

Efficient Delivery of Antisense Oligodeoxyribonucleotide G3139 by Human Serum Albumin-Coated Liposomes

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Abstract: Human serum albumin (HSA)-coated liposomal formulations were synthesized and evaluated for the delivery of antisense oligodeoxyribonucleotide (ODN) G3139 in KB human oral carcinoma cells. Liposomes composed of dimethyldioctadecyl ammonium bromide/egg phosphatidylcholine/ α -tocopheryl polyethylene glycol 1000 succinate (58:40:2 molar ratio) complexed with G3139 and coated with HSA were investigated for *Bcl-2* downregulating activity. Cellular uptake of HSA-coated liposome–ODN complexes was more efficient than the uncoated liposome–ODN complexes. Treatment of the cells with HSA-coated liposome–ODN complexes resulted in efficient *Bcl-2* mRNA downregulation that was approximately 3-fold greater than with uncoated liposomes ($p < 0.05$) and 6-fold greater than with free ODN. The transfection efficiency of liposome–ODN complexes coated with HSA was dependent on the concentration of HSA used and on the contents of α -helix and β -strand in HSA. HSA-coated liposomes are effective delivery vehicles for antisense ODN.

Keywords: Liposomes; antisense oligonucleotide; G3139; *Bcl-2*; lipoplex; human serum albumin; drug delivery; cancer

1. Introduction

G3139, also known as genasense and oblimersen sodium, is an 18-mer phosphorothioate ODN complemen-

tary to the initial coding region of *Bcl-2* mRNA and is designed for inhibiting *Bcl-2* expression in cancer cells.¹ In clinical studies, G3139 is given by continuous iv infusion and has so far produced mixed results, possibly due to inefficient delivery. Improving delivery, therefore, is regarded as a critical potential strategy for improving therapeutic efficacy of ODN drugs.^{2–6} Cationic liposomes can form electrostatic complexes (lipoplexes) with ODNs

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and have shown some promise as delivery vehicles.^{7,8} Nonetheless, further improvements in delivery efficiency may be required for clinical application of liposomal ODN.

Human serum albumin (HSA) is the most abundant protein in human plasma. It has been reported to improve the delivery of plasmid DNA.^{9,10} Plasma proteins have also been shown to enhance cellular uptake of liposomal ODN.¹¹ In this study, we prepared formulations consisting of liposome–ODN complexes coated with HSA and investigated the effects of HSA on transfection efficiencies. We investigated the effect of HSA concentration on the stability and transfection efficiencies of liposome–ODN complexes, and examined the effect of the secondary structure in HSA on the transfection efficiencies of liposome–ODN complexes coated with HSA. We demonstrated that treatment of the cells with HSA-coated liposome–ODN complexes resulted in significant enhanced *Bcl-2* mRNA downregulation. The transfection efficiency of liposome–ODN complexes coated with HSA was found to be dependent on the concentration of HSA and the content of α -helix and β -strand in HSA.

2. Materials and Methods

2.1. Materials. Dimethyldioctadecyl ammonium bromide (DDAB) and HSA were purchased from Sigma-Aldrich (St. Louis, MO). Egg phosphatidylcholine (PC) was purchased from Lipoid GMBH (Ludwigshafen, Germany). α -Tocopheryl polyethylene glycol 1000 succinate (TPGS) was obtained as a gift from Eastman Chemical Ltd. (Llangefni, Anglesey, U.K.). RPMI-1640 media, fetal bovine serum (FBS), trypsin–EDTA and penicillin–streptomycin were purchased from Invitrogen (Grand Island, NY).

2.2. Antisense Oligonucleotides. G3139, a fully phosphorothioated 18-mer oligonucleotide (sequence 5′–3′: TCT CCC AGC GTG CGC CAT), G3622, a reversed sequence control for G3139 (sequence 5′–3′: TAC CGC GTG CGA CCC TCT), and FITC-labeled G3139 (sequence 5′–3′: FITC-TCT CCC AGC GTG CGC CAT) were obtained from Alpha DNA (Quebec, Canada).

