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Inhibition on hepatitis B virus e-gene expression of 10–23 DNAzyme delivered by novel chitosan oligosaccharide–stearic acid micelles

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ABSTRACT

10–23 DNAzyme (DrzBC) could block the expression of HBV e-gene with application limit of dependence on exogenous delivery. Stearic acid grafted chitosan oligosaccharide (CSSA) could self-aggregate to form micelles in aqueous medium and bind with DrzBC by electrostatic interaction. Compared with Lipofectamine™ 2000, the CSSA micelles showed much lower cytotoxicity (412.5 μg/mL) and advantaged target in subcellular organelles-cytoplasm. Both including DrzBC of 1 μmol/L, Lipofectamine™ 2000/DrzBC complex showed maximum inhibition rate (IR) on HBeAg expression of 46.53 ± 2.00% at 48 h, and then decreased rapidly, while CSSA/DrzBC complex showed maximum IR of 82.51 ± 1.28% at 72 h, and hold on IR above 70% until 96 h. Moreover, within a concentration range of 0.1–2.0 μmol/L, and equally incubating for 48 h, the IR of Lipofectamine™ 2000/DrzBC complex was from 23.20 ± 1.61% to 66.27 ± 1.96%, while the IR of CSSA/DrzBC complex increased from 40.23 ± 3.28% to 80.95 ± 1.69%. CSSA/DrzBC complex is a promising effective system for inhibiting HBeAg expression.

1. Introduction

DNAzyme is a specifically structured DNA sequence with catalytic RNA-cleaving activity. 10–23 DNAzyme was the 23rd clone from round 10 during the course of 10 rounds in vitro selection by Santoro and Joyce (1997). The enzyme consists of a catalytic domain formed by 15 deoxynucleotide in the center and recognition arms formed by 7–10 nucleotides on both sides. In a highly sequence-specific manner through its recognition arms, 10–23 DNAzyme combines specifically with target mRNA, while the central catalytic domain cleaves mRNA target at the purine–pyrimidine junctions (A·U sites), and thereby blocks the expression of corresponding mRNA. 10–23 DNAzyme has been reported in a number of paper for the inhibition of various target virus gene expression and has showed promising results (Hou et al., 2006; Kumar, Chaudhury, Kar, & Das, 2009; Sood, Gupta, Bano, & Banerjea, 2007; Takahashi, Hamazaki, & Habu, 2004; Wo, Hou, Li, & Liu, 2006).

Hepatitis B virus (HBV) is an attractive target for the 10–23 DNAzyme, because the HBV replicative cycle starts from pregenomic RNA, besides, the HBV genome consists of overlapping genes. Thus anti-HBV 10–23 DNAzyme can cleave multiple viral mRNAs and viral pregenomic RNA at the same time. Our previous research (Wo et al., 2006) has showed that the HBV-specific 10–23 DNAzyme named DrzBC was able to efficiently block the expression of HBV e-gene within cells, resulting in the suppression of corresponding antigen proteins, suggesting that DrzBC can be developed into a powerfully specific and effective form of anti-HBV gene therapy. However, a major challenge facing the application of 10–23 DNAzyme is that it cannot replicate endogenously and, consequently, has to rely on exogenous delivery. This usually gives rise to poor intracellular uptake and affects the stability of the DNAzyme. Therefore, efficient gene transfer is crucial in gene therapy. Up to now, two main types of vectors used in gene therapy are viral and non-viral gene delivery systems. Due to safety concerns, non-viral gene delivery systems such as cationic liposome (Liu et al., 2005; Mark, Kevin, Duncan, Karen, & Hazel, 2007), nature and synthetic polymers (Mao, Sun, & Kissel, 2010; Kim & Kim, 2011) have been chosen as alternatives against viral systems. However, the high toxicity of cationic liposomes and the low efficiency of gene delivery by cationic polymers restrict their application (Jiang et al., 2008).

