Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal of Materials Chemistry B

Dynamic Article Links ►

Cite this: DOI: 10.1039/c0xx00000x

ARTICLE TYPE

www.rsc.org/xxxxxx

Co-delivery of thioredoxin 1 shRNA and doxorubicin by folate-targeted gemini surfactants-based cationic liposomes to sensitize hepatocellular carcinoma cells

Wenjie Li,[a1] Jing Shi,[a1] Chun Zhang,[a] Min Li,[a] Lu Gan,[*a] Huibi Xu[a] and Xiangliang Yang[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The overexpression of thioredoxin (Trx) 1 is one of the mechanisms for drug resistance in hepatocellular carcinoma (HCC) treatment. The co-delivery of drugs and a short hairpin RNA (shRNA) against Trx1 to silence its expression might provide a promising approach to improve drug sensitivity to chemotherapeutic agents. In this paper, cationic liposomes containing gemini surfactants, soybean lecithin and DOPE were firstly constructed as gene transfection carriers. The length and symmetrical degree of aliphatic chains of gemini surfactants were found to affect gene transfection efficiency of cationic liposomes. Cationic liposome containing gemini surfactant with symmetrical C16 aliphatic chains (L16-2-16) showed the highest transfection efficiency in HCC Bel7402 cells and also displayed the strongest DNA condensation capacity and the highest cellular uptake. Folic acid (FA) targeting further enhanced the transfection efficiency of L16-2-16 by binding to folate receptor (FR) in FR-overexpressing Bel7402 cells and lipid raft/caveolae-dependent endocytosis might be involved in FA-L16-2-16-mediated gene delivery. Then the multifunctional co-delivery system for doxorubicin (DOX) and Trx1 shRNA was developed via electrostatic interactions between FA-L16-2-16 and Trx1 shRNA/DOX complex. Co-delivery of Trx1 shRNA and DOX by FA-L16-2-16 inhibited the cell viability and induced apoptosis of Bel7402 cells more efficiently than co-delivery of control shRNA and DOX. The system for co-delivering DOX and Trx1 shRNA could simultaneously achieve gene transfection and drug release in the same cell and increase the intracellular as well as intranuclear DOX concentrations. These results demonstrated that gemini surfactants-based cationic liposomes were promising carriers for the co-delivery of nucleic acids and chemotherapeutic agents to sensitize HCC chemotherapy.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most fatal diseases in the world with a five-year survival rate of less than 5 %, particularly in developing countries.[1] Although surgery, transarterial chemoembolization (TACE) and local ablative therapy were commonly used to treat HCC, these operations were limited by tumor size or hepatic functional reserve due to diagnosis at a late stage.[2] Systemic chemotherapy must be one of the important possibilities of multimodal treatment for advanced HCC, however no chemotherapy drugs such as doxorubicin (DOX) or 5-fluorouracil (5-FU) were found to significantly improve the overall survival of HCC patients.[3] Chemoresistance and nonspecific toxicity are the major problems affecting chemotherapy failure in HCC.[4,5] Therefore, the development of drug delivery systems that can enhance the chemosensitivity of HCC cells and reduce the adverse effects to normal cells is urgently required.

Recent studies have shown that drug resistance is mainly caused by malfunction of genes owing to chromosomal alternations in cancer cells.[6,7] Co-delivery of gene and drug for synergistic therapy has provided a promising strategy to sensitize cancer cells to chemotherapeutics.[8,9] Thioredoxin (Trx) 1, a ubiquitously expressed small redox protein which has a conserved Cys-Gly-Pro-Cys redox catalytic site, plays critical roles in the regulation of cellular redox homeostasis.[10] Trx1 has been shown to be overexpressed in a wide variety of human tumors including lung, pancreas, colon, gastric, breast and liver cancers.[11-14] Trx1 expression in cancer cells is associated with aggressive tumor growth,[14] clinical resistance to cisplatin and docetaxel,[15,16] et al., which makes Trx1 an attractive target for cancer therapy.[17] Inhibition of Trx1 by small interfering RNA (siRNA) or a Trx1 inhibitor PX-12 was found to significantly inhibit cancer cell growth and sensitize cancer cells to DOX-induced cell growth inhibition.[18] Thus, the combination of Trx1 gene therapy and chemotherapy might increase the therapeutic efficacy in HCC therapy.

It has been reported that cationic liposomes have been widely used to deliver DNAs, siRNAs, antisense oligonucleotides and even drugs into the cells due to low immunogenicity and ease of preparation,[19] which makes them successful co-delivery systems for genes and drugs. However, relatively low transfection efficiency of cationic liposomes limited their use in gene delivery.[20] Numerous kinds of cationic lipids have been designed as gene delivery carriers to improve the transfection efficiency. Gemini surfactants are a relatively new class of amphiphilic molecules containing two polar headgroups and two aliphatic chains linked by a spacer.[21] In view of the presence of the multivalent positively charged polar headgroups which may

efficiently condense the negatively charged DNA molecules and having several advantages compared with classic monovalent surfactants, such as lower critical micelle concentration (cmc), higher efficiency in reducing surface tension and greater tendency to self-assemble,[22] gemini surfactants have been shown to be highly effective in delivering genetic materials to cells, and also have been shown promising as cationic components of liposome formulations for gene delivery.[23-25] However, the structure-activity relationship on the gene transfection efficiency of gemini surfactants-based cationic liposomes and their roles as gene and drug co-delivery systems remain to be elucidated.

DOX has been commonly used as a chemotherapeutic drug for HCC treatment which works by intercalating DNA. However severe systemic toxicity, such as cardiotoxicity limits its use in the clinical treatment.[26] Reduction of its usage doses but maintenance of its high efficacy will be needed. In this paper, cationic liposomes containing gemini surfactants, soybean lecithin and Dioleyl-sn-glycero-3-phosphoethanolamine (DOPE) were constructed as co-delivery systems for a short hairpin RNA (shRNA) to silence Trx1 expression and DOX. A relationship between the structure of gemini surfactants with different lengths and symmetrical degrees of aliphatic chains and transfection efficiency of cationic liposomes was firstly investigated in HCC cells, and a tumor-homing strategy targeting folate receptor (FR) overexpressed in HCC was further prepared to enhance the transfection efficiency. The multifunctional co-delivery systems for DOX and Trx1 shRNA were developed via DOX intercalation into Trx1 shRNA and then electrostatic interactions between Trx1 shRNA/DOX complex and gemini surfactant-based cationic liposomes. The co-delivery systems were thoroughly characterized, and the therapeutic efficacy in HCC cells and the potential mechanisms were systematically evaluated.

2. Experimental section

2.1. Materials

DOPE and soybean lecithin were purchased from Lipoid (Ludwigshafen, Germany). Folic acid (FA) was obtained from Aladdin Reagent Co (Shanghai, China). FITC, chlorpromazine, cytochalasin D and methyl-β-cyclodextrin (MβCD) were purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 medium, FA-free RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (Grand Island, NY, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals used were of analytical grade commercially available.

2.2. Cell culture

The human HCC cell lines (Bel7402 and HepG2) and human normal liver cell line (HL7702) were purchased from China Center for Type Culture Collection (Wuhan, China). The cells were cultured in RPMI 1640 medium supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5 % CO_2 in a humidified atmosphere.