2.3. Preparation of Cationic Liposomes and Liposome–ODN Complexes. Cationic liposomes were prepared from DDAB, PC and TPGS by ethanol dilution as described previously¹² with minor modification. Briefly, DDAB, PC and TPGS were dissolved in ethanol at a molar ratio of 58:40:2 and injected into a stirring HEPES buffered solution (20 mM HEPES, pH 7.4) at room temperature. Ethanol was removed by dialysis using a MWCO 10,000 Da Float-A-Lyser (Spectrum Laboratories Inc., Rancho Dominguez, CA) and against HEPES buffer (20 mM HEPES, pH 7.4) for 2 h at room temperature. The resulting liposomes were then sterilized using a 0.22 μ m filter (Fisher Scientific, Pittsburgh, PA). Cationic liposome–ODN complexes were prepared by mixing cationic liposomes with an equal volume of ODN in HEPES buffer (20 mM HEPES, pH 7.4) and incubated at room temperature for 15 min. HSA was directly added to the liposome–ODN complexes at molar ratios to lipids of 0.15:100, 0.75:100, 1.5:100, 3:100, 4.5:100 and 9:100, and incubated for 15 min before use in transfection studies. The concentration of ODN was fixed at 0.9 μ M. The model structure of HSA-coated liposome–ODN complexes is presented in Figure 1.¹³

2.4. Size and Zeta Potential Measurements. The particle size of liposome–ODN complexes was determined by dynamic light scattering using a particle sizer (NICOMP 370, Santa Barbara, CA) in the volume weighing mode. The liposome–ODN complexes was prepared in 20 mM HEPES, pH 7.4 (300 μ L). Prior to measurement the samples were diluted with the same buffer to 500 μ L. The zeta potential of liposome–ODN complexes was determined by phase

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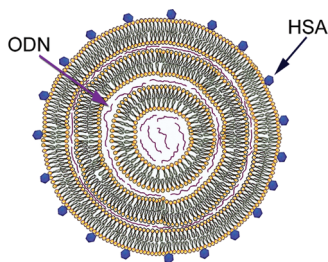


Figure 1. A model structure of HSA-coated liposome-ODN complex.¹³

analysis light scattering using a PALS zeta potential analyzer (Brookhaven Instrument Corp, Holtsville, NY).

2.5. Encapsulation Efficiency. Liposome-ODN complexes were prepared in 100 μL of 20 mM HEPES, pH 7.4. Their encapsulation efficiency was determined by centrifugation (14,000 rpm, 20 min) through a Microcon centrifugal filter device, MWCO 10,000 (Millipore Corporation, Bedford, MA). The content of unencapsulated ODN in the supernatant was determined by measuring OD at 260 nm on a Nanodrop spectrometer (ND1000, NanoDrop Technologies, Inc., Wilmington, DE). The encapsulation efficiency (%) was calculated as follows. Empty liposomes were used as a background control. A standard curve was generated between 0.5 and 15 μg of ODN per mL in 20 mM HEPES, pH 7.4.

$$\text{encapsulation efficiency (\%)} = \frac{\text{total ODN added} - \text{unencapsulated ODN}}{\text{total ODN added}}$$

2.6. Agarose Gel Electrophoresis. The liposome-ODN complexes were analyzed on a 1.2% agarose gel stained with ethidium bromide (0.5 $\mu\text{g}/\text{mL}$) in $1\times$ TBE buffer (Invitrogen, Calsbad, CA). Electrophoresis was carried out at 100 V for 30 min. The volume of the sample loaded in the gel well was 15 μL of liposome-ODN complexes containing 2.5 μg of ODN. The ODN was visualized using a UV transilluminator (Electronic UV Transilluminator, Ultra-Lum, Claremont, CA) and imaged using a Kodak DC 290 digital camera (Eastman Kodak, Rochester, NY).

2.7. Preparation of Heated HSA. HSA was dissolved at a concentration of 5 mg/mL in 1 mL of phosphate buffer, pH 7.4 and heated to different temperatures (50 $^{\circ}\text{C}$, 70 and 90 $^{\circ}\text{C}$) in a thermal cycler (Techne PHC-3, Pegasus Scientific Inc., Frederick, MD), incubated for 30 min and cooled to room temperature. The heated HSA was directly used to coat the liposome-ODN to study the effect of heated HSA on the *Bcl-2* downregulation in KB cells.

2.8. Circular Dichroism (CD) Spectra Measurement. The content of α -helix in HSA solution was determined using CD spectra on a circular dichroism spectrometer model 62A DS (AVIV Associates, Lakewood, NJ), using a 1 cm path length cuvette in phosphate buffer, pH 7.4. The concentration of HSA was 1 mg/mL. The settings for measurement were 180–260 nm scanning wavelength, 1.0 nm bandwidth, 5 s time constant, 25 $^{\circ}\text{C}$, and three accumulations. The content of α -helix and β -strand (%) in HSA was quantified by using

K2D2 software publicly accessible at <http://www.ogic.ca/projects/k2d2/>.¹⁴

2.9. Cell Culture. KB (originally identified as a human oral carcinoma cell line, later identified as a subline of human cervical cancer HeLa cell line) cells was grown in RPMI-1640 (Invitrogen, Grand Island, NY) containing 10% FBS, 100 $\mu\text{g}/\text{mL}$ streptomycin and 100 U/mL penicillin. The cells were maintained at 37 $^{\circ}\text{C}$ in a humidified incubator with 5% CO_2 .

