOPE liposome/AS ODN complexes at lipid-to-ODN ratios of 1.25, 2.5, 5, 10, 20, 40 and 80, with increasing the amount of added cationic liposomes to a fixed amount of pEGFP and AS ODN were investigated. As shown in (Fig. **1**), when DDAB:DOPE liposomes were mixed with pEGFP and AS ODN, the amount of free pEGFP and AS ODN decreased proportionally with increasing amounts of DDAB:DOPE liposomes.

pEGFP and AS ODN showed different nucleic acid condensation patterns. In the case of pEGFP, the complete complexes of DDAB:DOPE liposomes and pEGFP were formed at lipid-to-DNA ratios above 6. On the other hand, the complete complexes of liposomes and AS ODN were formed at lipid-to-ODN ratios above 20. In all experiments, DDAB: DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes was chosen at lipid-to-DNA and lipid-to-ODN ratios below complete complexes due to the high cytotoxicity of high amount of cationic lipids.

Particle Size and Zeta Potential

The particle size and zeta potential of DDAB:DOPE liposomes and DDAB:DOPE liposome/nucleic acid complexes were determined with a Zetasizer Nano ZS. The particle size of DDAB:DOPE liposomes were 166.3±3.3 nm with polydispersity index of 0.533. As cationic liposomes, they had a positive charge. The zeta potential of DDAB:DOPE liposomes were +25.9±1.4 mV. This demonstrates that the liposomes with nanosized and positive zeta potential could be suitable for cellular entry of plasmid DNA and AS ODN, and thus they were employed for complex formation with nucleic acids.

The particle size and zeta potential of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were plotted versus lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10, respectively (Fig. **2**). The particle size of DDAB:DOPE

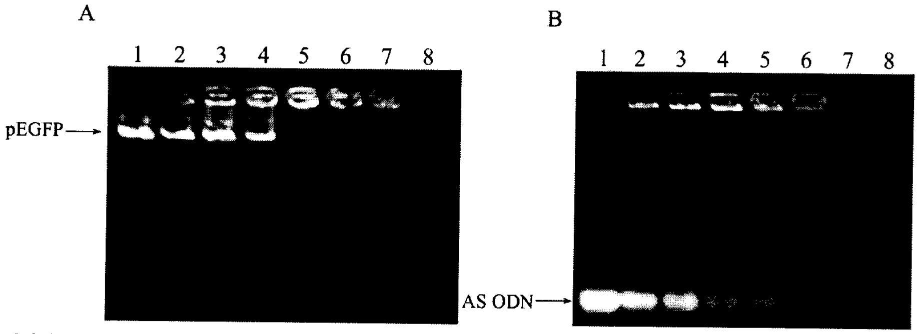

Fig. (1). Gel electrophoresis analysis of (**A**) DDAB:DOPE liposome/pEGFP complexes on 1.2% agarose gel. Lane 1: free pEGFP (0.25 µg). Lanes 2-8: DDAB:DOPE/pEGFP complexes at lipid-to-DNA ratios of 1.5, 3, 6, 12.5, 25, 50 and 100, respectively, and (**B**) DDAB:DOPE liposome/AS ODN complexes on 1.2% agarose gel. Lane 1: free AS ODN (0.3125 µg). Lanes 2-8: DDAB:DOPE liposome/AS ODN complexes at lipid-to-ODN ratios of 1.25, 2.5, 5, 10, 20, 40 and 80, respectively.

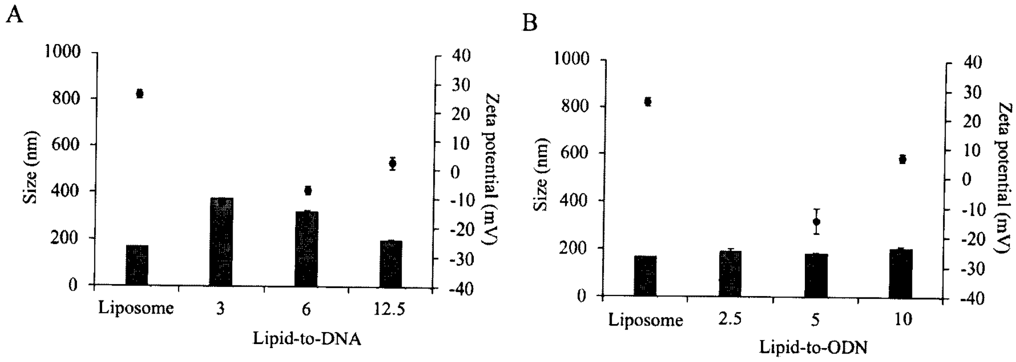

Fig. (2). Particle size (■) and zeta potential (•) of (**A**) DDAB:DOPE liposome/pEGFP complexes at varying lipid-to-DNA ratios, and (**B**) DDAB:DOPE liposome/AS ODN complexes at varying lipid-to-ODN ratios in deionized water. Each value represents the mean±S.D. of three measurements.