2.3. Plasmid DNA

The reporter plasmids of pCMV-Luc encoding a luciferase gene and pEGFP-N1 encoding enhanced green fluorescent protein (EGFP) were obtained from Clontech (Mountain View, CA, USA). For construction of Trx1 shRNA plasmid, a 21-bp sequence used to knock down endogenous Trx1 (5'-atgactgtcaggatgttgc-3') was inserted into pCMS4-H1p-EGFP (provided by Dr. Haojie Huang, Mayo Clinic, Rochester, MN, USA) which contained an H1 promoter for shRNA expression, a CMV promoter for expression of shRNA-resistant cDNAs, and an SV40 promoter controlling EGFP expression and then identified by sequencing.[27] To amplify these plasmids, plasmids were proliferated firstly in *Escherichia coli* DH5α strain and then purified using QIAGEN plasmid purification kits (QIAGEN Sciences Inc, Germantown, MD, USA) following the manufacturer's instructions. The concentration and purity of plasmids were determined by UV spectrophotometry.

2.4. Preparation of cationic liposomes and cationic liposome/DNA complexes

Cationic liposomes containing soybean lecithin, DOPE and various kinds of gemini surfactants with different lengths and symmetrical degrees of aliphatic chains (synthesized and identified in college of Life Science and Technology, Huazhong University of Science and Technology, according to an established procedure [21)] were prepared by the film hydration method.[28] Briefly, the lipid mixtures containing soybean lecithin: DOPE: gemini surfactants (1:1:1, w/w/w) were dissolved in chloroform. The solvent was then evaporated at 37 °C under vacuum in a rotary evaporator to obtain a thin lipid film. The lipid film was maintained under vacuum for at least 30 min to remove any residual solvent, and then hydrated with ultrapure water for 30 min to achieve a final lipid concentration of 1 mg/ml. This preparation was followed by sonication for 15 min to obtain a solution of multilamellar vesicles. FITC-labeled or FA-targeted cationic liposomes were prepared in the same way except that FITC-labeled DOPE (soybean lecithin: DOPE: gemini surfactant: FITC-DOPE=1:1:1:0.005, w/w/w/w) or different amounts of FA-PEG-DOPE was added. The synthesis and identification of FITC-DOPE or FA-PEG-DOPE were performed as described,[29,30] respectively.

Cationic liposome/DNA complexes were obtained by mixing plasmid DNA with appropriate amounts of cationic liposomes at the desired N/P ratios (the ratios of moles of the amine groups of gemini surfactants to those of the phosphate ones of DNA). The mixtures of cationic liposome/DNA were incubated for 30 min at room temperature.

2.5. Preparation of cationic liposome/DNA/DOX complexes

A complex between DNA and DOX was formed as described.[31] Briefly, different concentrations of plasmid DNA were added to a fixed concentration of DOX (2 μg/ml) in phosphate-buffered saline (PBS, pH 7.4) and then the mixtures were incubated for 10 min. The fluorescence intensity of DOX was scanned with the excitation wavelength at 502 nm and emission wavelength at the interval of 520-695 nm (1.0 mm slit) on an F-4500 FL spectrophotometer (Hitachi, Tokyo, Japan). Cationic liposome/DNA/DOX complexes were prepared by adding cationic liposomes to DNA/DOX complexes at N/P ratio of 2 and the DNA/DOX mass ratio of 9. The mixtures were incubated for 30 min at room temperature.

2.6. Characterization of cationic liposome/DNA/DOX complexes

The effective hydrodynamic diameter and zeta potential of cationic liposome/DNA and cationic liposome/DNA/DOX complexes were determined by photon correlation spectroscopy technique using a "ZetaSizer" Nano Series (Malvern Instrument, Malvern, UK). The morphology of cationic liposome/DNA/DOX complexes was examined under a transmission electron microscope (JEM-2010, JEOL, Japan). *In vitro* DOX release from cationic liposome/DNA/DOX and DNA/DOX complexes was performed using dialysis method. Briefly, 1ml of the complexes (25 μg DOX/ml) was placed in the dialysis bag (MWD 3500) and the end-sealed dialysis bag was fully submerged into 20 ml of PBS (pH 7.4). At the indicated time points, 1 ml of the sample was withdrawn and replaced with an equal volume of fresh medium. The absorbance of DOX released was measured on an F-4500 FL spectrophotometer with the exicitation wavelength at 502 nm and emission wavelength at 556 nm.

2.7. Circular dichroism (CD) spectroscopy

After the mixtures of different formulations of cationic liposomes and DNA (50 μg/ml) at N/P ratio of 2 were incubated for 30 min at room temperature, CD binding experiments were performed using a JASCO J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Samples were measured from 190 to 350 nm with a scan rate of 100 nm/min, a step resolution of 0.2 nm, a 1.0 nm bandwidth and a sensitivity of 10 mdeg. The spectra were obtained as the average of three scans and corrected by subtracting the buffer baseline spectrum.

2.8. Displacement and exclusion assays

Ethidium bromide (EB), a DNA-intercalating dye, was used to examine the association of DNA with different formulations of cationic liposomes. In the displacement assay, 1 μg DNA and EB solution (1 μg/ml) were mixed in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) and allowed to equilibrate for 10 min. Then different amounts of cationic liposomes were added to the solution and mixed to obtain the desired N/P ratios. The fluorescence was measured after 10 min incubation with the excitation wavelength at 531 nm and emission wavelength at 590 nm.

In the exclusion assay, DNA (1 μg) and varying amounts of cationic liposomes were mixed to obtain the desired N/P ratios in TE buffer and allowed to incubate at room temperature for 30 min. Then EB solution (1 μg/ml) was added and the sample was mixed. After 10 min incubation, the fluorescence was measured with the excitation wavelength at 531 nm and emission wavelength at 590 nm.

2.9. *In vitro* transfection

Cells (Bel7402, HepG2 and HL7702 cells) were seeded at 5×10^4 cells/well in 24-well plates and incubated for 24 h. Then the cells were treated with the indicated complexes of cationic liposome/pEGFP-N1, cationic liposome/pCMV-Luc or cationic liposome/pCMV-Luc/DOX in FA-free and serum-free RPMI 1640 medium for 4 h at 37 °C, respectively. The mixtures were removed and then the cells were rinsed once with serum-containing medium and cultured in fresh medium containing FBS for 24 h. cells were washed with PBS and EGFP expression was observed under an Olympus 1X71 inverted fluorescence microscope (Melville, NY, USA). The luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI, USA) in a GloMax 20/20 single-tube luminometer (Promega, Madison, WI, USA). The relative light units (RLU) were normalized with respect to the total protein concentrations of the cell extracts. The protein content was determined using the BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) according to the manufacturer's instructions.

2.10. Western blot

Bel7402 cells were grown at 3×10^5 cells/well in 6-well plates and transfected with the indicated cationic liposome/control shRNA or cationic liposome/Trx1 shRNA complexes. After 36 h transfection, the cells were harvested and subjected to western blot. Briefly, the cells were lysed in the ice cold lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2 mM EGTA, 2 % SDS, 10 % glycerol, 10 mM NaF, 0.1 % Triton X-100, 2 mM Na_3VO_4 and complete protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The insoluble materials were centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatants were collected. The proteins from each sample (40 μg) were separated by 15 % SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5 % skin milk in TBST for 1 h and then probed with anti-Trx1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, respectively. The membrane was washed with TBST 3× for 5 min. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, protein signals were detected using enhanced chemiluminescence (Perice, Rockford, IL, USA). The expression of Trx1 protein was normalized to that of β-actin as a control.