2.10. Transfection Study of Liposome-ODN Complexes. KB cells were seeded into 6-well plates at a density of 2×10^4 cells/cm² in 2 mL of growth medium and grown to 60–70% confluent on the day of the experiment. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4), and then supplied with 850 μL of fresh culture medium without FBS. The cells were incubated with 150 μL of liposome-ODN complexes (N/P ratio of 3) at a final ODN concentration of 0.9 μM for 4 h. Untreated cells, cells transfected with free G3139, and cells transfected with Oligofectamine Reagent (15 μL per well) were used as controls. After transfection, the medium was replaced with 2 mL of fresh growth medium, and the cells were incubated for 48 h at 37 $^{\circ}\text{C}$ under 5% CO_2 atmosphere. All transfection experiments were performed in triplicate.

2.11. Intracellular Delivery of Liposome-ODN Complexes. KB cells were seeded into 6-well plates at a density of 2×10^4 cells/cm² in 2 mL of growth medium. Liposomes complexed with ODN spiked with 10% fluorescent ODN FITC-G3139 were added to the cells. After 4 h incubation at 37 $^{\circ}\text{C}$, the cells were washed with PBS three times, fixed with 3% paraformaldehyde and imaged using a confocal laser scanning microscope (ZEISS LSM 510 META, ZEISS GmbH, Jena, Germany). ODN uptake by KB cells was measured by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.12. RNA Extraction. Total RNA from cultured cells was extracted 48 h after incubation with the liposome-ODN formulation. Cells were harvested by trypsinization with 0.25% trypsin-EDTA and washed with PBS, pH 7.4. The cells were then lysed with 250 μL of Trizol reagent (Invitrogen, Grand Island, NY) for 5 min and total RNA was extracted by adding 100 μL of chloroform. The cell lysate was centrifuged at 12,000 rpm at 4 $^{\circ}\text{C}$ for 10 min and the supernatant was collected. The total RNA was further purified by ethanol precipitation¹⁵ and total RNA concentrations were quantified by measurement of UV absorbance at 260 nm using a NanoDrop spectrophotometer (ND1000, NanoDrop Technologies, Inc., Wilmington, DE).

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2.13. Determination of *Bcl-2* mRNA Expression by Quantitative RT-PCR. The level of *Bcl-2* mRNA expression was measured using quantitative RT-PCR. Synthesis of cDNA was performed using the first-strand cDNA synthesis kit (Invitrogen Carlsbad, CA) as follows. Two micrograms of total mRNA was mixed with 1 μ L of 50 ng/ μ L random hexamer primer and 1 μ L of 10 mM dNTP mix, and heated to 65 °C for 5 min using a thermal cycler (Biorad, Hercules, CA), and then cooled to 4 °C for 5 min. Ten microliters of cDNA synthesis mix containing 1 μ L of 200 U/ μ L SuperscriptIII RT, 1 μ L of 40 U/ μ L RNaseOUT, 2 μ L of 10 \times RT buffer, 4 μ L of 25 mM MgCl₂ and 2 μ L of 0.1 M DTT were added into each sample, and the samples were then incubated in a thermal cycler (Biorad, Hercules, CA) at 25 °C for 10 min and heated to 50 °C for 50 min followed by heating to 85 °C for 5 min and cooling to 4 °C for 5 min. The resulting cDNA was amplified by quantitative real-time PCR StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using Taqman Universal Master Mix (Applied Biosystems, Foster City, CA). Primer and probe sequences were designed by the Primer Express program (Applied Biosystems): *Bcl-2* forward primer 5' CCCTGTGGATGACTGAGTACCTG, *Bcl-2* reverse 5' CCAGCCTCCGTTATCCTGG, *Bcl-2* probe 5' FAM-ACCGGCACCTGCACACCTGGA-TAMRA, ABL forward 5' TGGAGATTAACACTCTAAGCATAACTAAAGGT, ABL reverse 5' GATGTAGTTGCTTGGGACCCA, ABL probe 5' FAM-CCATTTTTGGTTTGGGCTTACACCATT-TAM-PRA. Relative gene expression values were determined by the $\Delta\Delta C_T$ method using the StepOne v2.0 software (Applied Biosystems, Foster City, CA). *Bcl-2* expression was normalized to a housekeeping gene ABL as an endogenous reference in the corresponding samples, and relative to the untreated control cells.