Stearic acid grafted chitosan oligosaccharide (CSSA), a kind of novel drug delivery carrier, synthesized by a coupling reaction between the amino groups of chitosan oligosaccharide and carboxyl group of stearic acid (Hu, Liu, Du, & Yuan, 2009). The CSSA could self-aggregate to form micelles in the aqueous phase and encapsulate chemotherapeutical drugs. In our previous research, CSSA has been used to deliver the anti-HBV chemotherapeutics such as Lamivudine stearate (Li et al., 2010) and acyclovir (Huang

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et al., 2010) and obtained enhanced anti-HBV activity. However, the anti-HBV biomacromolecule targeted at subcellular region of host cells and cleaved the target viral RNA directly and effectively, such as 10–23 DNAzyme, has not been mentioned. The chemical conjugation of CSSA both has the merits of chitosan, such as biocompatibility, biodegradability (Muzzarelli, 2010), and also has the merits of stearic acid which could be rapidly internalized into cancer cells and accumulated in cytoplasm with suitable substitution degree of amino groups (You, Hu, Du, & Yuan, 2007a; You, Hu, Du, Yuan, & Ye, 2007b). In addition, CSSA owns the characteristics of good solubility in water, narrow size distribution, low cytotoxicity and positive surface charge to bind with biomacromolecule of passive charge by electrostatic interaction (Du, Lu, Zhou, Yuan, & Hu, 2010), all of which make it possible to use CSSA as an efficient carrier for 10–23 DNAzyme.

In this paper, we designed and synthesized a kind of 10–23 DNAzyme specific to HBV e-gene ORF A^{1816}UG, namely, DrzBC. Since the CSSA could bind with DrzBC by electrostatic interaction to form CSSA/DrzBC complex, the CSSA was synthesized and further used as a novel and advanced gene delivery system for DrzBC. Herein, the characteristics of CSSA and CSSA/DrzBC complex, the DrzBC stability in CSSA/DrzBC complex were investigated. The cytotoxicity of CSSA, the Internalization of CSSA micelles, and the inhibition rate on HBeAg expression of DrzBC delivered by CSSA or Lipofectamine^{TM} 2000 (as control) were further observed in HepG2.2.15 cell lines as model HBV-transfected tumor cells.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 450.0 kDa, 95% deacetylation degree, Yuhuan Marine Biochemistry Co., Ltd., Zhejiang, China); chitosanase (Chemical Industries Co., Ltd., Japan); Stearic acid (Shanghai Chemical Reagent Co., Ltd., China); 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA); Pyrene (Aldrich Chemical Co., Ltd., USA); Fluorescein isothiocyanate (FITC) (Acros Organic, USA); Lipofectamine^{TM} 2000 and OPTI-MEMI medium (Invitrogen Corporation, CA, USA); Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and G418 (GIBCO-BRL, Gaithersberg, MD, USA). All other solvents were analytical or chromatographic grade.

2.2. Design and synthesis of 10–23 DNAzyme

We designed 10–23 DNAzyme named DrzBC specific to HBV e-gene (Genbank gi: 59429) ORF A^{1816}UG by Watson–Crick base pairing and consigned synthesis to Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, China). The sequence GGCTAGCTACAACGA was a specific catalytic domain of 10–23 DNAzyme molecule, with both its side sequences were substrate-recognition domains of nine deoxynucleotides, respectively (Fig. 1).

2.3. Synthesis of CSSA

As reported in previous papers (Hu, Ren, Yuan, Du, & Zeng, 2006; Hu et al., 2008), chitosan oligosaccharide was prepared by enzymatic degradation of chitosan. The molecular weight of chitosan oligosaccharide with 18.4 kDa molecular weight used in this paper was determined by gel permeation chromatography (GPC) with TSK-gel column (G3000SW, 7.5 mm i.d. × 30 cm).

CSSA was synthesized according to previous research (Ye et al., 2008). Fristly, 2 g chitosan oligosaccharide was dissolved in 50 mL distilled water. 1.75 g stearic acid and EDC (10 mol/mol of stearic acid) were dissolved in 40 mL ethanol by sonicate treatment (Sonic Purger CQ250, Academy of Shanghai Shipping Electric Instrument) in water bath at room temperature for 30 s. Then the mixture was added into the chitosan oligosaccharide solution, and was stirred with 400 rpm at 80 °C for 5 h. Secondly, the reaction solution was cooled to room temperature and dialyzed against distilled water using a dialysis membrane (MWCO: 3.5 kDa, Spectrum Laboratories, Laguna Hills, CA) for 72 h to remove water-soluble by-products, and then the reaction solution was lyophilized. Finally, the lyophilized product was washed twice with 20 mL ethanol to remove un-reacted SA, and further lyophilized to receive the CSSA powder.