2.11. Endocytic pathway

To study the endocytic pathway involved in the internalization of the indicated cationic liposome/DNA complexes, Bel7402 cells were preincubated in FA-free and serum-free RPMI 1640 medium containing 10 mM sodium azide and 50 mM 2-deoxyglucose (NaN_3/DOG) (1 h), MβCD (10 mM, 1 h), chlorpromazine (10 μg/ml, 1 h), or cytochalasin D (5 μg/ml, 1 h), respectively. The medium was then changed to fresh FA-free and serum-free medium containing the inhibitors plus cationic liposome/pCMV-Luc complexes and further incubated for 4 h at 37 °C. The mixtures were removed and the cells were cultured in fresh medium containing FBS for 24 h. Then the cells were washed with PBS and the luciferase activity in cell extracts was measured using a luciferase assay kit in a GloMax 20/20 single-tube luminometer.

2.12. Cell cytotoxicity analysis

Bel7402 cells were seeded at a cell density of 5×10^3 cells/well in 96-well plates and incubated for 24 h. The cells were treated with the indicated complexes of cationic liposome/pCMV-Luc, cationic liposome/control shRNA/DOX or cationic liposome/Trx1 shRNA/DOX, respectively. After 24 h incubation, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) solution was added to the cells in each

well. Plates were incubated for an additional 4 h at 37 °C. The medium containing MTT was removed and 150 μl DMSO was added to dissolve the formazan crystals formed by living cells. Absorbance was measured at 490 nm using a 318c Eliasa microplate reader (Shanghai Sanco Instrument co., Ltd, Shanghai, China). Results were expressed as the percentage of viability with respect to control cells in which no complexes were added.

2.13. *In vitro* apoptosis detection

Bel7402 cells were seeded at a density of 3×10^5 cells/well in 6-well plates and incubated for 24 h. The cells were treated with FA-L16-2-16/control shRNA/DOX or FA-L16-2-16/Trx1 shRNA/DOX complex at a final DOX concentration of 350 nM for 24 h, respectively. Then the cells were collected and washed with PBS. After fixation with 70 % ethanol, cells were washed twice with PBS and stained with a solution containing 50 μg/ml propidium iodide (PI) and 20 μg/ml RNase A. Cells were incubated for 30 min at room temperature and the proportions of apoptotic cells in the sub-G1 phase were determined by flow cytometry (FC500 model, Beckman Coulter, Fullerton, CA, USA).

2.14. Intracellular uptake and distribution of DOX

Bel7402 cells were seeded at a density of 3×10^5 cells/well in 6-well plates and incubated overnight. The cells were treated with FA-L16-2-16/control shRNA/DOX or FA-L16-2-16/Trx1 shRNA/DOX complex at a final DOX concentration of 350 nM for 24 h, respectively. For qualitative analysis of the intracellular uptake and the distribution of DOX, the culture media were removed and the cells were washed with PBS twice. Then the cells were fixed with 4 % polyformaldehyde for 30 min at room temperature and the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The images were acquired using an Andor Revolution spinnig disk confocal microscope (Andor Technology, Belfast, UK). For quantitative analysis of the intracellular uptake of DOX, the treated cells were washed, harvested and subjected to flow cytometry.

2.15. Statistical Analysis

Experiments were carried out with three or four replicates. Statistical analyses were performed by Student's t test. Values with $P<0.05$ are considered significant.

3. Results and discussion

3.1. Transfection efficiency and cytotoxicity of gemini surfactants-based cationic liposomes

The construction of carriers having high gene transfection efficiency and low cytotoxicity is necessary for the design of a drug and gene co-delivery system. Four formulations of cationic liposomes containing gemini surfactants differing in the length and symmetrical degree of aliphatic chains in combination with neutral helper lipids, soybean lecithin and DOPE were prepared (Fig. 1A) and the structure-activity relationship was determined in HCC Bel7402 cells using a luciferase reporter gene. As shown in Fig. 1B, at the same N/P ratios, cationic liposome containing gemini surfactant with symmetrical C16 aliphatic chains (L16-2-16) showed the highest transfection efficiency, followed by liposomes containing gemini surfactant with symmetrical C14 aliphatic chains (L14-2-14), unsymmetrical C14 aliphatic chains (L14-2-6, L14-2-2), respectively, suggesting that the transfection efficiency of cationic liposomes might be dependent upon the length and symmetrical degree of aliphatic chains of gemini surfactants. The transfection effeciency of L16-2-16 was the highest at N/P ratio of 2. Furthermore, the effects of transfection with different cationic liposome/DNA complexes on the viability of Bel7402 cells were determined by MTT assay. As shown in Fig. 1C, all the liposome/DNA complexes caused relatively low toxicity at N/P ratio below 2. However, the cytotoxicity induced by the complexes increased with the increase of N/P ratios. Consequently, we used L16-2-16 at N/P ratio of 2 in the gene transfection and drug delivery studies.

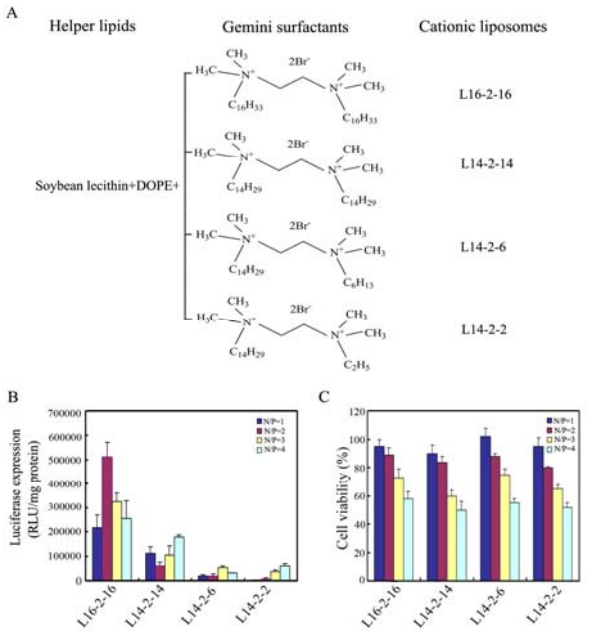

Fig. 1 The transfection efficiency and cytotoxicity of gemini surfactants-based cationic liposomes. (A) The formulations of cationic liposomes containing soybean lecithin, DOPE and gemini surfactants with different lengths and symmetrical degrees of aliphatic chains. (B) The luciferase activities in Bel7402 cells transfected with pCMV-Luc using different formulations of cationic liposomes at different N/P ratios. (C) Cell viabilities of Bel7402 cells transfected with pCMV-Luc using different formulations of cationic liposomes at different N/P ratios. Data as mean values± S.D. (n = 3).

3.2. The condensation efficiency of DNA by gemini surfactants-based cationic liposomes

DNA condensation is a necessary prerequisite for gene delivery and expression. To investigate the mechanisms of the difference in transfection efficiency, several methods were used to determine the ability of different formulations of cationic liposomes to condense plasmid DNA. Firstly, size and zeta potential measurements were performed to study DNA condensation by cationic liposomes. As shown in Fig. 2A and 2B, the size and zeta potential of different cationic liposome/DNA complexes were dependent on the given N/P ratios. Following addition of cationic liposomes, the size of the complexes decreased and the zeta potential increased. However, cationic liposome L16-2-16

showed the strongest capacity to decrease the size and increase the zeta potential of the complexes, followed by L14-2-14, L14-2-6 and L14-2-2, respectively. Here we noticed that the size and zeta potential of L16-2-16/DNA complexes nearly came to the plateau at N/P ratio of 2. The strong DNA condensation capability at N/P ratio of 2 and relatively high cytotoxicity at N/P ratio above 2 might explain the highest transfection efficiency of L16-2-16 at N/P ratio of 2.