liposome/pEGFP complexes decreased with increasing lipid-to-DNA ratio from 3 to 12.5. For DDAB:DOPE liposome/AS ODN complexes, the particle size was relatively constant in the range of the lipid-to-ODN ratios of 2.5 to 10. The particle size of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were in the range of 197±3.0 to 372±2.4 nm for DDAB:DOPE liposome/pEGFP complexes and of 180±2.0 to 203±7.7 nm for DDAB:DOPE liposome/AS ODN complexes. Zeta potential of DDAB:DOPE liposome/pEGFP complexes increased with increasing lipid-to-DNA ratio and lipid-to-ODN ratio in both DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes. An initial negative value of the zeta potential was observed at the low lipid-to-DNA ratios of 3 and 6, and lipid-to-ODN ratios of 2.5 and 5. At the lipid-to-DNA ratio of 6 and lipid-to-ODN ratio of 5, the zeta potential reached a positive value of +2.3±2.2 mV for DDAB:DOPE liposome/pEGFP complexes and of 6.8±0.4 mV for DDAB:DOPE liposome/AS ODN complexes.

AS ODN Uptake

The AS ODN uptake of the co-delivery of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were assessed in HeLa cells with the pEGFP and AS ODN concentrations of 4 and 5 µg/ml using Cy3-labeled AS ODN (Fig. **3A**, Fig. **4B**). DDAB:DOPE liposomes were complexed with pEGFP and AS ODN mixed with Cy3 labeled AS ODN at lipid-to-DNA ratios of 3, 6 and 12.5, and lipid-to-ODN ratios of 2.5, 5 and 10, respectively. The untreated cells yielded Cy3 positive cells of 1.71±1.2% fluorescence intensity. The fluorescence intensity of the cells treated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/Cy3-labeled AS ODN complexes examined by flow cytometry was significantly increased from 6.8±0.9 to 10.8±2.7% fluorescence intensity with increasing lipid-to-DNA ratio from 3 to 6 and lipid-to-ODN ratio from 2.5 to 5 ($p < 0.05$) and decreased to 6.2±1.5% fluorescence intensity at a lipid-to-DNA ratio of 12.5 and lipid-to-ODN ratio of 10 (Fig. **3A**). The fluorescent of Cy3-labeled AS ODN was observed mainly in the cytoplasm of

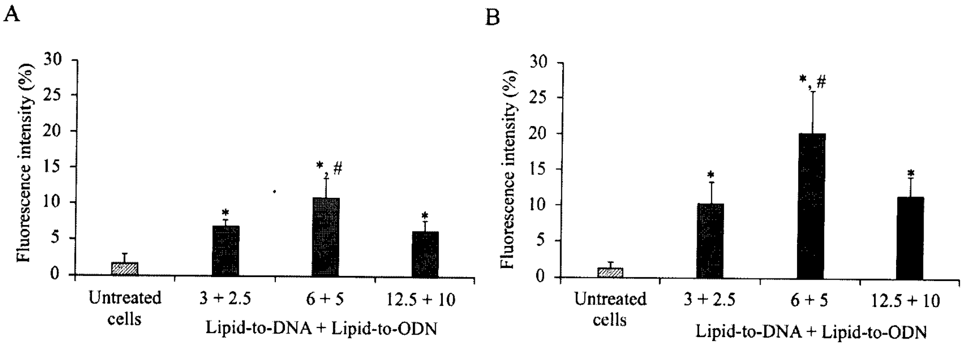

Fig. (3). **(A)** AS ODN uptake, and **(B)** GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at varying lipid-to-DNA ratios and DDAB:DOPE liposome/AS ODN complexes at varying lipid-to-ODN ratios in HeLa cells. In AS ODN uptake experiment, cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN for 4 h at 37°C. In GFP expression experiment, cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h at 37°C and in growth medium for 20 h at 37°C. The fluorescent intensity were measured on a FACSCanto™II flow cytometer. *$p < 0.05$ when compared with untreated cells; $^{\#}p < 0.05$ when compared with cells treated with the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5, and cells treated with the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 12 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 10. Each value represents the mean±S.D. of three measurements.