2.4. Preparation of CSSA/DrzBC complex

CSSA solutions with different concentration (25 mM sodium acetate buffer, pH 5.5) were firstly purified by 0.22 μm millipore filter. The stable CSSA/DrzBC complexes with various N/P (The N/P was the ratio of the number of unreacted free primary amines of CSSA and the number of phosphate groups of DrzBC) were prepared by mixing the appropriate volume of CSSA micelle solution and DrzBC solution (500 μg/mL) vortically for 30 s, and then incubating for 30 min at 37 °C.

Electrophoresis was then carried out with a current of 100 V for 20 min in TAE buffer solution (40 mM Tris–HCl, 1% (v/v) acetic acid, 1 mM EDTA). The retardations of the CSSA/DNA complexes with various N/P ratios were visualized by the staining of ethidium bromide.

2.5. Characterization of CSSA and CSSA/DrzBC complex

2.5.1. Substitution degree of amino groups (SD) of CSSA

The substitution degree of amino groups (SD), defined as the molar ratio of stearate to anhydroglucosidic units in chitosan oligosaccharide, was determined by the TNBS method. 2 mL of 4% $NaHCO_3$ and 2 mL of 0.1% TNBS solution were added into 2 mL of CSSA solution with 125 μg/mL CSSA, and the mixture was incubated at 37 °C for 2 h. 2 mL of HCl (2 N) was then added into the mixture to neutralize the remaining $NaHCO_3$. The ultra-violet (UV) absorbance of final reaction mixture at 344 nm was measured by UV spectroscopy (TU-1800PC, Beijing Purkinje General Instrument Co., Ltd., China).

2.5.2. Determination of critical micelle concentration (CMC)

The critical micelle concentration (CMC) of CSSA was estimated by fluorescence spectroscopy using pyrene as a probe. The excitation wavelength was 337 nm, and the slit was set at 2.5 nm (excitation) and 10 nm (emission). The intensities of the emission were monitored by a fluorometer (F-2500, Hitachi Co., Japan) at a wavelength range of 360–450 nm. The concentrations of CSSA solution containing 5.93×10^{-7} M of pyrene were varied from 1.0×10^{-3} to 1.0 mg/mL. The intensity ratio (I_1/I_3) of the first peak (I_1, 374 nm) to the third peak (I_3, 385 nm) in the pyrene emission spectra was analyzed to calculate CMC.

2.5.3. Size and zeta potential determination

The size and zeta potential of CSSA micelles and CSSA/DrzBC complex were measured by Dynamic Light Scattering (Zetasizer 3000HSA, Malvern Instruments Ltd., UK). Size presented in this section is volume average diameter.

2.6. DNase I protection assay

The DNase I protection assay was performed to investigate the efficiency of CSSA micelles protecting the DrzBC from enzymatic degradation by DNase I. The CSSA/DrzBC complexes with 1.2 and 10

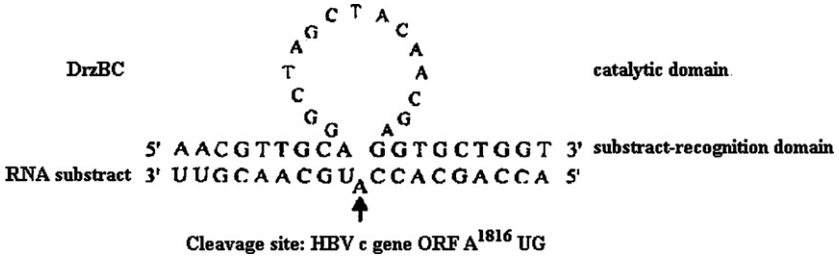

Fig. 1. Sequence of DrzBC and RNA substrates (arrow indicating cleavage site).

of N/P ratios were incubated in a buffer solution (10 mM Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.4) containing 25 units of DNase I. The UV absorbance change at 260 nm was measured at different intervals by using a UV spectrophotometer (UV-2450, Shimadzu, Japan).

