Pharm Res (2013) 30:1215–1227
DOI 10.1007/s11095-012-0960-9

RESEARCH PAPER

Ternary Complexes with Core-Shell Bilayer for Double Level Targeted Gene Delivery: *In Vitro* and *In Vivo* Evaluation

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Received: 30 July 2012 / Accepted: 7 December 2012 / Published online: 27 December 2012

ABSTRACT

Purpose Hyaluronic acid (HA)/polyethyleneimine-dexamethasone (PEI-Dex)/DNA ternary complexes with "core-shell" bilayer were developed for double level targeted gene delivery. A PEI_{1800}-Dex, as a core, was applied to compact DNA into a nano-sized structure and facilitate the nuclear translocation of DNA after endocytosis into tumor cells, and a polyanion HA, as the outer corona, was employed to improve targeted tumor delivery and reduce cytotoxicity.

Methods PEI-Dex was synthesized and characterized by ^1H NMR. The characterizations of ternary complexes were investigated. Their biological properties, including transfection efficiency, cytotoxicity, cellular uptake and *in vivo* efficacy were evaluated systemically.

Results Ternary complexes with the size of about 160 nm exhibited the lowest cytotoxicity and the highest transfection efficiency in B16F10 cells among investigated complexes. The subcellular localization study confirmed that ternary complexes could facilitate more efficient cell uptake and nuclear transport of DNA than binary complexes. Moreover, Cy7-labeled ternary complexes obviously accumulated in the tumor after *i.v.* administration, indicating that ternary complexes could assist the DNA targeting to the tumor. In *in vivo* studies, HA/PEI_{1800}-Dex/DNA ternary complexes showed confirmed anti-inflammation activity, and could significantly suppress tumor growth of tumor-bearing nude mice.

Conclusions HA/PEI-Dex/DNA ternary complexes might be a promising targeted gene delivery system.

KEY WORDS dexamethasone · hyaluronic acid · *in vivo* efficacy · nuclear translocation · tumor targeting

INTRODUCTION

Gene therapy has been emerging as a promising method for curing a wide range of inherited and acquired genetic disorders, cancers or viral infections based on its tremendous specificity of action and enormous potential for individualized treatment (1). Recently, some cationic polymers have been exploited as promising carriers for gene delivery to improve the stability of DNA, penetrate across the cell membrane through charge attraction and escape from the endosomal degradation. In addition, they also have outstanding superiorities in terms of active chemical reactivity, and are stable in storage and easy to produce in large quantities. However, some problems still remain such as the lack of targeted delivery of polymeric delivery systems as well as higher cytotoxicity and relatively low transfection efficiency (2,3).

At present, some advances on enhancing transfection efficiency and decreasing the cytotoxicity was confirmed by coupling the targeted ligands such as hyaluronic acid (HA), folic acid and RGD to cationic polymers. Our previous studies have indicated that anionic HA-based formulation could facilitate the intracellular delivery of DNA complexes by HA

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receptor-mediated endocytosis as well as reduce the cytotoxicity (4), in agreement with other studies (5–7). However, nuclear transport turns out to be another major barrier for efficient gene therapy (8). It has been reported that only 1–5% of the applied dose of DNA is able to enter the nucleus, and the nuclear membrane presents the major hurdle to entrance (9). Some studies have described that the glucocorticoid could significantly enhance the nuclear access of polycation complexes by the glucocorticoid receptor and dilating the nuclear pore (10,11). It was also reported that dexamethasone (Dex) could make more pronounced enhancement of the transfection efficiency in the group of glucocorticoid (12). Choi *et al.* developed Dex conjugated poly(amidoamine) (PAMAM) dendrimer (PAM-Dex) for efficient nuclear translocation (13). Compared to PAMAM, PAM-Dex showed approximately two-fold higher transfection efficiency in 293 cells due to Dex-mediated nuclear transport. Bae *et al.* described that Dex-conjugated polyethyleneimine (PEI) 2 kDa increased the gene expression level by 1 order of magnitude for HepG2 and at least 2 orders for 293 cells compared to unmodified PEI (14). However, *in vivo* efficacy has not been investigated so far. It is well known that *in vivo* therapeutic efficacy of the gene was particularly restrictive due to rapid plasma clearance and accumulation by reticuloendothelial system (RES) sites. Thus, it is essential to justify the *in vivo* efficacy of non-viral gene carriers in this study. In addition, immunological response following non-viral DNA delivery decreases the magnitude and duration of transgene expression. Dex, as an anti-inflammatory drug, is expected to display pharmacological activity to reduce the potential inflammation response after administration of the complexes.

In this study, we synthesized a low molecular weight PEI (1800 Da) derivative (PEI_{1800}-Dex) using an imine reaction between Dex-21-mesylate and thiols of PEI at first. It is expected to boost the nuclear access, enhance the transfection efficiency, and also reduce the side effect by combining the anti-inflammation activity of Dex with lower cytotoxicity of low molecular weight PEI. Furthermore, considering that molecular weight of approximately 10 kDa is reported to be more efficient for HA receptor-mediated endocytosis (15), 10 kDa HA was coated on the outer layer of PEI-Dex/DNA complexes to increase the affinity of complexes to tumor cells as well as shielding excessive positive charge of PEI to further reduce the cytotoxicity. These ternary complexes integrated multiple functional devices into one complex for therapeutic gene delivery in a convenient and relatively simple way. They also realize double level targeting effect by HA-mediated targeting to tumor cells and Dex-mediated nuclear translocation. The favorable properties of Dex and HA on biophysical properties and delivery of PEI-based complexes were investigated systematically. Especially, the *in vivo* efficacy of HA/PEI_{1800}-Dex/DNA complexes and anti-inflammation activity were also evaluated. The plasmid pIRES2-EGFP-p53 (pEGFP-p53) was used as model therapy gene in this study.

MATERIALS AND METHODS

Materials

Polyethyleneimine (molecular weight = 1800 Da) was obtained from Nippon Shobukai (Japan). Dexamethasone was obtained from GuoGuang Co. Ltd (Hangzhou, China). N,N-dimethylsulfoxide (DMSO) and Traut's reagent was purchased from Sigma-Aldrich (MO, USA). Hyaluronic acid (molecular weight = 10 kDa) was obtained from Shandong Freda Biochem Co,.Ltd (Shandong, China). Goldview™ were purchased from Saibaisheng (Beijing, China). Lipofectamine™ 2000 was purchased from Invitrogen Corporation (CA, USA). RPMI 1640 and trypsin–EDTA (TE, 0.5% trypsin, 5.3 mM EDTA tetra-sodium) were obtained from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (Nanjing, China). MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Fluka (MO, USA). The plasmid pIRES2-EGFP-p53 (pEGFP-p53, ~13 kb) were amplified in DH-5α *Escherichia coli* and purified according to the manufacture's protocol with the Qiagen Plasmid Mega Kit (Qiagen, CA, USA).

Synthesis of PEI_{1800}–Dex

PEI_{1800}-Dex was synthesized as previously described with some modifications (13). Briefly, PEI 1800 Da was dissolved in 2 mL of anhydrous DMSO with a 3.8 equiv. of Dex-21-mesylate. After stirring for 30 min, 3.8 equiv. of Traut's reagent was added dropwise. The reaction was performed for 4 h under N_2 at room temperature and then quenched by the addition of an excess amount of cold ethyl acetate. The precipitated product was dried in vacuum and then solubilized in water. After being filtered by 0.8 μm micropore film, the product was dialyzed for 2 day against pure water using a dialysis membrane (MWCO 1,000). The dialysis medium was refreshed every 8 h. A pale yellow product was obtained after further lyophilized. The product was stored at 4°C until use. The degree of substitution (DS) of Dex was determined by ^1H NMR analysis (Avace AV-500, Bruker, Germany).