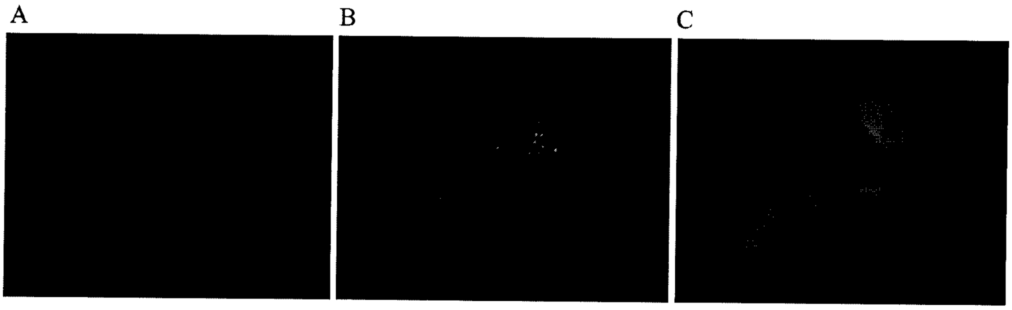

Fig. (4). Fluorescent images of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 6 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN of 5 in HeLa cells. **(A)** Untreated cells. **(B)** AS ODN uptake. Cells were incubated with DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposomes complexed with AS ODN mixed with 10% fluorescent Cy3 labeled AS ODN for 4 h at 37°C. **(C)** GFP expression. Cells were incubated with DDAB:DOPE liposome/ pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes for 4 h at 37°C and in growth medium for 20 h at 37°C.

the cells at 4 h after the addition DDAB:DOPE liposome/ pEGFP complexes and DDAB:DOPE liposome/ Cy3-labeled AS ODN complexes to the cells, indicating that DDAB: DOPE liposome/AS ODN complexes entered the cells and AS ODN delivered into the cytoplasm. (Fig. **4B**).

GFP Expression

Improving nucleic acid transfection efficiency is an ultimate goal for the development of nucleic acid delivery system. To investigate cationic liposomes mediated gene delivery, *in vitro* gene transfection was performed in HeLa cells using pEGFP-C2 plasmid DNA encoding green fluorescence protein.

The GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5, 5 and 10, used for AS ODN uptake study, were employed to assess the GFP expression of pEGFP. The GFP expression of all lipid-to-DNA and lipid-to-ODN ratios was higher than that of untreated cells. The GFP expression significantly increased with increasing lipid-to-DNA ratio from 3 to 6 and lipid-to-ODN ratio from 2.5 to 5 with the fluorescence intensity from 10.3±3.0 to 20.2±5.8% ($p < 0.05$) and decreased after a lipid-to-DNA ratio of 6 and lipid-to-ODN ratio of 5 to 11.3±2.7% fluorescence intensity (Fig. **3B**). Figure **4C** shows the GFP expression of the co-delivery of DDAB:DOPE liposome/pEGFP