2.7. Cell culture

HepG2.2.15 cells, hepatitis B virus-transfected human hepatoma cells, were maintained in our laboratory. This cell lines were propagated in DMEM medium supplemented with 10% (w/w) fetal bovine serum (FBS) and 500 μg/mL G418 in the air containing 5% (v/v) CO_2 at 37 °C.

2.8. Cytotoxicity assay

HepG2.2.15 cells were seeded in a 96-well plate at a seeding density of 10,000 cells per well and cultured at 37 °C for 48 h. The cells were exposed to a series of concentrations of Lipofectamine™ 2000 and CSSA micelles for another 72 h. After incubation, the metabolic activities of the cells were measured. A 100 μL of fresh growth medium containing 50 mg MTT was added to each well, and the cells were incubated for 4 h. After the unreduced MTT and medium were removed, each well was washed with 100 μL of PBS, and 180 μL of DMSO was then added to each well to dissolve the MTT formazan crystals. Plates were shaken for 20 min and the absorbance of formazan product was measured at 570 nm in a microplate reader (BioRad, Model 680, USA). Survival percentage was calculated as compared to mock-treated cells (100% survival). All the experiments were performed in triplicate.

2.9. Internalization of CSSA micelles

Fluorescent CSSA micelles were prepared by adding FITC ethanol solution into 5.0 mg/mL CSSA aqueous solution. The molar ratio of CSSA to FITC was set at 1:4. The mixture was stirred to react with 250 rpm overnight at room temperature under protection from light. Then the reaction solution was dialyzed against DI water using a dialysis membrane (MWCO: 3.5 kDa, Spectrum Laboratories, Laguna Hills, CA) for 6 h to remove the un-reacted FITC. Finally, the dialyzed product was lyophilized to receive FITC-labeled CSSA.

HepG2.2.15 cells were seeded in a 24-well plate at a seeding density of 1.0×10^5 cells per well in 1 mL of growth medium and allowed to attach for 48 h. Cells were incubated with FITC-labeled CSSA micelles and FITC-labeled CSSA/DrzBC complexes (N/P ratio is 10) (both of the CSSA concentration were 100 μg/mL) in growth medium for different time and were washed thrice with PBS (pH 7.4). The cellular uptakes of FITC-labeled CSSA micelles and FITC-labeled CSSA/DrzBC complexes were observed under a fluorescence microscope (OLYMPUS America, Melville, NY).

2.10. Inhibitory effect of DrzBC on HBeAg expression in HepG2.2.15 cells

HepG2.2.15 cells were seeded at 1.0×10^5 cells/well in a 24-well plate and grown for 48 h to allow the cells to attach. After the medium was replaced with 0.5 mL OPTI-MEMI, different amounts of CSSA/DrzBC complexes (N/P ratio is 10) and Lipofectamine™ 2000/DrzBC complexes performed as positive controls were added to each well, and incubated with cells for 6 h. The medium was then replaced with 1 mL of fresh complete medium, and the incubation was continued for further 12–96 h at 37 °C. All the experiments were performed in triplicate. The culture media from the wells were collected for the virological assessment. HBeAg productions were determined by commercial enzyme immunoassay kits (AXSYM System, Abbott, Wiesbaden, Germany).

3. Results and discussion

3.1. Characteristics of CSSA

By controlling reaction time or the charged amount of chitosan oligosaccharide, SA and EDC, CSSA copolymer with different SD could be obtained. To determine the graft ratio of SA, substitution degree of amino groups (SD) of CSSA was measured using TNBS method. Table 1 shows the SD of CSSA in present study was $37.92 \pm 0.62\%$.