CD spectroscopy has been widely used to study the conformation of DNA. As shown in Fig. 2C, the spectrum of naked DNA exhibited a characteristic B-type conformation with a positive signal near 270 nm and a negative signal near 240 nm, which is consistent with the literature.[32] A significant change in DNA conformation was observed when DNA was complexed with different formulations of cationic liposomes. The signal reduction at 270 nm and the increase at 240 nm of DNA complexed by L16-2-16 was the most obvious, followed by L14-2-14, L14-2-6 and L14-2-2, respectively.

The ability of cationic liposomes to interact with DNA was further assessed by EB displacement and exclusion assays. EB, a probe for DNA, displays a dramatic increase in fluorescence intensity when it intercalates into DNA. EB fluorescence is quenched by the addition of small molecules that compete with EB for DNA intercalation.[33] The ability of different formulations of cationic liposomes to displace or exclude EB from DNA was shown in Fig. 2D and 2E. As the concentrations of cationic liposomes increased, the decrease in the fluorescence intensity of EB was observed in all cases. However L16-2-16 was the most efficient to quench the fluorescence, followed by L14-2-14, L14-2-6 and L14-2-2, respectively.

It had been reported that the subtle changes in formulations, such as the configuration of a stereogenic center on the polar head group or the length of spacer of gemini surfactants affected the DNA condensation and the biological activity.[32,34,35] From the above data, it could be seen that the length and symmetrical degree of aliphatic chains of gemini surfactants incorporated in cationic liposomes influenced DNA condensation, which might result in different gene transfection efficiency.

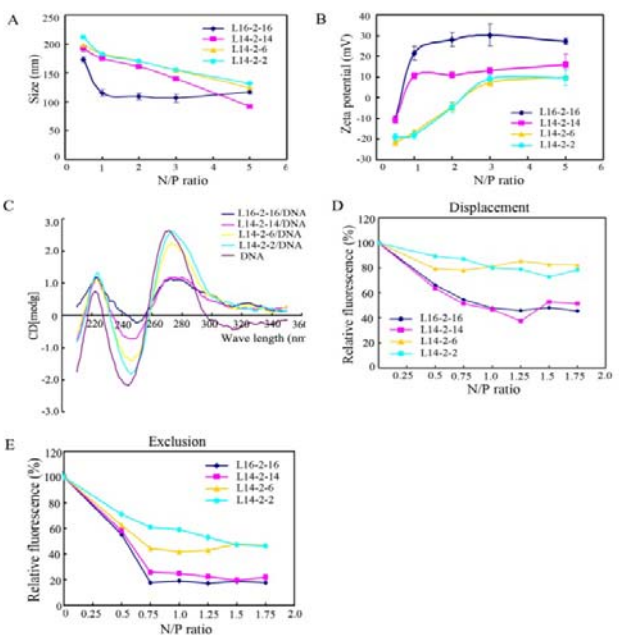

Fig. 2 DNA condensation efficiencies by gemini surfactants-based cationic liposomes. (A) The sizes of different cationic liposome/DNA complexes at different N/P ratios. (B) The zeta potential of different cationic liposome/DNA complexes at different N/P ratios. (C) CD spectra of different cationic liposome/DNA complexes at N/P ratio of 2. (D, E) EB displacement and exclusion assays of different cationic liposome/DNA complexes at different N/P ratios.

3.3. Cellular uptake of gemini surfactants-based cationic liposomes

Crossing cell membrane barrier must be overcome to achieve efficient gene delivery systems. To further explore the mechanism on the difference in transfection efficiency, different formulations of FITC-labeled cationic liposome/DNA complexes were incubated with Bel7402 cells and then the cellular uptake was determined by confocal microscopy and flow cytometry. As shown in Fig. 3A, the internalized cationic liposome L16-2-16 showed the strongest fluorescence in Bel7402 cells by confocal microscopy. The order of the internalized cationic liposomes in Bel7402 cells was L16-2-16>L14-2-14>L14-2-6≈L14-2-2. The quantitative results obtained from flow cytometry (Fig. 3B) were consistent with the qualitative results from confocal microscopy studies. It was reported that following binding to the cells, cationic liposome/DNA complexes might be internalized through endocytosis or fusion with the cell membrane.[36] The length and symmetrical degree of aliphatic chains of gemini surfactants affected the hydrophilic/hydrophobic properties of cationic liposomes, which might influence the interaction between cationic liposomes and cell membrane, resulting in different cellular uptake and gene transfection efficiency.

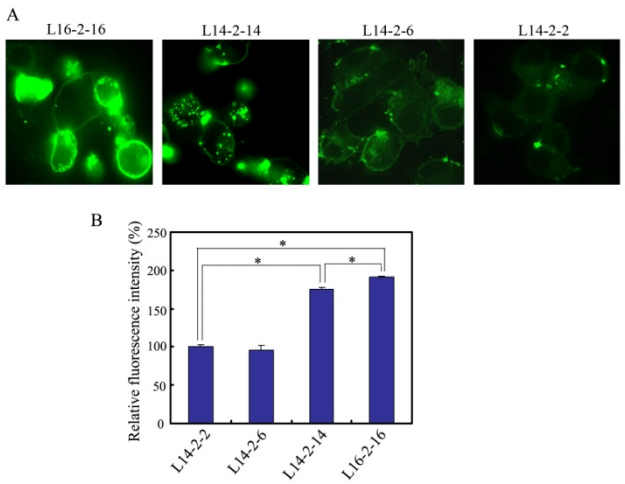

Fig. 3 Cellular uptake of gemini surfactants-based cationic liposomes in Bel7402 cells. (A) Confocal microscopy images of the cellular uptake of gemini surfactants-based cationic liposomes in Bel7402 cells after treatment with FITC-labeled cationic liposome/pCMV-Luc complexes for 4 h. (B) The quantitative analysis of cellular uptake of gemini surfactants-based cationic liposomes in Bel7402 cells after treatment with FITC-labeled cationic liposome/pCMV-Luc complexes for 4 by flow cytometry.