Preparation of HA/PEI_{1800}–Dex/DNA Complexes

The charge ratio (N/P ratio) of PEI_{1800}-Dex/DNA was expressed as the ratio of moles of amine groups of PEI_{1800}-Dex to moles of phosphate groups of DNA. The mass ratio of HA to PEI_{1800}-Dex was 10, thus the charge ratio of HA/PEI_{1800}-Dex was about 1:1. PEI_{1800}-Dex/DNA binary

complexes were induced to self-assemble in 5% glucose by mixing the DNA solution (0.1 mg/mL) with PEI_{1800}-Dex solution (0.1 mg/mL) at certain N/P ratio keeping the amount of DNA constant and standing for 30 min at room temperature before use. Ternary HA/PEI_{1800}-Dex/DNA complexes was induced to self-assemble in 5% glucose by mixing binary complexes with HA solution (1 mg/mL) keeping the mass ratio of HA to PEI_{1800}-Dex constant and standing for 30 min at room temperature before use.

Characterization of HA/PEI_{1800}–Dex/DNA Complexes

The condensation capability of PEI_{1800}-Dex with DNA was evaluated using the agarose gel electrophoresis. Binary and ternary complexes were prepared at various N/P ratios, and were electrophoresed on a 0.7% (w/v) agarose gel containing 0.05 μl/mL Goldview™ in TBE buffer. After electrophoresis, the gel was visualized under a UV illuminator to show the location of the DNA. In addition, as to the UV analysis, the complexes solutions were centrifuged at 3,000 rpm for 2 min, free DNA in supernatant was quantified by measuring the absorbance at 260 nm using a spectrophotometer (Purkinje Instrument, Beijing, China).

The size and zeta potential values of complexes at various N/P ratios were determined using the Malvern Zetasizer Nano ZS90 system (Malvern Instruments Ltd, U.K.). The measured value is presented as the average plus or minus a standard deviation of three assays.

Cytotoxicity Assay

MTT assay was performed to evaluate the cytotoxicity of polycations and complexes. B16F10 cells were seeded at a density of 2×10^4 cells/well in 96-well plates. Cells were treated with polymers solution or complexes for 48 h, respectively. The therapeutic gene-free plasmid (pDNA) was used in this experiment. Then 20 μL of 5 mg/mL MTT solution was added and incubated for 4 h at 37°C. The supernatant was aspirated off and 150 μL of DMSO was added. The absorbance values were measured at 570 nm using Microplate Reader (EL800, BIO-TEK Instruments Inc). The cell viability (%) was calculated as the absorbance ratio of samples compared with the control.

In Vitro Cell Transfection

The B16F10 cells were seeded at a density of 1×10^5 cells/well in six-well flat-bottomed plates and grown overnight to reach 70–80% confluence. The primary growth medium was removed and replaced with serum-free medium. Complexes were prepared at various N/P ratios, diluted with serum-free medium and added to the wells. The amount of plasmid DNA was fixed at a 4 μg/well.

After incubated for 6 h, the complexes were removed and replaced with fresh medium with FBS. The expression of GFP in B16F10 cells was observed after 48 h incubation under the fluorescent microscope (OlympusIX51, Japan) and the transfection efficiency of complexes were quantified for GFP-positive cells by flow cytometry (FACSCanto, Becton-Dickinson, USA). Transfection with Lipofectamine 2000/DNA complexes and PEI 25 kDa/DNA complexes was performed in the same manner in the same study as the control.

To inhibit the HA receptor-mediated tumor cell uptake, the cells were pretreated with HA solutions (4). Before transfection, the medium was replaced with fresh growth medium without FBS. HA solution at different concentration was added, and incubated for 1 h. After pretreatment, complexes were added to the cells. Then, cells were transfected for 6 h at 37°C. The GFP-positive cells were observed under fluorescent microscope and quantified by flow cytometry (FACSCalibur, BectoneDickinson, USA).

Confocal Microscopy Experiments

B16F10 cells were seeded at a density of 1×10^5 cells/well on a cover glass (18 mm sq) that was spread in each well of a six-well plate and incubated overnight at 37°C. FITC-labeled complexes were prepared at N/P ratio of 12. The amount of plasmid DNA was fixed at 4 μg per well. Complexes were added to the cells and incubated for 2 h, 6 h, 8 h and 12 h in 10% FBS-containing medium. After transfection, cells were washed and then fixed for 10 min with 4% paraformaldehyde at room temperature. For nucleus labeling, cells were stained with DAPI (2.9 nM) for 10 min. For lysosome labeling, cells were stained with LysoTracker (50 nM) for 30 min. Finally, cells were washed with the phosphate buffer solution (PBS) and observed using confocal laser-scanning microscopy (Leisca TCS-SP5, USA).

In Vivo Imaging Analysis

Nine BALB/c nude mice bearing HepG2 tumor were randomly divided into three groups and injected with 5% glucose solution (negative control), PEI 25 kDa-CY7/DNA complexes and HA/PEI_{1800}-Dex-CY7/DNA complexes (n=3 for each group), respectively. Images were performed 1, 2, 4, 6 h after *i.v.* injection by *In Vivo* Imaging System (FX PRO, Kodak, USA) equipped with CY7 filter sets (excitation/emission, 730/790 nm).

Anti-inflammation Activity of HA/PEI_{1800}–Dex/DNA Complexes

The anti-inflammatory effect of HA/PEI_{1800}–Dex/DNA complexes was examined in Thioglycollate (TG)-induced

mice models (16). Male, 6- to 8-week-old ICR mice, five per group were used. Mice were injected intraperitoneal (i.p.) with either PBS solution, Dex (a dose of 2 mg/kg), or an equimolar amount of ternary complexes. After 30 min, mice were injected *i.p.* with 1.5 ml of 3% TG. At 4 h post-TG, the *i.p.* fluid was collected and neutrophils were counted. All works performed with animals were conducted in compliance with the Regulations for Experiment Animals of Jiangsu Province.

Tumor Growth Inhibition Experiments

In order to investigate the influence of ternary complexes on the tumor growth, four groups of BALB/c nude mice bearing red fluorescent protein labeled HepG2 tumor were infected intratumorally with 5% glucose solution (negative control), PEI 25 kDa/DNA complexes, PEI_{1800}-Dex/DNA complexes and HA/PEI_{1800}-Dex/DNA complexes, respectively. The pEGFP-p53 gene was selected as a therapeutic gene. The dose was 10 mg pEGFP-p53 per mouse of 20 g. The initial day of administration was defined as day 0. Therapy was then continued 11 times at 2-day interval. The photos of tumor-bearing mice were taken by IFLUOR-100 *in vivo* bioluminescence imaging system (Nanjing East-Image Digital Technology Co. Ltd, Nanjing, China) and tumor volume and body weight of mice were measured every 4 days. Finally, terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay and Hematoxylin and Eosin (HE) staining assay of the isolated tumors were used to analysis the *in vivo* apoptosis and histopathology of the tumor.