The CMC value is one of the important characteristics for polymeric micelles as a drug delivery carrier, indicating the self-aggregation ability. Owing to the hydrophobic SA modification of water-soluble chitosan oligosaccharide molecules, the synthesized CSSA could self-aggregate to form micelles. The aggregation behavior of CSSA was investigated by fluorescence spectroscopy using pyrene as a probe. Fig. 2 shows the variation of fluorescence intensity ratio for I_1/I_3 (I_1, em = 374 nm; I_3, em = 385 nm) against logarithm of CSSA concentration. Before the micelles formed in

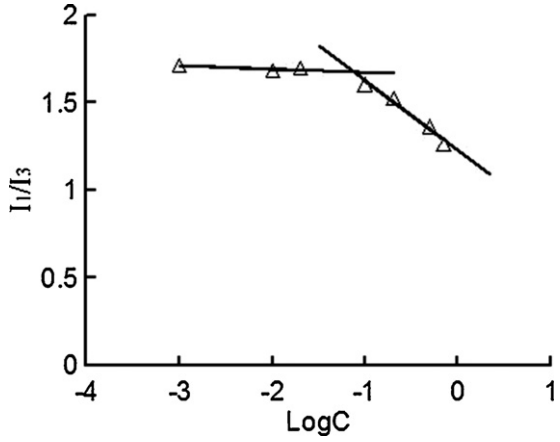

Fig. 2. Variation of intensity ratio (I_1/I_3) vs concentration of CSSA (Δ).

Table 1
Characteristics of CSSA micelles and CSSA/DrzBC complex.

	Volume average diameter (nm)	Zeta potential (mV)	SD (%)	CMC (mg/mL)
CSSA micelles	67.3 ± 3.4	48.3 ± 2.9	37.92 ± 0.62%	0.075
CSSA/DrzBC complex	164.0 ± 2.1	44.4 ± 3.7	–	–

aqueous environment, the value of I_1/I_3 remained constant. Once the CSSA concentration reaching above the CMC, pyrene molecules are preferably partitioned into the less polar hydrophobic core, thereby the value of I_1/I_3 deceased sharply, indicating the formation of micelles. The CSSA concentration causing the change of I_1/I_3 was the CMC value of CSSA, herein, determined to be 0.075 mg/mL in water (shown in Table 1). The low CMC value is desired to avoid the disassociation during the micelles drug delivery system were diluted by body fluid. The CMC value of CSSA is significantly lower than that of unmodified chitosan oligosaccharides (0.39 mg/mL) and low-molecular weight surfactants such as sodium dodecyl sulfate (2.47 mg/mL) in water (You et al., 2007a), which means the present CSSA with more stable self-aggregation can form micelles in highly diluted condition.

As shown in Table 1, the volume average diameter and zeta potential of CSSA micelles with 1.0 mg/mL concentration in DI water were 67.3 ± 3.4 nm and 48.3 ± 2.9 mV, respectively.

3.2. Characteristics of CSSA/DrzBC complexes

The DrzBC condensation capacity of CSSA micelles was analyzed by a gel retardation assay using an agarose gel electrophoresis. Fig. 3 shows the gel retardation result of CSSA/DrzBC complexes at different N/P ratios from 0.25 to 20. When the N/P ratio was above 1.2, the complete retardation of the complexes shown in Fig. 3, which suggested tight CSSA/DrzBC complexes started to form.

The size and zeta potential of CSSA/DrzBC complexes were also listed in Table 1, The CSSA micelle with 1.0 mg/mL of CSSA concentration in DI water were 164.0 ± 2.1 nm of volume average diameter and 44.4 ± 3.7 mV of zeta potential. The value of zeta-potential in CSSA micelles and CSSA/DrzBC complex are almost the same. The result suggests that the complex constitution is not affected by the surface charge of the particles, thus the zeta potential had no obvious change.

3.3. DNase I protection assay

Protection of DrzBC from nucleases is one of the crucial factors for its efficient delivery to inhibit HBV gene expression. Herein, DNase I was used as a model enzyme to examine the effect of protection to DrzBC in CSSA/DrzBC complex from DNase I degradation. Increasing the UV absorbance value at 260 nm with incubation time reveals the degradation of DrzBC backbone. Fig. 4 shows the naked DrzBC was significantly degraded in 5 min, whereas combined with CSSA micelles, DrzBC was protected from the attack of DNase I in some extent. The perfect protection relied on appropriate N/P ratio, as shown in Fig. 4, when the N/P ratio was 1.2, some extent of degradation could be observed, which was consistent with the gel retardation result of Fig. 3. However, when the N/P ratio was 10, just a little degradation could be observed, which suggested the DrzBC could be almost fully condensed with the polymeric micelles at the N/P ratio of 10.