3.4. FA-targeted cationic liposome L16-2-16 for gene delivery

It is well known that FR is frequently overexpressed in many

types of tumors including ovarian, kidney and liver cancers, and FA binds to FR with high affinity on the cancer cell surfaces.[37] To further increase the transfection efficiency and tumor-targeting of L16-2-16, FA-targeted L16-2-16 (FA-L16-2-16) was constructed with different amounts of FA-PEG-DOPE incorporation and the transfection activity was determined using EGFP or luciferase as a reporter gene in FR-overexpressing Bel7402 cells,[38] FR-negative HepG2 cells,[39] and normal liver HL7702 cells. As shown in Fig. S1, FA-L16-2-16 had much higher transfection activity compared with L16-2-16 using an EGFP reporter gene in Bel7402 cells, with a plateau at 2 % FA-PEG-DOPE incorporation. The increase in transfection activity mediated by FA-targeted L16-2-16 in Bel7402 cells was confirmed using a luciferase reporter gene (Fig. 4A). In contrast, there was no significant difference between L16-2-16- and FA-L16-2-16-mediated transfection in HepG2 cells and HL7702 cells (Fig. S2). Furthermore, the effects of knocking down Trx1 was determined by western blotting when Bel7402 cells were transfected with Trx1 shRNA using L16-2-16, FA-L16-2-16 and Lipofectamine 2000, a commercial transfection reagent. As shown in Fig. 4B, the protein expression of Trx1 in Trx1 shRNA-transfected Bel7402 cells using L16-2-16 did not change appreciably compared with that in control shRNA-transfected cells. However, Trx1 protein expression significantly decreased when Bel7402 cells were transfected with Trx1 shRNA using FA-L16-2-16 compared with that in control shRNA-transfected cells. Importantly, the effects of knocking down Trx1 in Bel7402 cells transfected with Trx1 shRNA using FA-L16-2-16 were comparable with those using Lipofectamine 2000. These data suggested that FA-L16-2-16-mediated transfection was facilitated by binding to FR. To verify the role of FR in the transfection activity of FA-L16-2-16, a FA competition experiment was performed using a luciferase reporter gene in the presence of 1 mM FA in Bel7402 cells. Consistent with the data in Fig. 4A, L16-2-16-mediated transfection activity was significantly enhanced using FA-targeted techniques in Bel7402 cells. However, free FA significantly reduced the transfection activity of FA-L16-2-16 (Fig. 4C), which strongly confirmed that FA-L16-2-16-mediated gene transfection was regulated by FR.

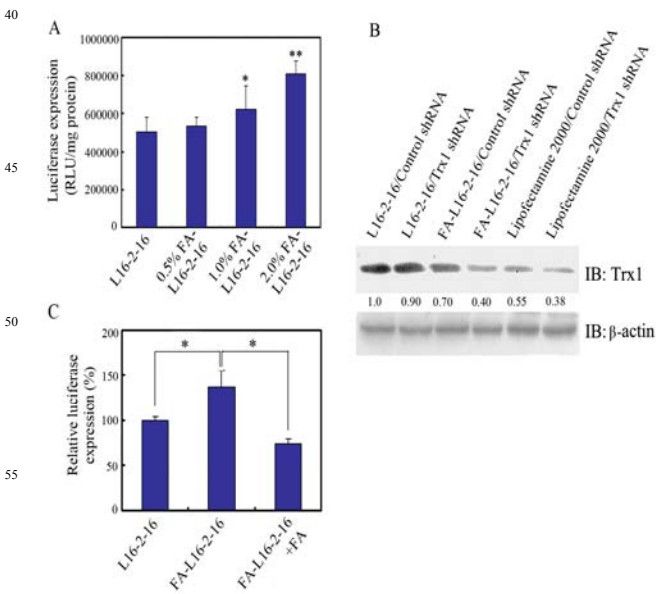

Fig. 4 FA-targeted L16-2-16 for gene transfection. (A) The transfection efficiency of FA-L16-2-16 with different amounts of FA-PEG-DOPE incorporation in Bel7402 cells using a luciferase reporter gene. Data as mean values± S.D. (n = 3). * P<0.05, ** P<0.01 compared with group transfected with pCMV-Luc by L16-2-16. (B) The knock-down efficiency of Trx1 when Bel7402 cells were transfected with Trx1 shRNA using L16-2-16, 2 % FA-L16-2-16 and Lipofectamine 2000, respectively. The number underneath each band in the immunoblot indicated the relative intensity of the corresponding band. (C) The transfection efficiency of 2 % FA-L16-2-16 pretreated with or without 1 mM free FA in Bel7402 cells using a luciferase reporter gene. Data as mean values± S.D. (n = 3). * P<0.05.

3.5. The endocytic pathways of L16-2-16- and FA-L16-2-16-mediated gene delivery

To gain insight into the endocytic pathways of L16-2-16- and FA-L16-2-16-mediated gene transfection, several specific endocytic inhibitors were used.[40] As shown in Fig. 5, ATP synthesis inhibitors NaN_3/2-deoxyglucose (NaN_3/DOG) almost completely inhibited the transfection efficiency of L16-2-16 and FA-L16-2-16 using a luciferase reporter gene, suggesting that both L16-2-16- and FA-L16-2-16-mediated gene transfection were an energy-dependent process. Furthermore, MβCD, a cholesterol-depleting agent to disrupt several lipid raft-mediated endocytic pathways and cytochalasin D, a potent inhibitor of actin polymerization to inhibit both caveolin and clathrin-mediated pathways as well as macropinocytosis, also significantly reduced L16-2-16- and FA-L16-2-16-mediated gene transfection. However no inhibition in L16-2-16- and FA-L16-2-16-mediated transfection was found in cells pretreated with chlorpromazine, an inhibitor to probe clathrin-mediated endocytosis. These results indicated that FA targeting did not change the endocytic pathway of L16-2-16 and lipid raft/caveolae-dependent internalization might be involved in L16-2-16- and FA-L16-2-16-mediated gene transfection.

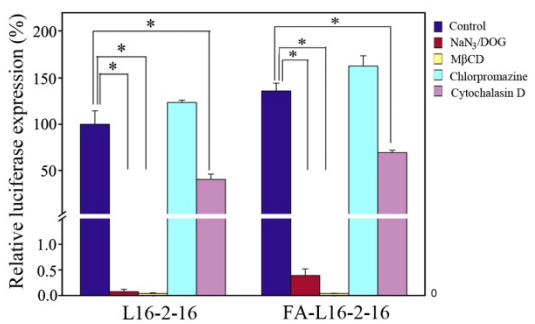

Fig. 5 The effects of endocytic inhibitors on the transfection efficiency of L16-2-16 and 2 % FA-L16-2-16 in Bel7402 cells when the cells were pretreated with the specific endocytic inhibitors followed by incubation with L16-2-16/pCMV-Luc or FA-L16-2-16/pCMV-Luc complex. * P<0.01.

3.6. Monitor of the formation of DNA/DOX complex

It is well known that DOX fluorescence is quenched after intercalation with double-stranded DNA.[31] To monitor the formation of DNA/DOX complexes, fluorescence spectroscopy was used to examine the binding of DOX to Trx1 shRNA plasmid

DNA. As shown in Fig. 6A, the spectrum of free DOX showed strong fluorescence in the range of 520-690 nm. Consecutive decreases in the fluorescence spectra of DOX were observed when a fixed concentration of DOX was incubated with an increasing concentration of Trx1 shRNA. DOX was almost totally intercalated into DNA at mass ratio of 1:9.

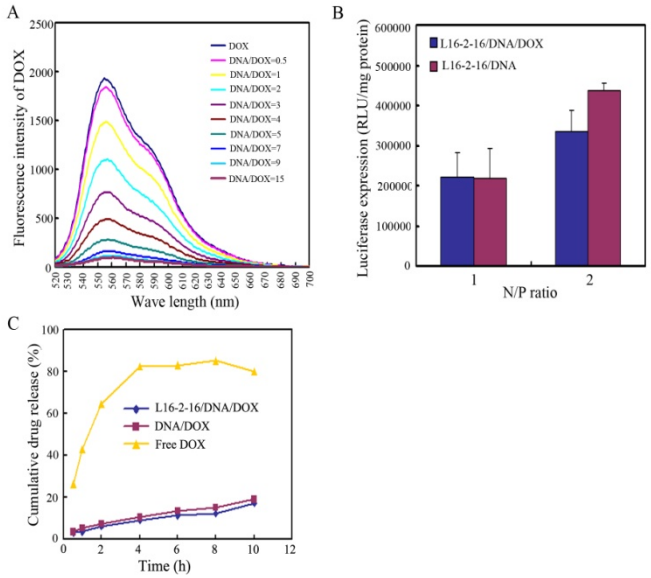

Fig. 6 Characterization of co-delivery system for gene and DOX by L16-2-16. (A) Fluorescence spectra of DOX with increasing mass ratios of plasmid Trx1 shRNA to DOX. (B) The transfection efficiency of L16-2-16/DNA and L16-2-16/DNA/DOX complexes at different N/P ratios using a luciferase reporter gene. (C) *In vitro* drug release profiles of DOX from free DOX, DNA/DOX and L16-2-16/DNA/DOX complexes at the DNA/DOX mass ratio of 9 and the N/P ratio of 2 in PBS solution (pH 7.4) at 37 ℃. Data as mean values± S.D. (n = 3).