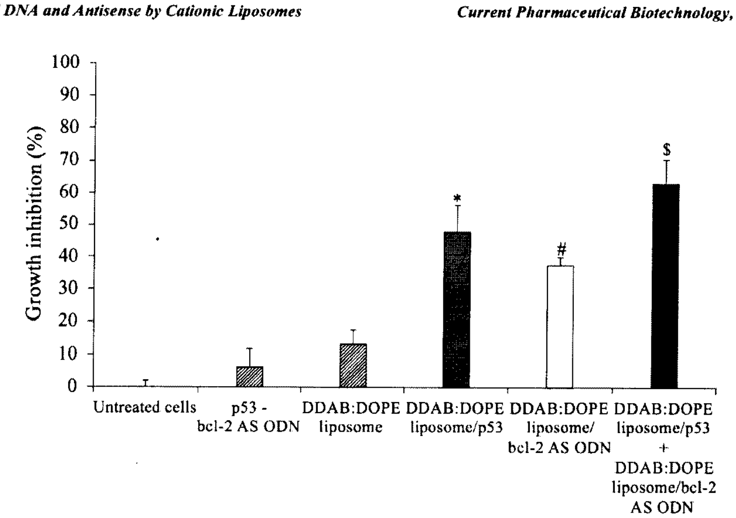

Fig. (5). Effect of the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 2.5, DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 6, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 5 on HeLa cell growth. Cells were incubated with the complexes at the p53 plasmid DNA and bcl-2 AS ODN concentrations of 4 and 5 µg/ml for 4 h and in growth medium for 20 h. $^{*}p <$ 0.05 when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, cells treated with DDAB:DOPE liposomes, and cells treated with DDAB:DOPE liposome/bcl-2 AS ODN; $^{\#}p < 0.05$ when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, and cells treated with DDAB:DOPE liposomes; $^{\$}p < 0.05$ when compared with untreated cells, cells treated with the p53 plasmid DNA and bcl-2 AS ODN, cells treated with DDAB:DOPE liposomes, cells treated with DDAB:DOPE liposome/p53 plasmid DNA complexes, and cells treated with DDAB:DOPE liposome/bcl-2 AS ODN complexes. Each value represents the mean±S.D. of three wells.

complexes and DDAB:DOPE liposome/AS ODN complexes in HeLa cells.

Co-delivery of p53 Plasmid DNA and bcl-2 AS ODN Using Cationic Liposomes on HeLa Cell Growth Inhibition

The growth inhibition on HeLa cells following 24 h incubation after co-transfection with DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 2.5, and after transfection with DDAB: DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 6, DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-to-ODN ratio of 5, free p53 plasmid DNA and bcl-2 AS ODN at a concentration of 4 and 5 µg/ml, respectively, and liposome alone is shown in (Fig. **5**). Free p53 plasmid DNA and bcl-2 AS ODN revealed negligible cell growth inhibition. DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 47.7±8.6% ($p < 0.05$) and 37.4±2.4% ($p < 0.05$) of the control, respectively. The p53 plasmid DNA and bcl-2 AS ODN co-delivered using DDAB:DOPE liposomes exerted growth inhibition level of 63.1±7.6% ($p < 0.05$), which was greater than either p53 plasmid DNA or bcl-2 AS ODN delivered using DDAB: DOPE liposomes. DDAB:DOPE liposomes alone had low toxicity to cells.

To study the effect of the lipid-DNA and lipid-ODN ratios of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes on the cell growth inhibition, different amounts of total lipids with a fixed p53 plasmid DNA concentration of 4 µg/ml and bcl-2 AS ODN concentration of 5 µg/ml were evaluated in HeLa cells. Figure **6** shows the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at lipid-to-ODN ratios of 2.5, 5 and 10, on HeLa cell growth inhibition. The co-delivery of DDAB: DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratios of 3, 6 and 12.5, and DDAB:DOPE liposome/ bcl-2 AS ODN complexes at lipid-to-ODN ratios of 2.5, 5 and 10, inhibited cell growth to 63.1±7.6 to 75.2±3.5% ($p<0.05$) that of the control. Free p53 plasmid DNA and bcl-2 AS ODN exhibited a low growth inhibition (6.2±5.5%). The cytotoxicity of DDAB:DOPE liposome alone increased with an increasing the amount of liposomes. The cytotoxicity of DDAB:DOPE liposome alone at the amount corresponding to DDAB:DOPE liposome/p53 plasmid DNA complexes at lipid-to-DNA ratio of 3, and DDAB:DOPE liposome/bcl-2 AS ODN complexes at lipid-to-ODN ratio of 2.5 was low with 13.2±4.3% growth inhibition.

Figure **7** shows the growth inhibition level of p53 plasmid DNA and bcl-2 AS ODN using cationic-based liposomes formulated with various cationic lipids including DDAB, DLPAP and DOTAP at a lipid-to-DNA ratio of 3 and lipid-to-ODN ratio of 2.5:1, and a lipid-based transfection reagent, Lipofectamine™2000. The co-delivery of DDAB:DOPE