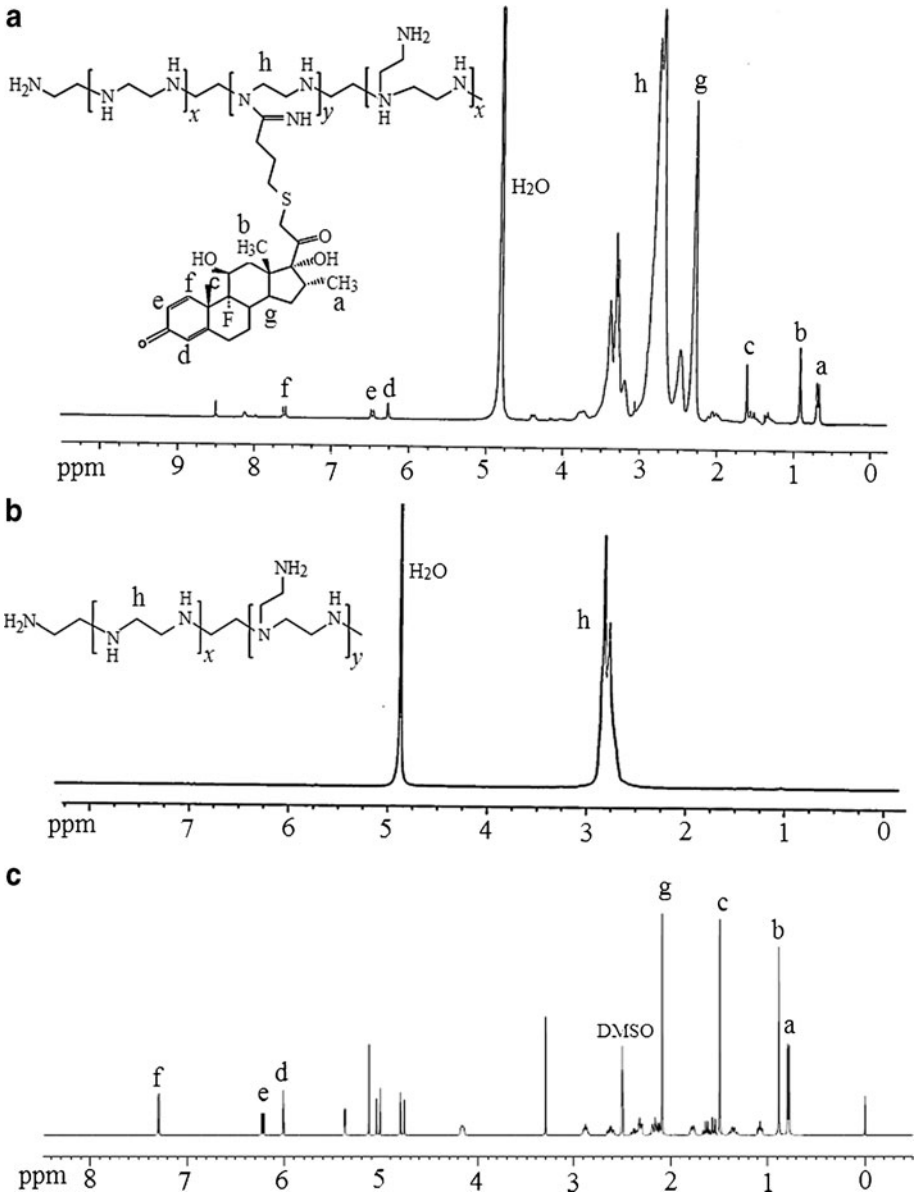

Fig. 1 ^{1}H NMR of (**a**) PEI_{1800}-Dex in D_2O, (**b**) PEI 1800 Da in D_2O and (**c**) Dex in DMSO.

Statistical Analysis

Results represent the average plus or minus a standard deviation from multiple experiments performed in a parallel manner unless otherwise indicated. Statistical analysis was performed using two-way unweighted mean analysis of variance (ANOVA) and a value of $p<0.05$ was considered significant.

RESULTS

Synthesis of PEI_{1800}-Dex

PEI_{1800}-Dex was conjugated through amidine bond between Dex-21-mesylate and thiols of PEI which were introduced by reacting of Traut's reagent (2-iminothiolane) with amines on PEI. The composition of synthesized polymer was analyzed by ^{1}H-NMR (Fig. 1). The proton peaks of PEI ($-NHCH_2CH_2-$) from the products appeared at 2.7–3.4 ppm (peak g in Fig. 1a), which was different with that from the native PEI only appeared at about 2.7 ppm (peak h in Fig. 1b). In addition, the characteristic peaks from Dex appeared at peak a (δ 0.812, d, 3H, CH_3), peak b (δ1.027, s, 3H, CH_3), peak c (δ1.47, d, 3H, CH_3), peak d (δ6.24, s, 2H, CH_2), peak e (δ6.44, d, 2H, CH_2), peak f (δ7.58, d, 2H, CH_2) and peak g (δ2.09, s, H, CH) (in Fig. 1a). The results indicated that Dex was grafted to the PEI. According to the ^{1}H NMR spectrum, the DS of Dex was 16.19%, i.e. each mole of PEI conjugated to 1.06 mol of Dex.

In addition, we also synthesized PEI-Dex by coupling PEI with O-Dex instead of Dex-21-mesylate in our previous study (17). However, the maximum DS of Dex could only reach to 10.23%, which was much lower than that of products in this study. Higher DS of Dex benefited the transfection efficiency to a certain extent. Therefore, we focused on the product synthesized in this study for further investigation.

Characterization of Ternary Complexes

The condensation capability of PEI_{1800}-Dex with DNA was evaluated using the agarose gel electrophoresis as shown in Fig. 2. The result indicated that migration of DNA was retarded completely at the N/P ratio of 4 either by PEI 1800 Da or PEI_{1800}-Dex, suggesting that there was no significant change in the retardation behavior after coupling of Dex to PEI. In addition, the migrating of DNA was completely retarded at the N/P ratio of 5 for HA/PEI_{1800}-Dex/DNA complexes, indicating that anionic HA may slightly weaken the compaction of cationic polymers and DNA as compared to PEI alone. It will facilitate the DNA release in the tumor cells while it doesn't lead to leakage of DNA from complexes before cell uptake (18). In addition, the formation of ternary complexes was also investigated by UV spectroscopy. Before complete DNA condensation, free DNA was quantified by UV spectrometer. The amount of remained DNA was less than 3% at charge ratio of 4 (for two binary complexes) and 5 (for ternary complexes) while there was no significant difference in amount of remained DNA when the charge ratio was further increased, indicating the DNA was condensed completely.

The particle sizes of complexes were presented in Fig. 3a. The sizes of the complexes decreased by an increase of the charge ratios in three groups as expected, indicating more amount of cationic polymers would contribute to compacter condensation of DNA. The HA coating resulted in the slight