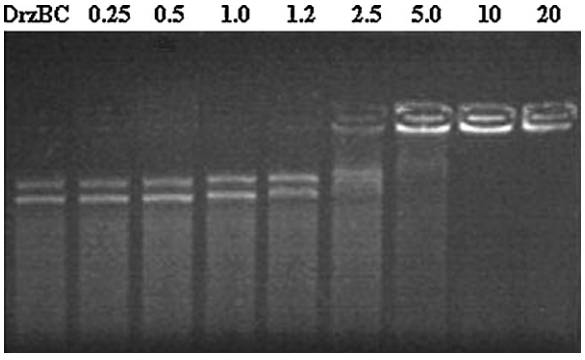

Fig. 3. Gel retarding analysis of CSSA/DrzBC complex nanoparticles. Lane 1 is naked DrzBC; lanes 2–9 are CSSA/DrzBC complex nanoparticles with N/P ratio of 0.25, 0.5, 1.0, 1.2, 2.5, 5, 10 and 20, respectively.

3.4. Cytotoxicity

The cytotoxicity of Lipofectamine™ 2000 or CSSA micelles were determined in HepG2.2.15 as model HBV-transfected tumor cells. Fig. 5A and B represent the cell viabilities incubated with Lipofectamine™ 2000 or CSSA micelles at various concentrations for 72 h, respectively. By MTT assay, the cellular growth inhibition (IC_{50}) of Lipofectamine™ 2000 was measured to be 6.8 μg/mL, while the IC_{50} of CSSA was measured to be 412.5 μg/mL. The results revealed that CSSA possessed much lower cytotoxicity than Lipofectamine™ 2000, which indicated the CSSA micelles system is much safer carrier for DrzBC than traditional Lipofectamine™ 2000.

3.5. Internalization of CSSA

Fig. 6A and B show the fluorescence images of FITC-labeled CSSA micelles and FITC-labeled CSSA/DrzBC complexes (both of the CSSA concentration were 100 μg/mL) incubated with HepG2.2.15 cells for 2 h, respectively. Significant fluorescence in cells was observed after incubating with fluorescent CSSA micelles. It was also observed that FITC-labeled CSSA accumulated in cytoplasm, suggesting CSSA micelles possess the cytoplasm-targeting localization and delivery. Moreover, the property of cytoplasm targeting was not changed after DrzBC was oaded into the CSSA micelles (Fig. 6B). Since DrzBC exerts inhibitory effect on HBeAg expression

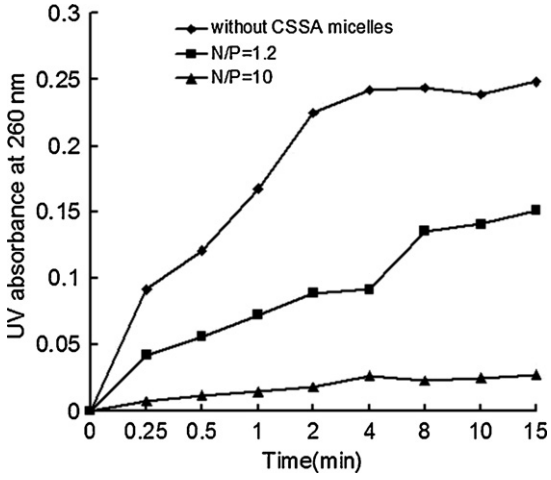

Fig. 4. Variation of UV absorbances at 260 nm against reaction time in DNase degradation experiments with or without CSSA micelles.