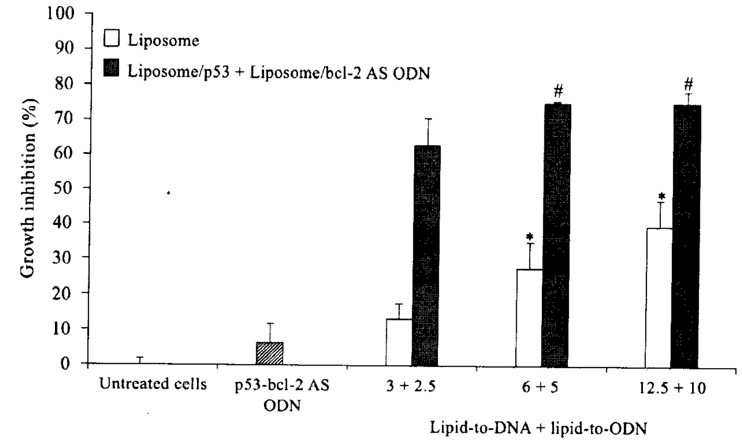

Fig. (6). Effect of lipid-to-DNA and lipid-to-ODN ratios of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes at the p53 plasmid DNA and bcl-2 AS ODN concentrations of 4 and 5 μg/ml on HeLa cell growth. Cells were incubated with DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes for 4 h and in growth medium for 20 h. $^{*}p < 0.05$ when compared with cells treated with p53 plasmid DNA and bcl-2 AS ODN; $^{\#}p < 0.05$ when compared with cells treated with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5. Each value represents the mean±S.D. of three wells.

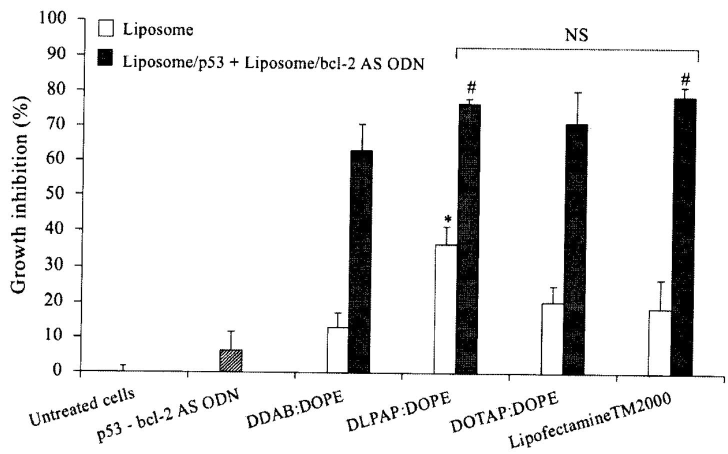

Fig. (7). Growth inhibition of the co-delivery of cationic liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3 and cationic liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5 in HeLa cells by DDAB:DOPE liposomes, DLPAP:DOPE liposomes, DOTAP:DOPE liposomes, and a lipid-based transfection reagent, Lipofectamine™2000. $^{*}p < 0.05$ when compared with cells treated with DDAB:DOPE liposomes, DOTAP:DOPE liposomes, and Lipofectamine™2000; $^{\#}p < 0.05$ when compared with cells treated with the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes; NS, not significant. Each value represents the mean±S.D. of three wells.

liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 63.1±7.6%, whereas the co-delivery of DLPAP:DOPE liposome/p53 plasmid DNA complexes and DLPAP:DOPE liposome/bcl-2 AS ODN complexes, and the co-delivery of DOTAP:DOPE liposome/p53 plasmid DNA complexes and DOTAP:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth to 76.7±1.3% and 71.3±8.8% respectively. The cytotoxicity of DDAB:DOPE liposomes, DLPAP:DOPE liposomes and DOTAP:DOPE liposomes was 13.2±4.3, 36.5±4.3 and 20.5±4.6%, respectively. The co-delivery of Lipofectamine™2000 (1.84 μl/ml)/p53 plasmid DNA complexes and Lipofectamine™2000 (1.84 μl/ml)/bcl-2 AS ODN complexes inhibited cell growth to 78.7±2.6%. Lipofectamine™2000 alone inhibited cell growth to 18.7±8.3%.