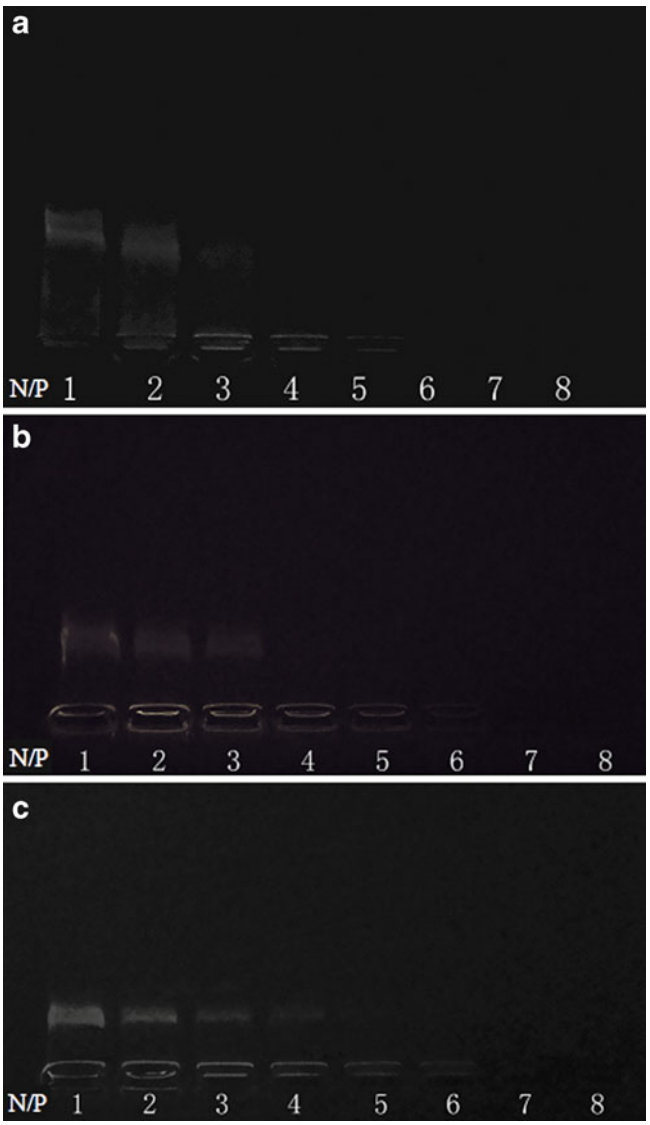

Fig. 2 Gel retardation assay of complexes at various N/P ratios (lane 1–8: N/P=1,2,3,4,5,6,7,8). (**a**) PEI 1800 Da; (**b**) PEI_{1800}-Dex/DNA binary complexes; (**c**) HA/PEI_{1800}-Dex/DNA ternary complexes.

increase of particle sizes of complexes at the same charge ratio. The sizes of HA/PEI_{1800}-Dex/DNA ternary complexes were approximately 160 nm at N/P ratio of 12, and were not significantly varied after being kept under ambient temperature for 24 h (Fig. 3c), suggesting a good physical stability. On the contrary, the size of binary complexes was greatly increased after the storage due to cationic PEI-induced aggregation. The results indicated that HA might prevent the particles aggregation and increase the stability of complexes, which is consistent with our previous studies (4). The zeta potential of complexes was also investigated. Figure 3b showed that both PEI 1800 Da/DNA and PEI_{1800}-Dex/DNA binary complexes had a cationic charge of 16–22 mV at N/P ratio of 12. Ternary complexes showed anionic charges and dramatically lower zeta potential than binary complexes ($p<0.01$). HA is negatively charged due to the ionization of carboxyl groups of the glucuronic acid constituents at physiological pH. Therefore, the ternary complexes had the outer corona of HA segment loops around the particles [19], which resulted in the negatively charged surface of ternary complexes. Moreover, the density of HA molecular on the surface of PEI-Dex/DNA core was increased as the N/P ratio increased, which contributed to the gradually decreased zeta potential. It has been reported that positive charge-shielding would reduce the nonspecific interaction and avoid the aggregation among the complexes (5,19), which contributes to the enhanced gene transfection.

In addition, ternary complexes had high protection of DNA from nuclease at charge ratio of 12 by nuclease resistance assay (data not shown).

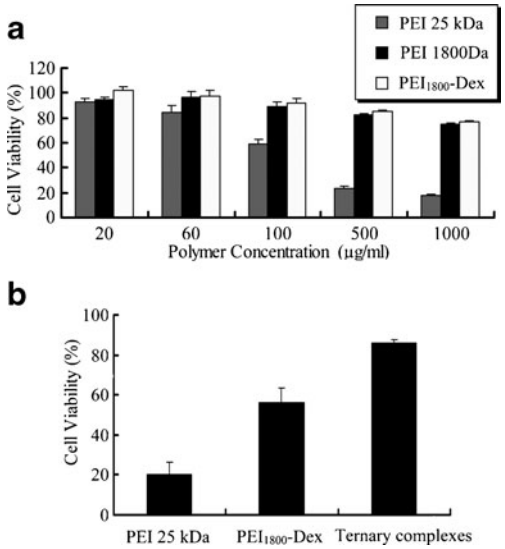

Fig. 4 Cytotoxicity assay of PEI 25 kDa, PEI 1800 Da and PEI_{1800}-Dex and their complexes in B16F10 cells. (**a**) Cells were treated with cationic polymers at various concentrations. (**b**) Cells were treated with PEI 25 kDa/DNA, PEI_{1800}-Dex/DNA and HA/PEI_{1800}-Dex/DNA ternary complexes at N/P ration of 12. Cell viability was calculated assuming the absorbance at 570 nm of intact control cells to be 100%. The data were expressed as mean±S.D. ($n=3$).

Cytotoxicity Assay

The cytotoxicity of polymers and complexes were examined by MTT assay in B16F10 cells. As shown in Fig. 4a, no significant cytotoxicity was found among the PEI_{1800}-Dex especially at<500 μg/mL, which was similar to the

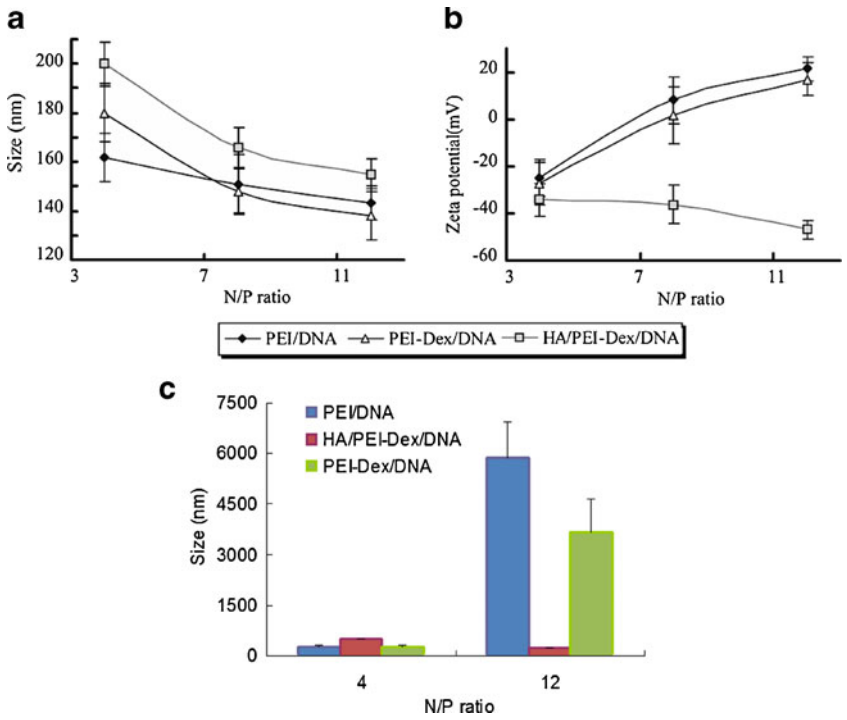

Fig. 3 (**a**) Average particle size and (**b**) zeta potential of PEI_{1800}/DNA binary complexes, PEI_{1800}-Dex/DNA binary complexes and HA/PEI_{1800}-Dex/DNA ternary complexes with N/P ratio of 4, 8 and 12. (**c**) Average particle size of the complexes with N/P ratio of 4 and 12 after 24 h. ($n=3$, mean±S.D.).

PEI 1800 Da. On the contrary, PEI 25 kDa showed a dose-dependent cytotoxicity with increasing concentrations. The effect of complexes on the cell viability was shown in Fig. 4b. The cell viability loss after being incubated with PEI 25 kDa/DNA complexes, PEI 1800 Da/DNA complexes and HA/PEI_{1800}-Dex/DNA ternary complexes were 79.7%, 43.6% and 13.9%, respectively. The improvement of ternary complexes in cell viability could be owing to low molecular weight of PEI and anionic surface charge (20).