2.14. Protein Extraction. Liposome-ODN transfection of KB cells was performed as described above. After 48 h incubation, the cells were harvested by trypsinizing with 0.25% trypsin-EDTA and washing with PBS, pH 7.4. The cells was then lysed with 50 μ L of 25 mM HEPES lysis buffer, pH 7.4 (Boston BioProducts, Worcester, MA) containing protease Inhibitor Cocktail Set III (CalBiochem, San Diego, CA) 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 was collected. The total protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

2.15. Evaluation of *Bcl-2* Protein by Western Blot Analysis. Cell lysate containing 20 μ g of total protein was separated on a 4–20% Precast Gel Tris HCl-polyacrylamide gel (Bio-Rad, Hercules, CA) at 180 V for 1 h 30 min and then blotted to polyvinylidene difluoride membranes (Hybond-P, Amersham Bioscience, GE Healthcare, Piscataway, NJ) at 180 mA overnight 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 antihuman *Bcl-2* (Dakocytomation, Glostrup, Denmark) at a dilution of 1:1000 or polyclonal goat

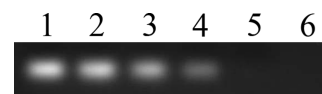


Figure 2. Gel electrophoresis analysis of ODN in liposome formulation. Lane 1: free G3139 (2.5 μ g). Lanes 2–6: liposome-ODN complexes at lipid-to-ODN ratios of 0.4, 2, 4, 6, and 8, respectively.

antihuman actin antibody (Santa Cruz, Santa Cruz, CA) at a dilution of 1:2000 overnight at 4 °C. Following four washing steps, the membranes were incubated with horseradish peroxidase linked antimouse IgG (Amersham Bioscience, GE Healthcare, Piscataway, NJ) at a dilution of 1:2000 or rabbit antigoat IgG (Pierce, Rockford, IL) at a dilution of 1:4000 for 2 h at room temperature. The membranes were then developed with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and imaged with an autoradiography film (Hyblot CL, Denville Scientific, Inc., Metuchen, NJ). The *Bcl-2* protein level was quantified using ImageJ software and normalized with the β -actin level from the same sample.

2.16. Statistical Analysis. The results were presented as the mean \pm standard deviation (SD) of three repeat studies unless otherwise indicated. Statistical significance of the differences in fluorescent intensity data obtained by flow cytometry were examined using the Student's *t*-test. Statistical significance of the differences in *Bcl-2* mRNA and *Bcl-2* protein expressions was examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < 0.05$.

3. Results

3.1. Liposome Characterization. Cationic liposomes prepared by ethanol dilution had an average diameter of 28.06 ± 4.06 nm as determined by dynamic light scattering. We then investigated the ability of our liposome formulation to form electrostatic complexes with the ODN and retard its migration during agarose gel electrophoresis. Figure 2 shows liposome-ODN complexes formed at lipid-to-ODN ratios of 0.4, 2, 4, 6, and 8. Complexes were completely retarded at lipid-to-ODN ratios above 4. Liposome-ODN formulation with a lipid-to-ODN ratio of 6 was then selected for further investigation. The particle size of the liposome-ODN complexes was 91.1 ± 11.6 nm and the efficiency of oligonucleotide encapsulation was $87.2 \pm 1.7\%$.

3.2. Intracellular Delivery of HSA-Coated Liposome-ODN Complexes. Intracellular delivery of HSA-coated liposome-ODN complexes was examined by confocal fluorescence microscopy (Figure 3A). FITC-ODN was incorporated into liposome-ODN complexes or liposome-ODN complexes coated with HSA, which were incubated with KB cells for 4 h at 37 °C. Cells exhibited ODN uptake as shown by its fluorescence. The brighter fluorescence in the cytoplasm of the cells treated with HSA-coated liposome-ODN complexes suggested more efficient endosomal escape of the ODN into the cytoplasm. The fluorescence intensity of the cells treated with HSA-coated