3.7. Characterization of cationic liposome/DNA/DOX co-delivery systems

The co-delivery systems for DOX and DNA were constructed by combining cationic liposome L16-2-16 or FA-L16-2-16 with DNA/DOX complex at N/P ratio of 2 and the DNA/DOX mass ratio of 9. As shown in Table 1, co-delivery of DOX and DNA by L16-2-16 or FA-L16-2-16 slightly increased the hydrodynamic diameter of L16-2-16/DNA or FA-L16-2-16/DNA complex. FA-PEG-DOPE incorporation further slightly increased the hydrodynamic diameters of cationic liposomes or the cationic liposome/DNA complexes. DOX intercalation into DNA decreased the zeta potential of liposome/DNA complexes. TEM imaging showed that the complexes of L16-2-16/DNA/DOX and FA-L16-2-16/DNA/DOX were monodisperse and spherical (Fig. S3).

To investigate whether DOX intercalated into DNA affected the gene delivery, *in vitro* transfection efficiency of L16-2-16/DNA/DOX complex was assessed using pCMV-Luc as a reporter gene in Bel7402 cells. The analyses of luciferase activities showed that L16-2-16/pCMV-Luc/DOX complex still exhibited high gene transfection efficiency, just slightly lower than that mediated by L16-2-16/pCMV-Luc (Fig. 6B). Furthermore, the *in vitro* DOX release behaviors from free DOX, pCMV-Luc/DOX and L16-2-16/pCMV-Luc/DOX complexes were investigated. As shown in Fig. 6C, the release profile of DNA/DOX and L16-2-16/DNA/DOX complexes was similar and both showed a sustained slow release over a prolonged time. Nearly 20 % of DOX was released from pCMV-Luc/DOX and L16-2-16/pCMV-Luc/DOX complexes during 10 h. However, free DOX showed a rapid release and nearly 80 % of free DOX was released during 10 h.

Table 1 Mean diameter and zeta potential of liposomes

Liposomes	Diameters (nm)	Zeta potential (mV)
L16-2-16	121.5±3.4	47.1±8.4
FA-L16-2-16	133.8±3.2	39.8±1.8
L16-2-16/DNA	123.6±0.67	18.4±0.13
FA-L16-2-16/DNA	138.1±1.4	28.3±1.9
L16-2-16/DNA/DOX	136.3±1.0	9.81±0.73
FA-L16-2-6/DNA/DOX	148.0±1.0	5.29±0.68

3.8. *In vitro* cytotoxicity and cell apoptosis assay

To study whether the co-delivery system carrying Trx1 shRNA and DOX achieved a synergistic antitumor effect, the antiproliferative activities of various kinds of cationic liposome/Trx1 shRNA/DOX and cationic liposome/control shRNA/DOX complexes were evaluated by MTT assay. As shown in Fig. 7A, the cytotoxic effects of L16-2-16/control shRNA/DOX and L16-2-16/Trx1 shRNA/DOX against Bel7402 cells were similar at the same concentrations of DOX. However, FA-L16-2-16/Trx1 shRNA/DOX complex resulted in a significant reduction in cell viability compared with FA-L16-2-16/control shRNA/DOX complex, suggesting that FA-targeted co-delivery of DOX and Trx1 shRNA significantly increased the treatment efficiency against Bel7402 cells. In the meanwhile, the cell viability induced by FA-L16-2-16/Trx1 shRNA/DOX complex was found to be about 20 % less than that induced by FA-L16-2-16/control shRNA/DOX complex at the indicated DOX concentration, which is superior to the reduction in DOX-induced cell viability between Trx1 shRNA-transfected cells and control shRNA-transfected cells by Lipofectamine 2000 (the difference in cell viability between Trx1 shRNA–transfected cells and control shRNA-transfected cells by Lipofectamine 2000 is less than 20 % at the indicated DOX concentration). Here we noticed that DOX treatment after transfection with control shRNA by Lipofectamine 2000 in Bel7402 cells showed stronger cell viability inhibition than L16-2-16/control shRNA/DOX and FA-L16-2-16/control shRNA/DOX complexes at the same DOX concentrations (Fig. 7A). The reason might be due to the difference in cytotoxicities of these carriers themselves, which was shown that Lipofectamine 2000 had much higher cytotoxic effects than L16-2-16 and FA-L16-2-16 (Fig. S4). Considering that FA-L16-16 was specifically targeted to FR-overexpressing HCC cells with low cytotoxicy, FA-L16-2-16 might be a more suitable carrier for drug and gene co-delivery in HCC therapy.

To further confirm the advantages of co-delivery of Trx1 shRNA and DOX by FA-L16-2-16, the percentages of cell apoptosis treated with FA-L16-2-16/control shRNA/DOX and

FA-L16-2-16/Trx1 shRNA/DOX were determined by flow cytometry. Consistent with MTT results, more apoptosis was observed in FA-L16-2-16/Trx1 shRNA/DOX-treated cells compared with FA-L16-2-16/control shRNA/DOX-treated cells (Fig. 7B).

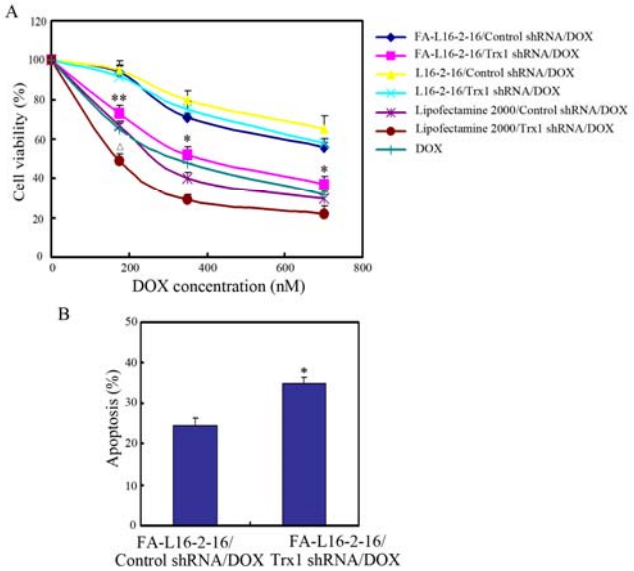

Fig. 7 The antitumor effects of co-delivering Trx1 shRNA and DOX by FA-L16-2-16 in Bel7402 cells. (A) Cell viabilities of Bel7402 cells when the cells were treated with L16-2-16/control shRNA/DOX, L16-2-16/Trx1 shRNA/DOX, 2% FA-L16-2-16/control shRNA/DOX or 2 % FA-L16-2-16/Trx1 shRNA/DOX complex at the DNA/DOX mass ratio of 9 and the N/P ratio of 2 for 24 h, respectively, or the cells were transfected with Lipofectamine 2000 and then treated with DOX for 24 h. Data as mean values± S.D. (n = 3). * P<0.05, ** P<0.01 compared with 2 % FA-L16-2-16/control shRNA/DOX-treated group. △ P<0.05 compared with Lipofectamine 2000/control shRNA/DOX-treated group. (B) Cell apoptosis induced by 2 % FA-L16-2-16/control shRNA/DOX or 2 % FA-L16-2-16/Trx1 shRNA/DOX complex in Bel7402 cells at the final DOX concentration of 350 nM for 24 h. Data as mean values± S.D. (n = 3).* P<0.05 compared with 2 % FA-L16-2-16/control shRNA/DOX-treated group.