In addition, the study on interaction of erythrocytes also suggested much lower toxicity of HA-coated ternary complexes than PEI 25 kDa/DNA complexes and PEI_{1800}-Dex/DNA complexes, where no agglutination was observed in HA-coated ternary complexes (data not shown).

In Vitro Transfection Efficiency

Transfection efficiency of binary and ternary complexes was investigated in B16F10 cells (Fig. 5). B16F10 cells which are confirmed to over-express the HA receptors were used to evaluate the affinity of ternary complexes to tumor cells in this study. The control groups were commercial Lipofectamine™ 2000 and PEI 25 kDa which is widely used as 'gold standard' for transfection of non-viral carriers. Naked DNA was used as negative control. As shown in Fig. 5a, cells treated with PEI 1,800 Da/DNA complexes showed a little green fluorescence, which is consistent with previous studies (21).By comparison, PEI_{1800}-Dex/DNA binary complexes showed significantly increased fluorescence expression, which was also comparable with that of PEI 25 kDa/DNA complexes. The results indicated that conjugation of Dex into PEI molecular could significantly improve the transfection efficiency of low molecular weight PEI (1800 Da). Moreover, the HA/PEI_{1800}-Dex/DNA ternary complexes were found to be the most efficient, yielding higher transfection efficiency than both PEI 25 kDa/DNA complexes ($p<0.01$) and Lipofectamine 2000/DNA complexes ($p<0.01$).

The transfection efficiency was also determined by FACS analysis (Fig. 5b). The transfection efficiency of ternary complexes was 3.2-fold higher than that of PEI 25 kDa/DNA complexes ($p<0.01$) and 1.3-fold higher than that of Lipofectamine 2000/DNA complexes ($p<0.01$).

To evaluate targeting specificity, inhibition studies were performed with pretreatment of the cells by HA solution. The results showed that the pretreatment with 1.0 mg/mL, 2.0 mg/mL and 3.0 mg/mL of HA solutions decreased the

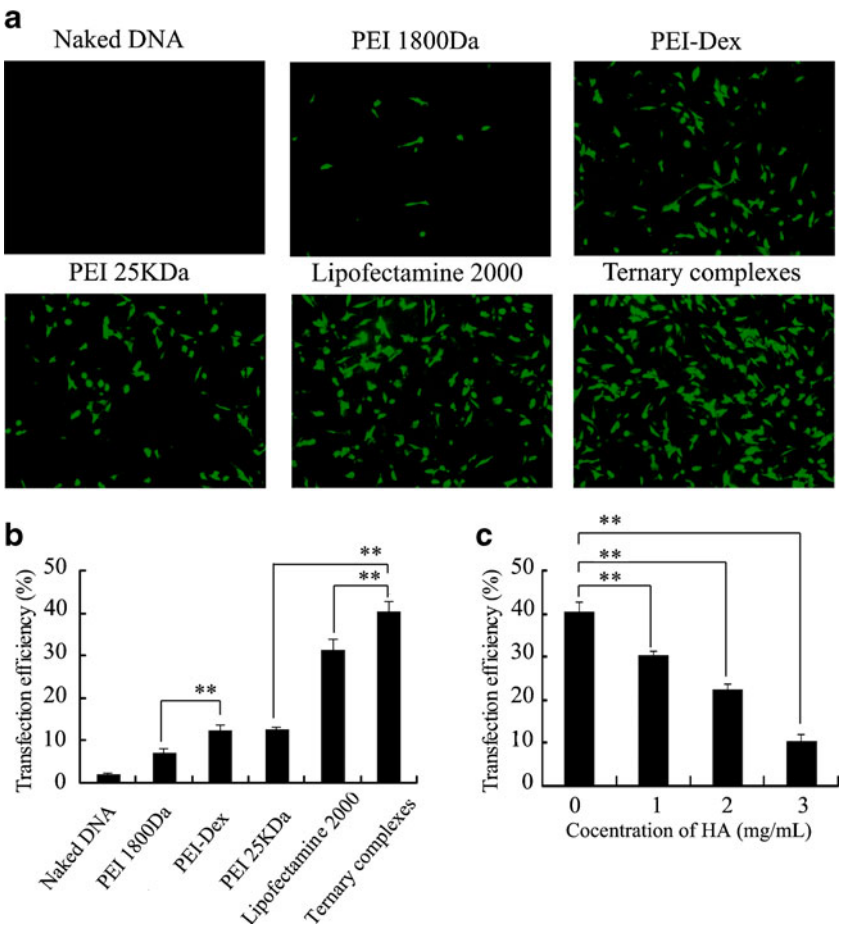

Fig. 5 Transfection efficiency in B16F10 cells at 48 h post-transfection with naked DNA, PEI 1800 Da/DNA complexes, PEI_{1800}-Dex/DNA binary complexes, PEI 25 kDa/DNA complexes, Lipofectamine 2000/DNA complexes and HA/PEI_{1800}-Dex/DNA ternary complexes. (**a**) Fluorescence microscopy photo. (**b**) Transfection efficiency of complexes determined by FACS analysis. The NP ratio of PEI/DNA complexes was 12. Lipofectamine 2000/DNA comlexes were prepared by the protocol of Invitrogen. (**c**) Transfection efficiency of complexes determined by FACS analysis after HA pretreatment for 1 h. The data were expressed as mean±S.D. ($n=3$). *$p<0.05$ and **$p<0.01$.

transfection efficiency of ternary complexes to 75.1%, 54.9% and 25.1% of the untreated control, respectively (Fig. 5c). These results suggested that the HA receptor-mediated endocytosis could facilitate the intracellular delivery of ternary complexes into CD44-positive B16F10 cells, as suggested in previous studies (22,23).

In Vitro Cell Uptake Studies

To identify the sub-cellular localization of complexes, confocal microscopy experiments were performed at different time points. The PEI or PEI-Dex was labeled with fluorescein isothiocyanate (FITC) (green). Late-endosome and lysosome were stained with LysoTracker (red), and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Figure 6 showed fluorescence microscopy photographs of cells treated with complexes. The results showed an entire distribution process of complexes in the cells. Only a little green fluorescence was observed in the cells for 2 h after incubation with FITC-labeled ternary complexes. After 6 h of incubation, part of green fluorescence was accumulated in the cytoplasm (yellow spots as the arrow indicates in emerged images of Fig. 6) while there was no remarkable distribution of green fluorescence in the nuclei. After 8 h of incubation, stronger fluorescence intensity was observed in the nuclei. These results suggested that ternary complexes could be translocated into the nuclei gradually. However, PEI/DNA binary complexes showed a little green fluorescence after 8 h of incubation. To further observe nuclear transport of the complexes, we also investigated the cell