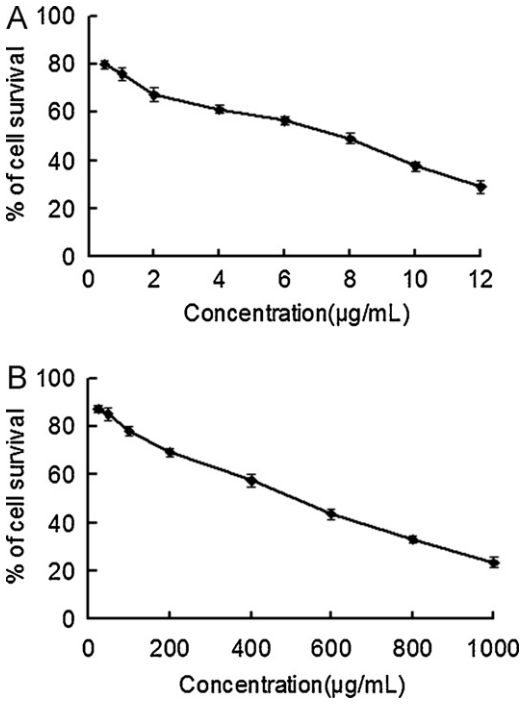

Fig. 5. Cell survival curves of HepG2.2.15 cells incubated with Lipofectamine™ 2000 (A) or CSSA micelles (B) at various concentrations for 72 h.

in the cytoplasm of HepG2.2.15 cells, CSSA micelles is suitable carrier to deliver DrzBC into target region of HepG2.2.15 cells.

3.6. Inhibitory effects of DrzBC on HBeAg expression in HepG2.2.15 cells

The effect of incubation time on the HBeAg inhibition rate (IR) in the same DrzBC concentration (1 μmol/L) was investigated. All experiments were repeated thrice. Fig. 7A shows the IR of Lipofectamine™ 2000/DrzBC complex on HBeAg expression increased sharply within 48 h and with maximum IR of 46.53 ± 2.00% at 48 h. After 48 h, the IR decreased rapidly which may be due to higher cytotoxicity of Lipofectamine™ 2000. On the other hand, when the CSSA complexed with DrzBC (N/P ratio is 10), the IR of CSSA/DrzBC complex on HBeAg expression was increased with the incubation time until 72 h. The incubation time reaching maximum IR of 82.51 ± 1.28% delayed to 72 h, may be related to lower release rate of DrzBC from CSSA and lower cytotoxicity than that of Lipofectamine™ 2000. After 72 h, the cells became multi-layers in the well due to the cellular growth, and the IR

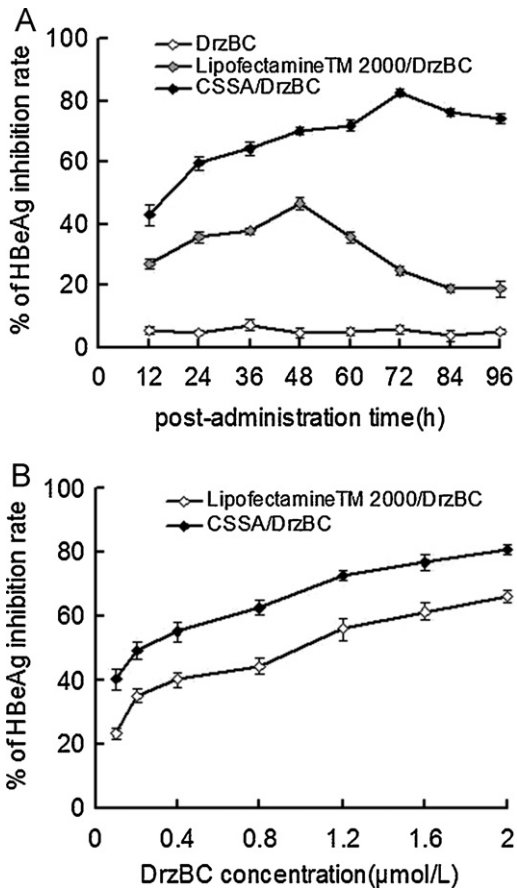

Fig. 7. HBeAg inhibition rate of DrzBC in different form: (A) in the same DrzBC concentration of 1 μmol/L for different incubation time; (B) for the same incubation time of 48 h with different DrzBC concentration.

was decreased. From Fig. 7A, it is obvious that DrzBC delivered by CSSA micelles exhibited higher efficiency for inhibiting HBeAg expression than that delivered by Lipofectamine™ 2000 after incubating with HepG2.2.15 cells in each and every time dot during 96 h (Fig. 7A).