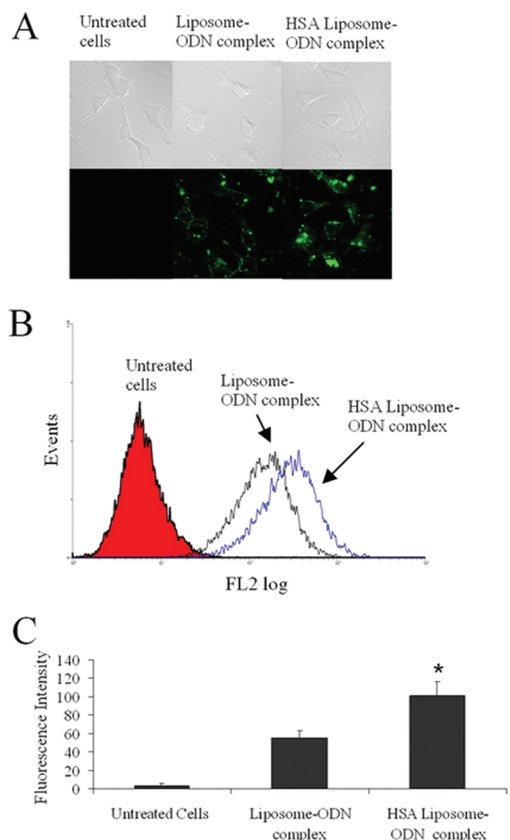


Figure 3. Intracellular delivery of HSA-coated liposome–ODN complexes in KB cells visualized on a confocal microscope (A) measured on a FACSCalibur flow cytometer (B) and fluorescent intensity (C). Cells were incubated with HSA-coated liposome complexed with ODN spiked with 10% fluorescent ODN FITC-G3139 at HSA to liposome molar ratio of 1.5:100 for 4 h at 37 °C. Differences values * were statistically significant ($P < 0.05$).

liposome–ODN complexes examined by flow cytometry was significantly higher than that treated with the uncoated liposome–ODN complexes ($p < 0.05$) (Figures 3B and 3C).

3.3. Effect of HSA Concentration on *Bcl-2* Downregulation. The role of protein-to-lipid ratio in transfection activity of HSA-coated liposome–ODN complexes was examined. Figure 4A shows the real-time RT-PCR analysis of liposome–ODN complexes coated with HSA at molar ratios of 0.15:100, 1.5:100, 3:100, 4.5:100 and 9:100. Oligofectamine–ODN complexes were used as a positive control and had a *Bcl-2* downregulation of $81.3 \pm 1.1\%$. The *Bcl-2* downregulation in KB cells by the uncoated liposome–ODN formulation was observed with a reduction of $51.3 \pm 2.8\%$. When HSA was added to the liposome–ODN complexes, the *Bcl-2* downregulation was significantly enhanced ($p < 0.05$). The degree of enhancement was affected by the molar ratio of HSA-to-lipid in the liposome formulation. The enhancement was most profound at the HSA to liposome molar ratio of 3:100 with $85.0 \pm 0.9\%$ reduction ($p < 0.05$) in cellular *Bcl-2* expression. The *Bcl-2* downregulation decreased to a value of $66.3 \pm 4.7\%$ at the high molar ratio of 9:100. Coincubation of HSA-coated liposome–ODN complexes with

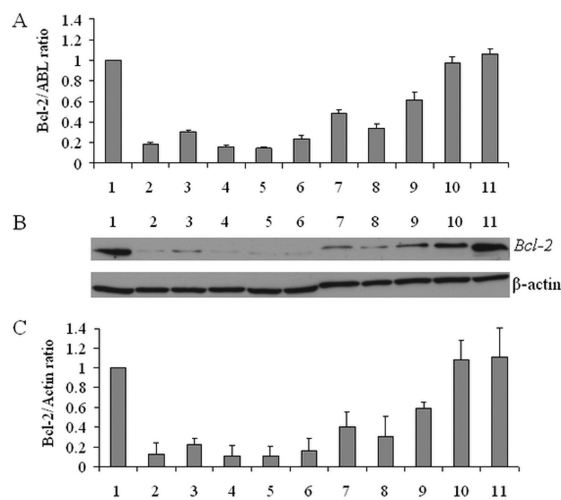


Figure 4. Effect of HSA concentration on transfection efficiency of liposome–ODN complexes in KB cells. *Bcl-2* mRNA expression determined by real-time RT-PCR (A), *Bcl-2* protein expression determined by Western blot (B) and quantification of *Bcl-2* protein level normalized by β -actin (C). (1) untreated cells; (2) Oligofectamine–ODN complexes; (3) liposome–ODN complexes coated with HSA at 0.15:100 molar ratio; (4) 1.5:100 molar ratio; (5) 3:100 molar ratio; (6) 4.5:100 molar ratio; (7) uncoated liposome–ODN complexes; (8) liposome–ODN complexes coated with HSA at 9:100 molar ratio; (9) liposome–ODN complexes coincubation with excess HSA (150:100 molar ratio); (10) free G3139; (11) free G3622. Each value represents the mean \pm SD of three repeat studies.

excess HSA (100 times) did not produce enhancement of *Bcl-2* downregulation.