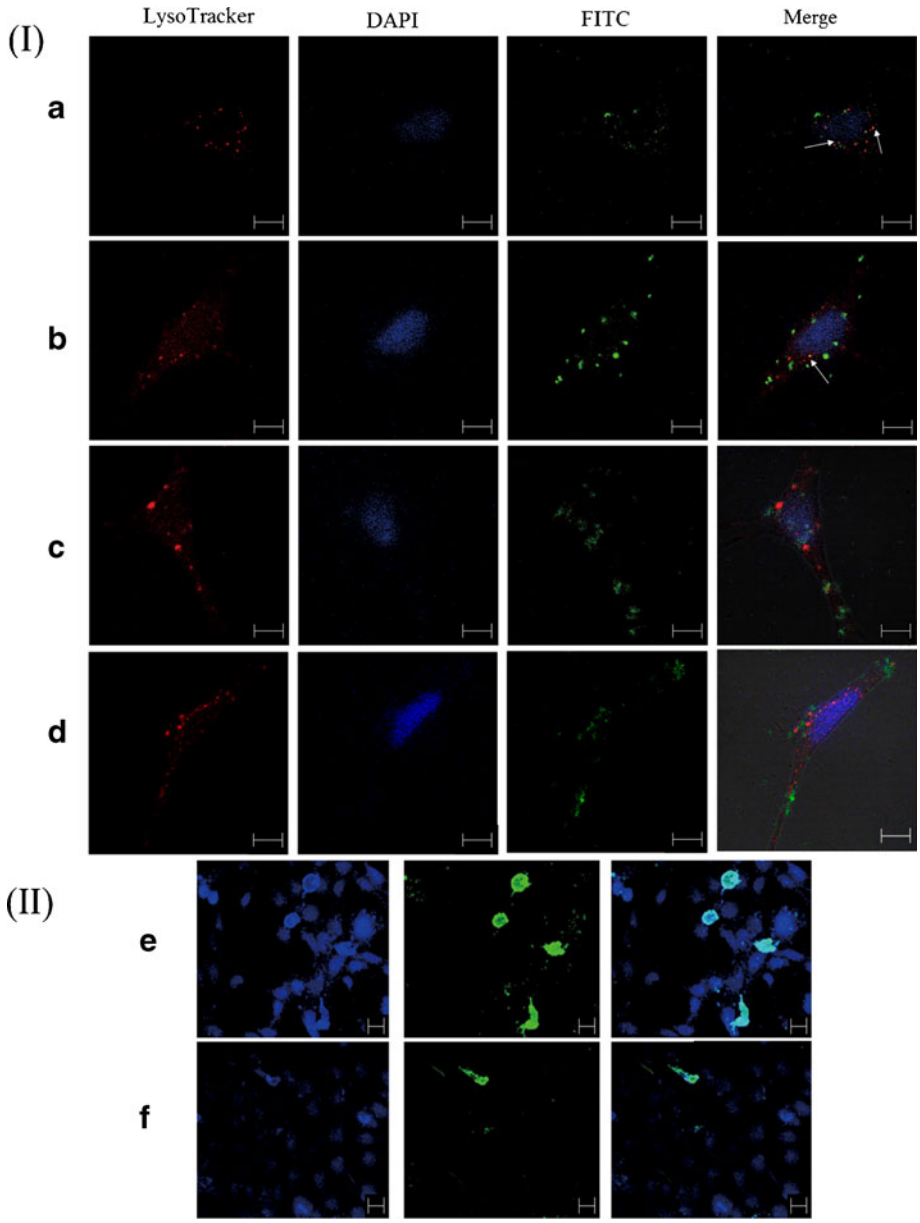

Fig. 6 Typical sub-cellular distribution of complexes was visualized by fluorescence confocal microsocopy. (**I**) Cells were treated with HA/PEI_{1800}-Dex/DNA ternary complexes for 2 h (**a**), 6 h (**b**) and 8 h (**c**), respectively while. Cells were treated with PEI/DNA binary complexes for 8 h (**d**). (**II**) Cells were treated with HA/PEI_{1800}-Dex/DNA ternary complexes (**e**) and PEI/DNA binary complexes (**f**) for 12 h, respectively. Scale bar is 10 μm (**a**–**d**) and 20 μm (**e**–**f**).

uptake after 12 h of incubation with HA/PEI_{1800}-Dex/DNA ternary complexes. As illustrated in Fig. 6e and f, more markedly increased nuclear transport efficiency was observed than binary complexes. Therefore, it could be concluded that HA/PEI_{1800}-Dex/DNA ternary complexes could facilitate the DNA translocation into the nuclear more efficiently, which might be attributed to the Dex-mediated nuclear transport.

In Vivo Imaging Analysis

In vivo fluorescent images were taken at 1, 2, 4, 6 h after *i.v.* administration, respectively. As shown in Fig. 7a, compared to PEI 25 kDa/DNA complexes, HA/PEI_{1800}-Dex/DNA complexes accumulated in the tumor (right armpit) was all markedly increased in the tumor-bearing nude mice at various time points. After 6 h of administration, it showed the strongest NIR fluorescent signal. Figure 7b shows the *ex vivo* images of the tumor. Stronger NIR fluorescent in the tumor of the nude mice after injected with HA/PEI_{1800}-Dex/DNA ternary complexes were observed than those with PEI 25 kDa/DNA complexes. The fluorescent intensity from the tumor in ternary complexes was significantly greater than that of binary complexes ($p<0.05$). Therefore, it can be indicated that the HA-coated ternary complexes could assist the DNA targeting to the tumor.

Anti-inflammation Activity of Ternary Complexes

Since Dex is an anti-inflammatory drug, PEI_{1800}-Dex is expected to reduce the inflammation response that was