Within a concentration range of 0.1–2.0 μmol/L and for the same incubation time of 48 h, the IR on HBeAg expression of DrzBC delivered by Lipofectamine™ 2000 was increased from 23.20 ± 1.61% to 66.27 ± 1.96%, while the IR on HBeAg expression of DrzBC delivered by CSSA micelles increased from 40.23 ± 3.28% to 80.95 ± 1.69%, both showing a distinct dose-dependent effectiveness. With additional increases of DrzBC admission doses, the IRs increased a little but platform-like effectiveness was observed. Moreover, DrzBC

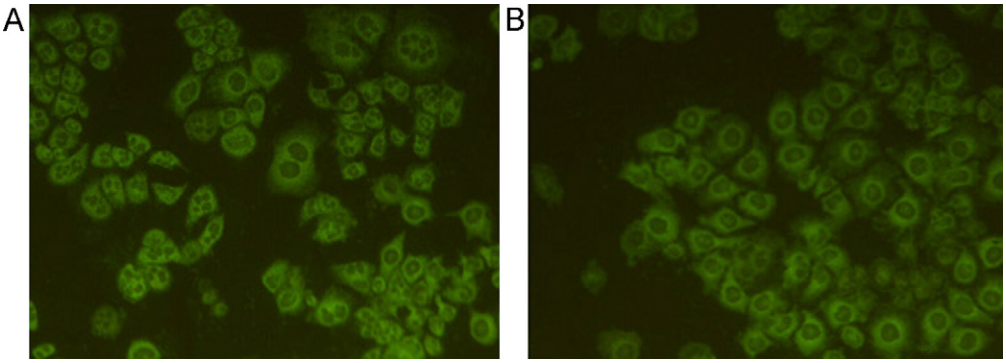

Fig. 6. Cellular uptake of (A) FITC-labeled CSSA micelles and (B) FITC-labeled CSSA/DrzBC complexes into HepG2.2.15 cells. Cells were incubated for 2 h with CSSA concentration of 100 μg/mL.

delivered by CSSA micelles exhibited higher efficiency for inhibiting HBeAg expression than that delivered by Lipofectamine™ 2000 in each and every concentration dot (Fig. 7B).

4. Conclusion

In this work, we designed a kind of 10–23 DNAzyme named DrzBC specific to blocking the expression of HBV e-gene with the application limit of dependence on exogenous delivery. The chemical conjugation of CSSA could self-aggregate above 0.075 mg/mL in aqueous medium to form cationic micelles with nano-size, and it indicated efficient ability to complex with DrzBC via electrostatic interaction to form CSSA/DrzBC complex nanoparticles, which can efficiently protect the condensed DrzBC from enzymatic degradation by DNase I. Furthermore, the CSSA micelles, with SD of 37.92 ± 0.62%, showed excellent internalization into HepG2.2.15 cells and accumulation in cytoplasm, where DrzBC could exerts inhibitory effect on HBeAg expression. Comparing with traditional gene transfer carrier-Lipofectamine™ 2000, CSSA micelles show much lower cytotoxicity (412.5 μg/mL), thus having an unhampered superior capacity of DrzBC condensation. By Electrophoresis and DNase I protection assay, CSSA/DrzBC complex with 10 of N/P ratio (volume average diameter, 164.0 ± 2.1 nm; zeta potential, 44.4 ± 3.7 mV) was chosen to investigate the anti-HBV activity. In the same concentration of 1 μmol/L, DrzBC delivered by Lipofectamine™ 2000 showed maximum inhibition rate (IR) of 46.53 ± 2.00% at 48 h, and then decreased rapidly, while DrzBC delivered by CSSA micelles showed maximum IR of 82.51 ± 1.28% at 72 h, and hold on IR above 70% until 96 h. Moreover, in the same incubation time of 48 h, and within a concentration range of 0.1–2.0 μmol/L, the IR of DrzBC delivered by Lipofectamine™ 2000 on HBeAg expression was from 23.20 ± 1.61% to 66.27 ± 1.96%, while the IR of DrzBC delivered by CSSA micelles on HBeAg expression increased from 40.23 ± 3.28% to 80.95 ± 1.69%. DrzBC delivered by CSSA micelles exhibit higher IR of HBeAg expression than that delivered by Lipofectamine™ 2000 in each and every concentration, which may contribute to the molecule target in cytoplasm. Overall, the study suggests that the CSSA/DrzBC complex nanoparticles have promising application for anti-HBV gene therapy.

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