The ability of HSA-coated liposome–ODN complexes to downregulate the *Bcl-2* protein in KB cells was examined by Western blotting. As shown in Figures 4B and 4C, cells treated with HSA-coated liposome–ODN complexes reduced *Bcl-2* protein level to $89.5 \pm 10.0\%$ at a molar ratio of 3:100, compared to $59.5 \pm 15.0\%$ by uncoated liposome–ODN complexes. The *Bcl-2* downregulation level was dependent on the molar ratio of HSA in the liposome formulation. At a concentration of 9:100, HSA-coated liposome–ODN complexes exhibited reduced *Bcl-2* downregulation level. HSA-coated liposome–ODN complexes coincubated with excess HSA (100 times) did not enhance *Bcl-2* protein downregulation. In summary, the observed effects on *Bcl-2* protein level correlated with the antisense ODN activity measured by the *Bcl-2* mRNA level as shown in Figure 4A.

3.4. Effect of HSA on the Stability of Liposome–ODN Complexes. Due to its negative charge, HSA may affect the stability of positively charged liposome–ODN complexes. Zeta potential measurement was performed following the addition of HSA to liposome–ODN. Figure 5 shows the zeta potential of the liposome–ODN complexes in the presence of HSA at the molar ratios of 0:100, 0.15:100, 0.75:100, 1.5:100, 3:100, 4.5:100 and 9:100. The zeta potential of plain liposome–ODN complexes had a positive value of $+22.0 \pm 1.2$ mV. The presence of HSA

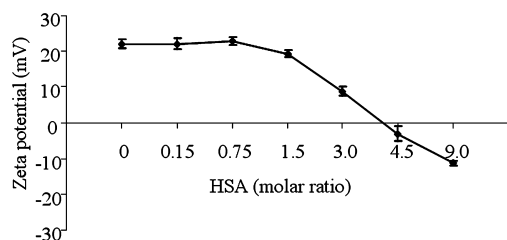


Figure 5. Zeta potential of liposome–ODN complexes formed at varying concentration of HSA. Each value represents the mean \pm SD of three repeat studies.

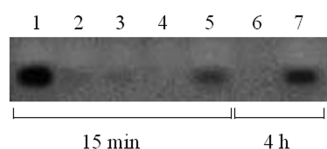


Figure 6. Gel electrophoresis of liposome–ODN complexes coated with HSA. Lane 1: free G3139 (2.5 μ g). Lane 2: uncoated liposome–ODN complexes. Lanes 3–5: liposome–ODN complexes coated with HSA at molar ratios of 1.5:100, 4.5:100 and 9:100, respectively. The incubation time of liposome–ODN complexes coated with HSA was 15 min. Lane 6: uncoated liposome–ODN complexes. Lane 7: liposome–ODN complexes coated with HSA at molar ratios of 9:100. The incubation time of liposome–ODN complexes coated with HSA was 4 h.

at the molar ratios of 0.15:100 and 0.75:100 did not affect the zeta potential value of liposome–ODN complexes. At a molar ratio greater than 0.75:100, the zeta potential value gradually decreased and turned to negative values of -3.1 ± 2.0 and -11.3 ± 0.5 mV at the molar ratios of 4.5:100 and 9:100, respectively.

Gel electrophoresis was used to evaluate the stability of liposome–ODN complexes coated with HSA. Figure 6 shows gel electrophoresis of liposome–ODN complexes incubated with HSA at 15 min and 4 h. Liposome–ODN complexes were destabilized by HSA at a molar ratio of 9:100 as free ODN could be detected on the gel. At HSA molar ratios of 1.5:100, 4.5:100, the formation of liposome–ODN complexes was not affected relative to the untreated liposome–ODN complexes.

3.5. Effect of Heated HSA on *Bcl-2* Downregulation.

HSA was heated to varying temperatures and then added to liposome–ODN complexes in order to study the effect of protein denaturation on ODN transfection. The *Bcl-2* mRNA downregulation by ODN in unheated and heated HSA-coated liposome–ODN complexes is shown in Figure 7A. The *Bcl-2* mRNA downregulation was decreased as the HSA was heated. The *Bcl-2* downregulation by unheated HSA-coated liposome–ODN formulation was observed with a reduction of $85.7 \pm 5.9\%$. In contrast, liposome–ODN complexes coated with HSA heated to 50 $^{\circ}$ C, 70 and 90 $^{\circ}$ C had a *Bcl-2* mRNA reduction of $76.8 \pm 7.4\%$, $8.4 \pm 14.1\%$ and $5.9 \pm 17.5\%$, respectively. The same trend was obtained based on *Bcl-2* protein level (Figure 7B and 7C). Treatment with liposome–ODN complexes coated with HSA heated to 50 $^{\circ}$ C had a *Bcl-2* protein reduction of $37.0 \pm 3.4\%$.