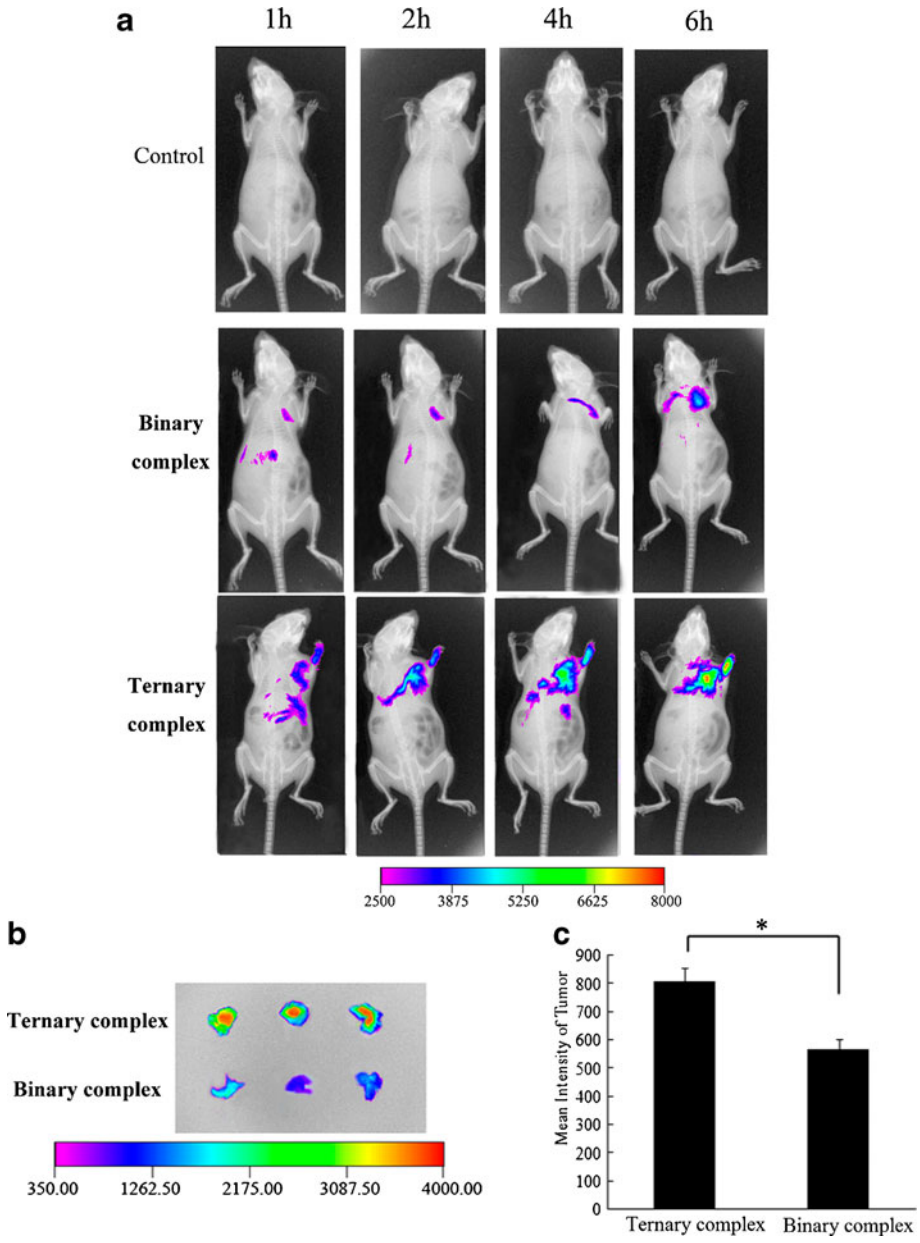

Fig. 7 *In vivo* imaging analysis of BALB/c nude mice bearing HepG2 tumor after administration with PEI 25 kDa-CY7/DNA binary complexes and HA/PEI_{1800}-Dex-CY7/DNA ternary complexes. (**a**) Typical *in vivo* non-invasive NIR fluorescence images taken at 1, 2, 4, 6 h after administration. (**b**) *Ex vivo* non-invasive NIR fluorescence images of tumors at 6 h after administration. (**c**) Tumor uptake expressed as mean intensity of tumor at 6 h after administration by the fluorescence intensity measurement method. All data are expressed as mean±S.D.($n=3$).

stimulated by exogenous polyplexes and/or lipoplexes. The anti-inflammatory effect of HA/PEI_{1800}-Dex/DNA ternary complexes was examined in TG-induced mice models. TG is frequently used in inflammation research to elicit the neutrophil and macrophage response *in vivo* (24). As shown in Fig. 8, at 4 h after TG challenge, Dex and HA/PEI_{1800}-Dex/DNA ternary complexes remarkably inhibited the accumulation of neutrophils in i.p. space compared to PBS solution ($p<0.05$). Interesting, HA/PEI_{1800}-Dex/DNA ternary complexes yielded the comparable anti-inflammation activity with that of Dex ($p>0.05$), which would be beneficial for gene therapy. More importantly, Since the HA/PEI_{1800}-Dex/DNA ternary complexes could easily transport into the tumor cells by HA receptor-mediated endocytosis compared to Dex as suggested in confocal microscopy experiment, the ternary complex was expected to have more superior in the *in vivo* efficacy.

In Vivo Growth Inhibition of Tumors

The *in vivo* antitumor activity of HA/PEI_{1800}-Dex/DNA ternary complexes was investigated. As shown in Figs. 9 and 10, compared to the control (5% glucose solution) and PEI 25 kDa/DNA complexes, HA/PEI_{1800}-Dex/DNA ternary complexes showed significant tumor growth inhibition of tumor-bearing nude mice, which was also superior to PEI_{1800}-Dex/DNA binary complexes. The tumor growth inhibitions at 21 days after the first administration were 23.9%, 53.4% and 70.9% in PEI 25 kDa/DNA complexes, PEI_{1800}-Dex/DNA binary complexes and HA/PEI_{1800}-Dex/DNA ternary complexes, respectively. The TUNEL assay is now commonly used to investigate apoptosis. As shown in Fig. 10(II), the most significant apoptosis of tumor cells in ternary complexes was observed, indicating that the tumor regression after multiple-dose injection of ternary complexes might be attributed to induction of apoptosis in the tumor cells. In addition, based on the results of pathological sections of HE stained tumor in Fig. 10(III), the tumor sections from the tumor-bearing nude mice treated with HA/PEI_{1800}-Dex/DNA ternary complexes showed the most obvious spotty necrosis and intercellular blank. These results indicated that HA/PEI_{1800}-Dex/DNA ternary complexes had superior *in vivo* antitumor efficacy.

DISCUSSION

It was well known that the nuclear membrane presents as a major obstacle to gene transfection (8). Many attempts have been made to overcome the nuclear membrane using nuclear localization signal (NLS) (25), transcription factor (26), DNA-binding proteins (11) and so on. However, only limited successes have been achieved due to difficultly controlling the topology and density of these functional macromolecules (27). Glucocorticoid receptor is a nuclear receptor, which served as shuttles to practice the uptake of cargos into the nucleus. Dex is a ligand for glucocorticoid receptor (28). Moreover, Dex may have some advantages over the macromolecules such as NLS and transcription factor. Especially, the density of Dex molecule could be controlled by adjusting the degree of Dex grafted to PEI, which benefits efficient nuclear translocation. Hydrophobic Dex could also increase the affinity of polycation to the lipophilic cell membrane (29). Therefore, in this study, Dex was introduced into the structure of low molecular weight PEI (1800 kDa) to overcome the obstacle of the nuclear membrane. In addition, it was reported that the reduction of amine groups in PEI molecular helped the dissociation of polyplexes in the cells, and thus increased transfection efficiency (30). Predictably, PEI_{1800}-Dex with reduced amine groups could contribute to improved intracellular trafficking and gene expression (31).

The transfection efficiency is a crucial factor to the success of gene therapy. The HA/PEI_{1800}-Dex/DNA ternary complexes with "core-shell" bilayer exhibited excellent transfection capability. There were several possible mechanisms for the increased transfection efficiency of ternary complexes. Generally, anionic complexes cannot be taken up well by cells due to the electrostatic repulsion and the lack of selectivity (3,32,33). In this study, HA-coated ternary complexes exhibited enhanced gene expression, which was attributed to HA-receptor mediated endocytosis based on the results of the markedly reduced transfection efficiency after HA pretreatment (Fig. 5c), as suggested in other reports (25,34–36). More importantly, Dex-mediated nuclear translocation might play a crucial role in increasing the transfection efficiency of ternary complexes based on the cell uptake (Fig. 6). Meanwhile, HA could improve the stability of nanoparticles, which also benefited the effective gene transfection. It has been reported that improvement of the nanoparticles' stability also benefited the effective gene transfection (19,37). In addition, ternary

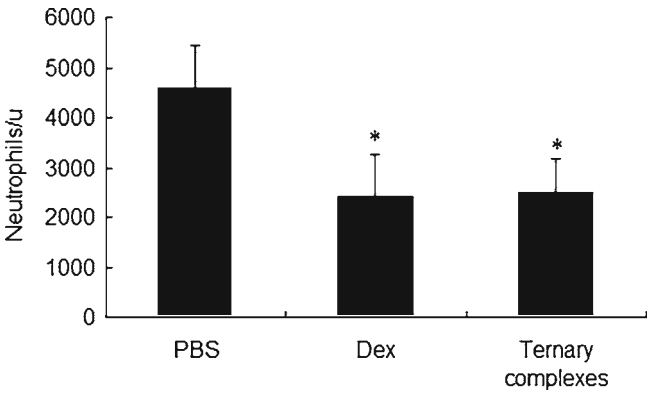

Fig. 8 Accumulation of neutrophils in intra-peritoneal space of ICR mice at 4 h after TG challenge. The PBS solution was used the negative control. Results was expressed as mean±S.D. ($n=5$). *$p<0.05$ and **$p<0.01$ *vs.* the PBS solution.