3.9. Cellular uptake and intracellular distribution of DOX

To investigate the cooperative mechanisms of co-delivery of DOX and Trx1 shRNA fused with EGFP by FA-L16-2-16, the co-delivery efficiency was firstly determined using confocal microscopy. As shown in Fig. 8A, EGFP expression (green channel) and cellular uptake of DOX (red channel) were clearly observed in Bel7402 cells when treated with FA-L16-2-16/control shRNA/DOX and FA-L16-2-16/Trx1 shRNA/DOX complexes for 24 h. The results showed that the complexes of FA-L16-2-16/control shRNA/DOX and FA-L16-2-16/Trx1 shRNA/DOX were internalized into the cells followed by intracellular DOX release from the complexes (red channel), considering that DOX fluorescence was almost completely quenched when DOX was intercalated into DNA at mass ratio of 9 (Fig. 6A). Concurrently, EGFP-fused Trx1 shRNA and control shRNA (green channel) were expressed in cells when cells were treated with these complexes. These results strongly demonstrated that intracellular drug release and gene transfection could be simultaneously achieved in the same cell using FA-L16-2-16/DNA/DOX complexes.

Cellular uptake and intracellular distribution of DOX may play a key role in its antitumor activity. As shown in Fig. 8A, DOX delivered by FA-L16-2-16/Trx1 shRNA/DOX complex was mostly localized in the nucleus of the cells where it induced cytotoxicity, while DOX released from FA-L16-2-16/control shRNA/DOX complex was mainly found in the cytoplasma. Importantly, FA-L16-2-16/Trx1 shRNA/DOX-treated cells showed enhanced intracellular DOX fluorescence compared with FA-L16-2-16/control shRNA/DOX-treated cells. The intracellular DOX fluorescence was further quantified by flow cytometry. As shown in Fig. 8B, the mean fluorescence intensity of DOX in Bel7402 cells treated with FA-L16-2-16/Trx1 shRNA/DOX complex was nearly 2 times higher than that in cells treated with FA-L16-2-16/control shRNA/DOX complex, which confirmed the qualitative results from confocal microscopy studies.

It had been shown that the chemoresistance effects of Trx1 were modulated by several key genes that were known to play a role in drug resistance. For example, Chen *et al*. reported that Trx1 suppressed cisplatin-mediated apoptosis of MCF-7 cells by upregulating p53 expression.[41] Li *et al*. found that knocking down Trx1 using siRNA downregulated the expressions of drug resistance genes, including ABCC1 which is a member of the superfamily of ATP-binding cassette (ABC) transporters, antiapoptotic gene bcl-2 and drug metabolism gene GSTpi.[18] In this study, the synergistic antitumor effects of the FA-L16-2-16/Trx1 shRNA/DOX complex might be due to the decrease of ABC transporter activity by knocking down Trx1, which explained the increase of DOX uptake in FA-L16-2-16/Trx1 shRNA/DOX-treated Bel7402 cells. Detailed cooperative antitumor mechanisms of co-delivering Trx1 shRNA and DOX by FA-L16-2-16 need to be further elucidated.

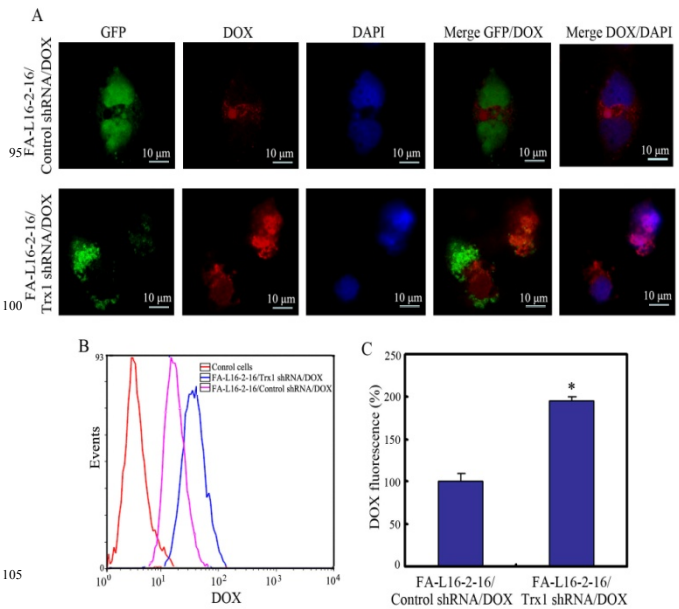

Fig. 8 The cellular uptake and intracellular localization of DOX when Bel7402 cells were treated with 2 % FA-L16-2-16/control shRNA/DOX or 2 % FA-L16-2-16/Trx1 shRNA/DOX complex. (A) Confocal microscopy images of Bel7402 cells treated with

2 % FA-L16-2-16/control shRNA/DOX or 2 % FA-L16-2-16/Trx1 shRNA/DOX complex at the DNA/DOX mass ratio of 9 and the N/P ratio of 2 (the final DOX concentration was 350 nM) for 24 h. (B) Fluorescence intensity of DOX in Bel7402 cells treated with 2 % FA-L16-2-16/control shRNA/DOX or 2 % FA-L16-2-16/Trx1 shRNA/DOX complex for 24 h at the final DOX concentration of 350 nM by flow cytometry. (C) The quantified values of DOX fluorescence intensity were given as percent with respect with 2 % FA-L16-2-16/control shRNA/DOX-treated group. Data as mean values± S.D. (n = 3). * P<0.01 compared with 2 % FA-L16-2-16/control shRNA/DOX-treated group.

Conclusions

In this study, cationic liposomes containing soybean lecithin, DOPE and gemini surfactants bearing different lengths and symmetrical degrees of aliphatic chains were constructed. The length and symmetrical degrees of aliphatic chains of gemini surfactants affected the gene transfection efficiency of cationic liposomes due to different DNA condensation capacity and cellular uptake. FA targeting further increased the transfection efficiency of cationic liposome L16-2-16 in FR-overexpressing Bel7402 cells. Co-delivery of Trx1 shRNA and DOX by FA-L16-2-16 could effectively mediate gene transfection and drug release in the same cell and furthermore increase the intracellular and intranuclear concentrations of DOX, which synergistically inhibited the cell viability and induced apoptosis in Bel7402 cells. This strategy could provide a promising approach for the increase of therapeutic efficacy in HCC treatments.

Acknowledgements

We thank Dr. Haojie Huang for the plasmids and Dr. Tao Xu's group for the help with confocal microscopy. We also thank the Analytical and Testing Center of Huazhong University of Science and Technology for related analysis. This work was supported by National Basic Research Program of China (973 Programs, 2012CB932500 and 2011CB933100), the National Natural Science Foundation of China (31070689 and 81372400) and the Fundamental Research Funds for the Center Universities (HUST: 2012TS013).