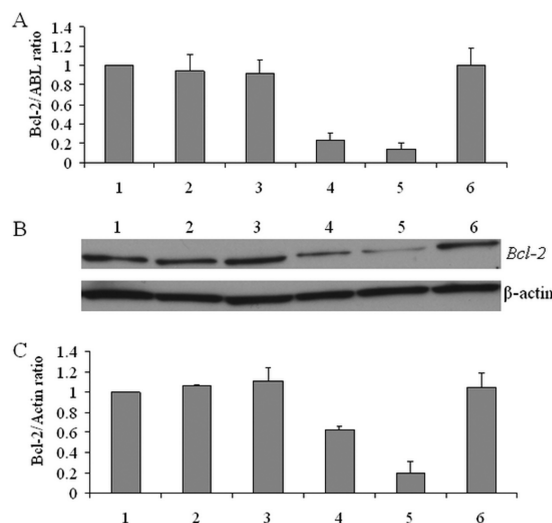


Figure 7. Effect of heat denaturation of HSA on transfection efficiency of liposome–ODN complexes in KB cells at different temperatures. Evaluation of *Bcl-2* mRNA expression by real-time RT-PCR (A), *Bcl-2* protein expression using Western blot (B) and quantification of *Bcl-2* protein level normalized by β -actin (C). (1) untreated cells; (2) HSA heated to 90 $^{\circ}$ C; (3) 70 $^{\circ}$ C; (4) 50 $^{\circ}$ C; (5) unheated HSA; (6) free ODN. Each value represents the mean \pm SD of three repeat studies.

Liposome–ODN complexes coated with HSA heated to 70 and 90 $^{\circ}$ C did not significantly reduce *Bcl-2* protein level. Unheated HSA-coated liposome–ODN complexes decreased *Bcl-2* protein level up to $81.0 \pm 12.5\%$.

3.6. Content of Secondary Structure in Heated HSA.

CD spectroscopy was performed to quantify the content of secondary structure in HSA heated to different temperatures. Figure 8A shows the CD spectra of unheated HSA and HSA heated to 50 $^{\circ}$ C, 70 $^{\circ}$ C and 90 $^{\circ}$ C. The CD spectra of unheated HSA and heated HSA have a negative broad band between 200 and 250 nm. Unheated HSA showed highest CD intensity. The CD intensity of HSA decreased with increasing the heating temperature. The content of α -helix and β -strand in unheated HSA and heated HSA was quantified using K2D2 software, and plotted with the corresponding HSA (Figure 8B). The content of α -helix in HSA had a tendency to decrease as the heating temperature increased. The content ranks of α -helix in HSA were as follows: unheated HSA ($45.5 \pm 4.1\%$) \sim HSA heated to 50 $^{\circ}$ C ($45.2 \pm 3.6\%$) $>$ HSA heated to 70 $^{\circ}$ C ($34.3 \pm 7.7\%$) $>$ HSA heated to 90 $^{\circ}$ C ($26.8 \pm 1.3\%$). On the other hand, the content of β -strand in HSA had a tendency to increase as the heating temperature increased. The content ranks of β -strand in HSA were as follows: unheated HSA ($9.7 \pm 0.02\%$) \sim HSA heated to 50 $^{\circ}$ C ($8.9 \pm 1.1\%$) $<$ HSA heated to 70 $^{\circ}$ C ($11.5 \pm 2.9\%$) $<$ HSA heated to 90 $^{\circ}$ C ($17.3 \pm 0.2\%$). This finding demonstrated that heating temperature affected the HSA conformation and specifically the content of the secondary structure in HSA.