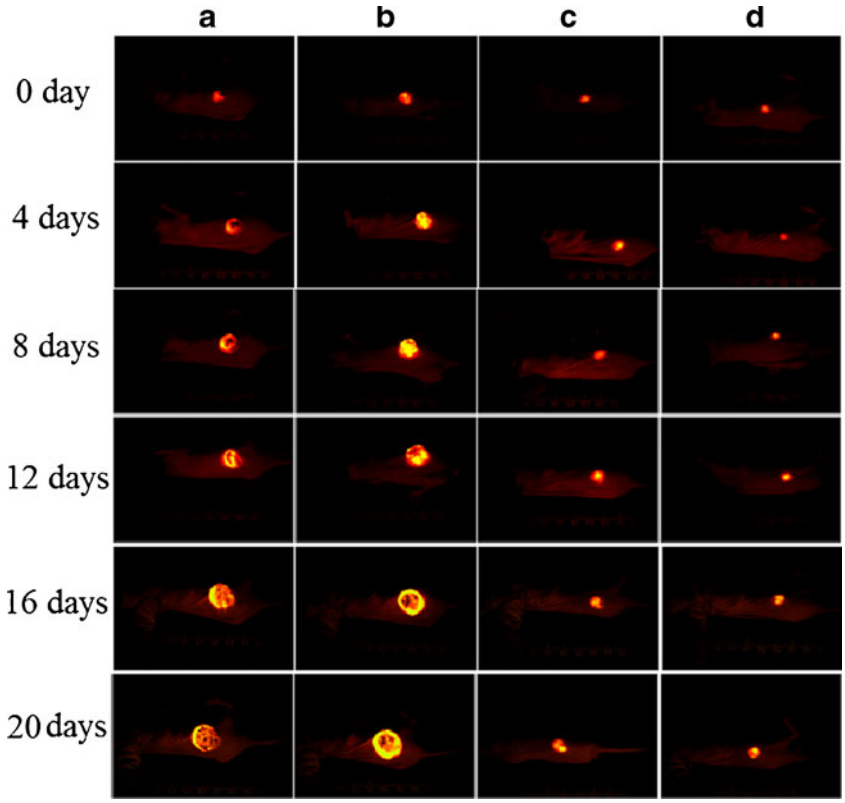

Fig. 9 Typical *in vivo* fluorescence images of BALB/c nude mice bearing HepG2 tumor expressing red fluorescence protein (RFP) after a schedule of multiple doses. (**a**) 5% glucose solution; (**b**) PEI 25 kDa/DNA complexes; (**c**) PEI_{1800}-Dex/DNA binary complexes; (**d**) HA/PEI_{1800}-Dex/DNA ternary complexes.

complexes had low cytotoxicity due to their negative surface charge, which was in favor for more efficient gene transfection (38).

The *in vivo* efficacy of the gene complexes has attracted great attention due to the discouraged outcomes in some cases. Fortunately, HA/PEI_{1800}-Dex/DNA ternary complexes exhibited significantly enhanced *in vivo* antitumor efficacy compared to PEI 25 kDa/DNA complexes and PEI_{1800}-Dex/DNA binary complexes, which may be owing to the synergistic effect of HA-mediated tumor localization and Dex-mediated nuclear translocation based on the above data from cell uptake and *in vivo* imaging analysis. It should also be noticed that mild host immune reaction and inflammatory response during transgene expression in common

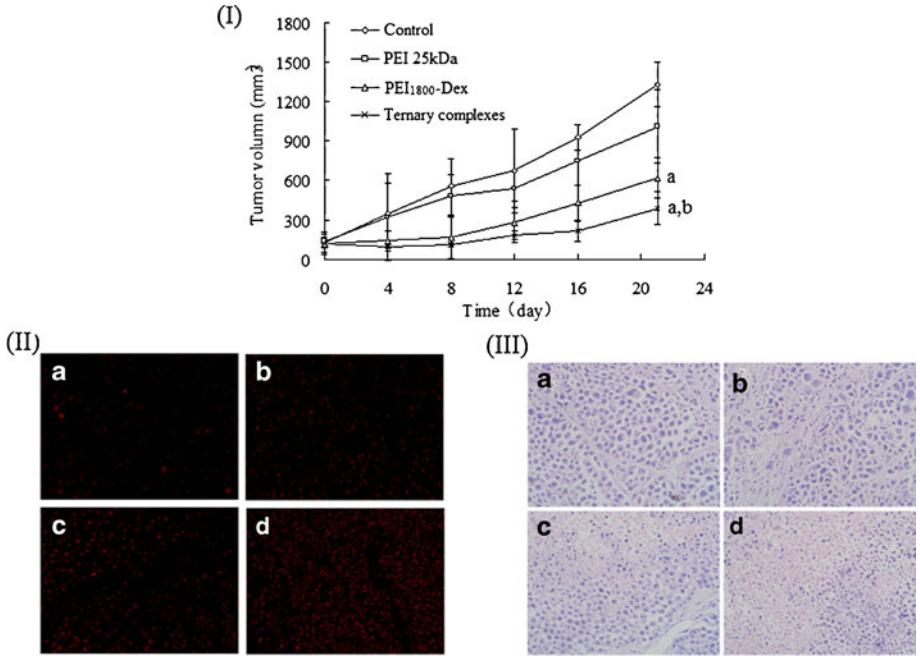

Fig. 10 *In vivo* anti-tumor effect assay. (**I**) Tumor grown curves of BALB/c nude mice bearing HepG2 tumor after a schedule of multiple doses. Data were expressed as mean±S.D. ($n=5$). [a] $p<0.01$ *vs.* the control group and [b] $p<0.01$ *vs.* PEI 25 kDa group. (**II**) TUNEL assay of tumor section after multiple-dose administration. The *red fluorescence* indicates TUNEL labeling. (**III**) HE section of tumor from BALB/c nude mice bearing HepG2 tumor. (**a**) 5% glucose, (**b**) PEI 25 kDa/DNA complexes, (**c**) PEI_{1800}-Dex/DNA complexes and (**d**) HA/PEI_{1800}-Dex/DNA ternary complexes.

clinical therapy might reduce the level and duration of transgene expression (39). In our studies, the coating of HA as non-immunogenic and nature biocompatible polysaccharide (40), may mitigate the occurrence probability of the immunity and inflammation. More importantly, Dex, a drug commonly used to treat inflammation and autoimmune diseases (16,41), gave the anti-inflammation activity of PEI_{1800}-Dex. Overall, HA/PEI_{1800}-Dex/DNA ternary complexes may offer unique benefits for gene delivery because it could not only augment the transfection efficiency but also inhibit the inflammatory response, and realize repetitive gene expression.

CONCLUSIONS

We developed HA/PEI_{1800}-Dex/DNA ternary complexes as a promising gene delivery tool. These ternary complexes showed low cytotoxicity, high transfection efficiencies and efficient cell uptake and nuclear transport of DNA. Moreover, HA/PEI_{1800}-Dex/DNA ternary complexes could obviously accumulate in the tumor after *i.v.* administration, and significantly inhibit tumor growth of tumor-bearing nude mice. The Dex-mediated nuclear translocation and HA-mediated tumor targeting may synergistically contribute to enhanced transfection efficiency and *in vivo* antitumor efficacy of the complexes, which also demonstrated that a combination of chemical modification and physical coating is effective to construct a multifunctional gene carrier.

ACKNOWLEDGMENTS AND DISCLOSURES

Ying Fan and Jing Yao contributed equally to this work and are both equally considered as first author. This work was supported by the Project Program of State Key Laboratory of Natural Medicines, China Pharmaceutical University (No. JKGQ201107), the National Natural Science Foundation of China (No. 30672548), the fifth of Six Talent Peak Foundation of Jiangsu Province, the Major Project of National Science and Technology of China for New Drugs Development (No.2009ZX09310-004), and the National Basic Research Program of China (No. 2009CB930303).

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