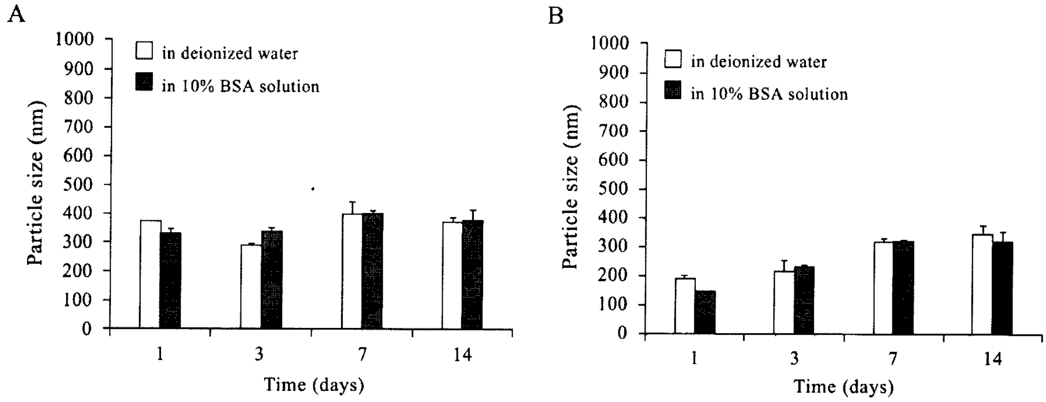

Fig. (8). Particle size of **(A)** DDAB:DOPE liposome/p53 plasmid DNA complexes at a lipid-to-DNA ratio of 3, and **(B)** DDAB:DOPE liposome/bcl-2 AS ODN complexes at a lipid-ODN-to ratio of 2.5 in 14 days. Liposome/nucleic acid complexes were kept at 4°C. Each value represents the mean±S.D. of three measurements.

Colloidal Stability

The colloidal stability of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes was monitored during storage at 4°C in deionized water and in 10% BSA solution. The particle size of DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage at 4°C was in the range of 288.7±5.1 to 472.2±10.0 nm and 149.2±0.5 to 398.4±5.0, respectively (Fig. **8**).

DISCUSSION

In this study, we investigated the co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/AS ODN complexes in HeLa cells, and evaluated the growth inhibition activity in HeLa cells. We found that the co-delivery of cationic liposome/plasmid DNA complexes and cationic liposome/AS ODN complexes were efficient in AS ODN delivery and gene expression, and effectively inhibited cell growth.

pEGFP plasmid DNA can be formed complexes with DDAB:DOPE liposomes better than AS ODN. This was because of the different size or MW of plasmid DNA and AS ODN. AS ODN has smaller size compared to plasmid DNA thus requires higher amount of the liposomes to form complete complexes. Previous studies showed that cationic polymer nanoparticles condensed plasmid DNA more efficiently than siRNA with a lower polymer-nucleic acid ratio [14, 15].

Particle size of cationic liposome/nucleic acid complexes is a crucial factor to control cellular uptake, and appropriate particle size of cationic liposome/nucleic acid complexes could enter cells [16, 17]. The particle size of DDAB:DOPE liposomes was 166.3±3.3 nm. The zeta potential of DDAB:DOPE liposomes was positive. Plasmid DNA and AS ODN were considered as large molecules. Mixing with pEGFP and AS ODN led to formation of nanosized DDAB:DOPE liposome/pEGFP complexes and liposome/AS ODN complexes, and decreased surface charge. This finding is in agreement with previous studies [18, 19]. The zeta potential of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes were negative and positive values depending on the lipid-to-DNA and lipid-to-ODN ratios. The lipid-to-nucleic acid ratio has been shown to determine the size and zeta potential of cationic liposome/nucleic acid complexes [19, 20].

Successful gene or antisense therapy depends on the delivery of nucleic acid molecules into cells. It has been reported that cationic liposomes are efficient for nucleic acid delivery [21, 22]. The AS ODN uptake and GFP expression in HeLa cells were efficient by DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes at lipid-to-DNA ratios of 3, 6 and 12.5 and lipid-to-ODN ratios of 2.5, 5 and 10. Our study shows that AS ODN uptake and GFP expression by the co-delivery of DDAB:DOPE liposome/pEGFP complexes and DDAB:DOPE liposome/AS ODN complexes in HeLa cells was lipid-to-DNA and lipid-to-ODN ratios dependent. Several reports showed that the cellular delivery and transfection efficiency of liposomes/nucleic acid complexes depended on the lipid-to-nucleic acid ratio [21-23]. The efficient AS ODN uptake and GFP expression could be due to the cellular uptake by cationic lipid, DDAB, and the endosomal rupture property of DOPE [18, 24, 25].