References

1 J. Bruix, L. Boix, M. Sala and J. M. Llovet, *Cancer Cell*, 2004, **5**, 215–219.
2 A. Forner, J. M. Llovet and J. Bruix, *Lancet*, 2012, **379**, 1245–1255.
3 M. Beaugrand, G. N'Kontchou, O. Seror, N. Ganne and J. C.Trinchet, *Semin. Liver Dis.*, 2005, **25**, 201–211.
4 S. H. Kerr and D. J. Kerr, *Cancer Lett.*, 2009, **286**, 114–120.
5 C. W. Chua and S. P. Choo, *Int. J. Hepatol*., 2011, **2011**, 348297.
6 R. Li, R. Hehlman, R. Sachs and P. Duesberg, *Cancer Genet. Cytogenet*., 2003, **163**, 44−56.
7 P. Duesberg, R. Li, R. Sachs, A. Fabarius, M. Upender and R. Hehlmann, *Drug Resist Updates*, 2003, **10**, 51−58.
8 S. Liu, Y. Guo, R. Huang, J. Li, S. Huang, Y. Kuang, L. Han and C. Jiang, *Biomaterials*, 2012, **33**, 4907−4916.
9 N. Wiradharma, Y. W. Tong and Y. Y. Yang, *Biomaterials*, 2009, **30**, 3100−3109.
10 M. K. Ahsan, I. Lekli, D. Ray, J. Yodoi and D. K. Das, *Antioxid. Redox. Signal*., 2009, **11**, 2741−2758.
11 A. Baker, C. M. Payne, M. M. Briehl and G. Powis, *Cancer Res*., 1997, **57**, 5162–5167.
12 M. Berggren, A. Gallegos, J. R. Gasdaska, P. Y. Gasdaska, J. Warneke and G. Powis, *Anticancer Res*., 1996, **16**, 3459–3466.
13 H. Nakamura, J. Bai, Y. Nishinaka, S. Ueda, T. Sasada, G. Ohshio, M. Imamura, A. Takabayashi, Y. Yamaoka and J. Yodoi. *Cancer Detect Prevent*., 2000, **24**, 53–60.
14 T. M. Grogan, C. Fenoglio-Prieser, R. Zeheb, W. Bellamy, Y. Frutiger, E. Vela, G. Stemmerman, J. Macdonald, L. Richter, A. Gallegos and G. Powis, *Hum. Pathol*., 2000, **31**, 475–481.
15 N. Kawahara, T. Tanaka, A. Yokomizo, H. Nanri, M. Ono, M. Wada, K. Kohno, K. Takenaka, K. Sugimachi and M. Kuwano, *Cancer Res*., 1996, **56**, 5330–5333.
16 S. J. Kim, Y. Miyoshi, T. Taguchi, Y. Tamaki, H. Nakamura, J. Yodoi, K. Kato and S. Noguchi, *Clin. Cancer Res*., 2005, **11**, 8425–8430.
17 G. Powis and D. L. Kirkpatrick, *Curr. Opin. Pharmacol*., 2007, **7**, 392–397.
18 C. Li, M. A. Thompson, A. T. Tamayo, Z. Zuo, J. Lee, F. Vega, R. J. Ford and L. V. Pham, *Oncotarget*, 2012, **3**, 314–326.
19 N. Smyth Templeton, *Curr. Med. Chem*., 2003, **10**, 1279–1287.
20 W. C. Tseng, F. R. Haselton and T. D. Giorgio, *J. Biol. Chem*., 1997, **272**, 25641–25647.
21 F. M. Menger and C. A. Littau, *J. Am. Chem. Soc*., 1991, **113**, 1451–1452.
22 P. Yang, J. Singh, S. Wettig, M. Foldvari, R. E. Verrall and I. Badea, *Eur. J. Pharm. Biopharm.*, 2010, **75**, 311–320.
23 S. D. Wettig, R. E. Verrall and M. Foldvari, *Curr. Gene. Ther*., 2008, **8**, 9–23.
24 A. J. Kirby, P. Camilleri, J. B. F. N. Engberts, M. C. Feiters, R. J. M. Nolte, O. Soderman, M. Bergsma, P. C. Bell, M. L. Fielden, C. L. García Rodríguez, P. Guédat, A. Kremer, C. McGregor, C. Perrin, G. Ronsin and M. C. van Eijk, *Angew. Chem. Int. Ed*., 2003, **42**, 1448–1457.
25 P. C. Bell, M. Bergsma, I. P. Dolbnya, W. Bras, M. C. A. Stuart, A. E. Rowan, M. C. Feiters and J. B. Engberts, *J. Am. Chem. Soc*., 2003, **125**, 1551–1558.
26 A. M. Rahman, S. W. Yusuf and M. S. Ewer, *Int. J. Nanomedicine*, 2007, **2**, 567–583.
27 S. M. Dehm, K. M. Regan, L. J. Schmidt and D. J. Tindall, *Cancer Res*., 2007**, 67**, 10067-10077.
28 R. Liu, L. Gan, X. Yang and H. Xu, *J. Biosci. Bioeng*., 2011, **111**, 98-103.
29 K. Murase, T. Fujiwara, Y. Umemura, K. Suzuki, R. Iino, H. Yamashita, M. Saito, H. Murakoshi, K. Ritchie and A. Kusumi, *Biophys. J*., 2004, **86**, 4075–4093.
30 R. J. Lee and P. S. Low, *Biochim. Biophys. Acta*., 1995, **1233**, 134–144.
31 V. Bagalkot, O. C. Farokhzad, R. Langer and S. Jon, *Angew. Chem. Int. Ed*., 2006, **45**, 1–5.
32 C. Bombelli, F. Faggioli, P. Luciani, G. Mancini and M. G. Sacco, *J. Med. Chem*., 2005, **48**, 5378–5382.
33 B. C. Baguley and M. Le Bret, *Biochemistry*, 1984, **23**, 937–943.
34 C. Bombelli, S. Borocci, M. Diociaiuti, G. Faggioli, L.

Galantini, P. Luciani, G. Mancini and M. G. Sacco, *Langmuir*, 2005, **21**, 10271–10274.
35 C. Bello, C. Bombelli, S. Borocci, P. di Profio and G. Mancini, *Langmuir*, 2006, **22**, 9333–9338.
36 S. W. Hui, M. Langner, Y. L. Zhao, P. Ross, E. Hurley and K. Chan, *Biophys. J.*, 1996, **71**, 590–599.
37 J. Sudimack and R. J. Lee, *Adv. Drug Deliv. Rev.*, 2000, **41**, 147–162.
38 G. B. Hong, J. X. Zhou and R. X. Yuan, *Int. J. Nanomedicine*, 2012, **7**, 2863–2872.
39 D. Hu, Z. Sheng, S. Fang, Y. Wang, D. Gao, P. Zhang, P. Gong, Y. Ma and L. Cai. *Theranostics*, 2014, 4, 142–153.
40 L. Xiao, X. Xiong, X. Sun, Y. Zhu, H. Yang, H. Chen, L. Gan, H. Xu and X. Yang, *Biomaterials*, 2011, **32**, 5148–5157.
41 X. P. Chen, S. Liu, W. X. Tang and Z. W. Chen, *Biochem. Biophys. Res. Commun.*, 2007, **361**, 362–366.

Notes

[a] *National Engineering Research Center for Nanomedicine, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China. Fax: +86 27 8779 2234; Tel:+86 27 8779 2147; E-mail: lugan@mail.hust.edu.cn*
[1] *These authors contributed equally to this paper.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