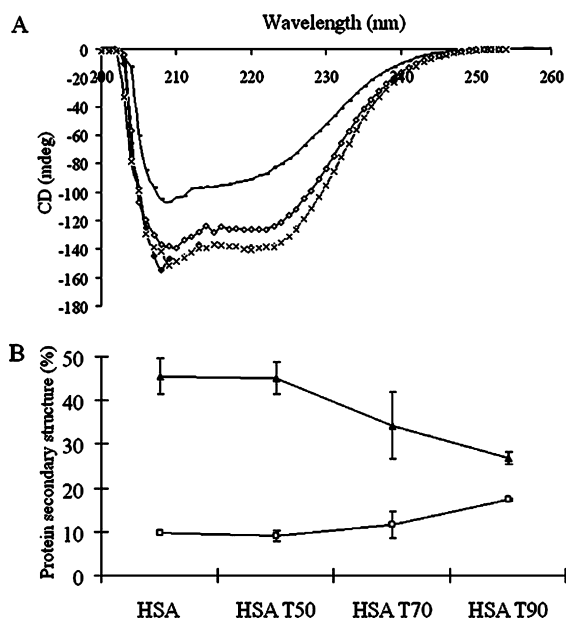


Figure 8. (A) CD spectra of HSA heated to different temperatures: (◆) unheated HSA; (○) HSA heated to 90 °C; (△) HSA heated to 70 °C; (×) HSA heated to 50 °C. (B) Protein secondary structure (%) in corresponding unheated HSA and heated HSA was quantified using K2D2 software. Each value represents the mean \pm SD of two repeat studies. (▲) α -Helix; (□) β -strand.

4. Discussion

The application of HSA and other serum albumin has been reported in delivery of drugs and genes.^{16,17} In this study, we formulated HSA-coated liposomes encapsulating the *Bcl-2* antisense oligonucleotide G3139, and evaluated the effect of the HSA concentration and HSA denaturation on the *Bcl-2* downregulation in KB cells. We found that HSA at an appropriate concentration could enhance the delivery of liposome-ODN complexes in KB cells. The enhanced transfection efficiencies depended on the HSA concentration and the secondary structure of HSA. Interestingly, HSA-coated liposome-ODN complexes exhibited high efficiency in *Bcl-2* mRNA and protein downregulation relative to Oligofectamine-ODN complexes.

Intracellular delivery of G3139 in KB cells showed that HSA can enhance delivery of liposome-ODN complexes. The increased transfection efficiency of HSA-coated liposome-ODN complexes could be due to both increased

cellular uptake and the fusogenic property of HSA. The enhancement of cell uptake by HSA is proposed to take place by binding of HSA to cell surface proteins followed by endocytosis.^{9,18} Albumin may exhibit fusogenic property at acidic pH in the endosomes.^{9,19} The carboxyls in human serum albumin can become protonated upon acidification of the endosomes, which may result in conformational changes that expose hydrophobic α -helices normally residing in the hydrophobic core of the folded structure of HSA. The protonated HSA can then interact with endosomal membrane and induce its fusion with the liposomes or destabilization.^{9,20} The degree to which HSA enhanced cellular uptake of liposome-ODN complexes depended on the amount of HSA in the HSA-coated liposome-ODN complexes and its secondary structure of the protein.

Our study showed that an excessively high level of HSA did not trigger transfection enhancement. This is because a high concentration of HSA can induce electrostatic destabilization of the liposome-ODN complexes. HSA possesses negative charge that can compete with the negatively charged ODN for binding to the positively charged liposomes and thus induce the release of ODN. Indeed, the addition of a high concentration of HSA to liposome-ODN complexes was shown to change the surface charge of the complexes to negative and cause ODN release. The negative charge of liposome-ODN complexes containing a high concentration of albumin may then inhibit their binding to the negatively charged cell surfaces, which leads to a decrease in ODN delivery activity.^{10,21} In addition, a high amount of albumin also can cause aggregation of the carrier system and thus decrease ODN delivery.²²

Heating of albumin at increased temperatures can alter its secondary structure.^{23,24} The decrease in *Bcl-2* downregulation could be due to a decrease in α -helix content and an increase in β -strand in heated HSA. This may affect HSA interaction with the cell surface as well as the endosomal activity of HSA. Our study on heated HSA demonstrated

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that heating to 70 °C and 90 °C greatly decreased the content of α -helix and increased the formation of β -strand relative to that heated to 50 °C and unheated HSA. This finding is in agreement with the reported denaturation of HSA taking place above 55 °C.²⁴ Therefore, HSA conformation, specifically the content of α -helix and β -strand, can affect the efficiency of ODN delivery.

5. Conclusion

The liposomes formulated in this study had the ability to complex to ODN and yielded nanosized particles. Liposome–ODN complexes coated with HSA effectively enhanced the transfection efficiency in KB oral carcinoma cells. The formulation of HSA-coated liposomes was very efficient in *Bcl-2* mRNA and protein downregulation. The transfection efficiency of liposome–ODN complexes coated with HSA was dependent on the concentration of

HSA and the content of α -helix and β -strand in HSA. Another advantage of HSA is that it is biocompatible and nontoxic. The cytotoxicity of lipoplexes was found to be decreased in the presence of human serum albumin.²⁵ This study suggests that HSA-coated liposome is a highly effective delivery vehicle for enhancing the delivery efficacy and therapeutic efficacy of antisense oligonucleotides and warrants further investigation.

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