Inhibition of tumors by p53 gene transfer in tumor xenografted mice treated with poly(lactide-*co*-glycolide)–p53 nanoparticles has been shown to be greater than with p53 alone [1]. Down regulation of bcl-2 protein has been shown to inhibit tumor growth [26]. Our study showed that DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes inhibited cell growth, and extensively by the co-delivery of DDAB:DOPE liposome/p53 plasmid DNA complexes and DDAB:DOPE liposome/bcl-2 AS ODN complexes. Uncomplexed p53 plasmid DNA and bcl-2 AS ODN, and DDAB:

DOPE liposomes slightly caused cytotoxicity to HeLa cells. Misra *et al.* [27] demonstrated that liposome/p53 plasmid DNA complexes exhibited cytotoxicity in H1299 and HEK293T cells. Weecharangsan *et al.* [18, 28] showed that the growth of KB human oral carcinoma cells were inhibited by disulfide-linked bcl-2 ODN liposomes and human serum albumin-coated liposome-bcl-2 AS ODN complexes. Ren *et al.* [29] showed that the growth of UM-UC-3 bladder cancer cells and UM-UC-3 bladder xenograft tumor was inhibited by Lipofectamine/bcl-2 AS ODN complexes. Rui *et al.* [8] showed that co-transfection of p53 and p16 gene using liposomes inhibited K562 human erythroleukemic cell proliferation stronger than either by p53 or p16 gene. He *et al.* [30] demonstrated that co-delivery of E7 antisense RNA and IL-12 gene using DC-Chol liposomes significantly improved C3 mouse tumor growth inhibition.

Increasing the lipid-to-DNA of 3, 6 and 12.5 of DDAB: DOPE liposome/p53 plasmid DNA complexes and the lipid-to-ODN of 2.5, 5 and 10 of DDAB:DOPE liposome/bcl-2 AS ODN complexes significantly increased HeLa cell growth inhibition. The growth inhibition of DDAB:DOPE liposome/ p53 plasmid DNA complexes and DDAB:DOPE liposome/ bcl-2 AS ODN complexes reached a high level of growth inhibition at the lipid-to-DNA above 3 and lipid-to-ODN above 2.5. The cytotoxicity of DDAB:DOPE liposomes increased with an increasing the amount of the liposomes could be due to high positive zeta potential of cationic liposomes [31].

Our study revealed that HeLa cell growth was significantly suppressed by the co-delivery of liposome/p53 plasmid DNA complexes and liposome/bcl-2 AS ODN complexes. Various cationic lipids including DDAB, DLPAP and DOTAP, and a lipid-based transfection reagent, Lipofectamine™2000, could be used for the co-delivery of p53 plasmid DNA and bcl-2 AS ODN.

DDAB:DOPE liposome/pEGFP complexes at a lipid-to-DNA ratio of 3 and DDAB:DOPE liposome/AS ODN complexes at a lipid-to-ODN ratios of 2.5 during 14 days storage in the presence and absence of BSA, exhibited particles less than 450 nm for DDAB:DOPE liposome/pEGFP complexes, and less than 400 nm for DDAB:DOPE liposome/AS ODN complexes.

CONCLUSION

Our study reveals that cationic liposomes effectively delivered both plasmid DNA and AS ODN. Co-delivery of p53 plasmid DNA and bcl-2 AS ODN by cationic liposomes significantly provided cell growth inhibition higher than either agent used alone. DDAB:DOPE liposomes at a low lipid-to-DNA and lipid-to-ODN ratios had low cytotoxicity, and their corresponding complexes with plasmid DNA and AS ODN yielded nanosized particles. These data suggest that co-delivery of plasmid DNA and AS ODN by cationic liposomes could be an attractive strategy for clinical application in cancer treatment.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflictsof interest.

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8. Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

Wanlop Weecharangsan, Praneet Opanasopit, Boon-ek Yingyongnarongkul, Prartana Kewsuwan, Robert J. Lee. Co-delivery of plasmid DNA and antisense oligodeoxyribonucleotide into human carcinoma cells by cationic liposomes. Current Pharmaceutical Biotechnology. 2014 15(9) 790-799.

8.2 Application

Co-delivery of plasmid DNA and AS ODN by cationic liposomes and PEI-CA could be an attractive strategy for clinical application in cancer treatment.

8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

The research was planned to be presented in AFPS Asian Federation for Pharmaceutical Sciences Conference, 25-27 November 2015, Bangkok, Thailand

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