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Research Paper

A Potential Targeting Gene Vector Based on Biotinylated Polyethyleneimine/Avidin Bioconjugates

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Purpose. To improve the gene delivery efficiency and safety of non-viral vector in liver cells, avidin, which exhibited good biocompatibility and remarkable accumulation in liver, was bioconjugated with biotinylated polyethylenimine to obtain a novel gene vector.
Materials and methods. Biotinylated polyethyleneimine/avidin bioconjugate (ABP) was synthesized through grafting biotin to high molecular weight branched polyethylenimine (PEI, 25 kDa) and then bioconjugating with avidin by the biotin-avidin interaction. Physiochemical characteristics of ABP/pDNA complexes were analyzed, and *in vitro* cytotoxicity and transfection of ABP were also evaluated in HepG2, Hela and 293 T cells by using 25 kDa PEI as the control.
Results. It was found that ABP was able to condense pDNA efficiently at N/P ratio of 4. The particle sizes of ABP/pDNA complexes were less than 220 nm, and the average surface charges were around 27 mV at the N/P ratio ranging from 2 to 60. Among three different cell lines, ABP and its DNA complexes demonstrated much lower cytotoxicity and higher transfection efficacy in HepG2 cells as compared with 25 kDa PEI.
Conclusion. ABP presented higher transfection efficacy and safety in HepG2 cells due to the biocompatibility of avidin and the specific interactions between avidin and HepG2 cells.

KEY WORDS: Avidin; biotinylated PEI; gene vector; targeting.

INTRODUCTION

The hepatocellular carcinoma (HCC) is one of the most common cancers, the incidence of which is increasing worldwide, and is the direct cause of millions of deaths annually (1). Only a few patients with early HCC undergo orthotopic liver transplantation; nonetheless, the vast majority of patients with advanced HCC remain untreated, since other conventional therapies are mostly palliative (2). Moreover, the prognosis of patients suffering from advanced HCC remains poor despite a large number of therapeutic options (3). Since advanced liver cancer lacks effective therapy in most cases, a considerable interest has been drawn towards the new HCC therapeutic strategies, particularly gene-based therapy (4). Despite gene therapy offering an exciting approach for treating liver cancer, it has largely been confined to preclinical and experimental settings (2,3). Fortunately, significant advances in gene therapy have been made over the past years, attributed to the development of effective gene transfer vectors (4,5).

Recently, non-viral vectors have garnered much attention from the biomedical research community engaged in liver gene therapy (6–8). Despite the high transfection efficacy of viral vectors, safety concerns have been raised in clinical trials. Non-viral vectors make a heterogeneous group of vehicles for gene transfer including cationic liposomes and polymers, which are considered to be less toxic, less immunogenic, and easier to handle than viral vectors, thus they are more attractive vectors for clinical applications (9–13). As the profound non-viral gene vector, polyethyleneimine (PEI) presents significantly high transfection capability in various cell cultures and *in vivo* gene transfer (14–17). The PEI polymer comes in two distinct forms: linear and branched, and the latter was utilized in our study since it is the standard form used for cell transfection (16,18).

In spite of their advantages, non-viral vectors lack cell specificity in gene delivery. In order to enhance the transfection efficiency of vectors, it is necessary to attach ligands to non-viral vectors that can be recognized by a specific mechanism on the target cells. According to the literature (19–25), cell-targeting ligands, including asialoglycoproteins, transferrin, folate, galactosidase, lactoferrin and RGD, were linked to cationic polymers and are being extensively investigated as targetable carriers for genes. Avidin is a 66 kDa highly glycosylated protein found in egg white and is known to contain four identical binding sites, which shows extremely high affinity for biotin (26). Due to this strong affinity, the avidin-biotin system has provided extremely useful and versatile intermediates and been employed as a linker for surface modification, biochemical techniques and recently in tumor targeting studies (27–30). It is known that avidin subunit contains 129 amino acid residues, four residues of mannose,

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and three residues of glucosamine (31); moreover, avidin exhibits a remarkable accumulation in liver (32,33).

In this study, avidin was bound to the biotinylated PEI (BP) through the biotin-avidin interactional system to form a novel vector biotinylated PEI/avidin bioconjugate (ABP). Physiochemical characteristics of ABP/pDNA complexes were analyzed by agarose gel electrophoresis, particle size, SEM morphology observation and ζ-potential measurements. The toxicity and transfection efficiency of ABP in different cells were evaluated systematically. Moreover, the cell internalization of the fluorescent labeled ABP was further investigated by confocal laser scanning microscopy.

MATERIALS AND METHODS

Materials

Branched 25 kDa polyethylenimine (PEI), d-biotin, N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich (Steinheim, Germany). Avidin with molecular weight of 66 kDa was purchased from Pierce (Rockford, IL, USA). Dimethyl sulphoxide (DMSO) was obtained from Shanghai Chemical Reagent Co. (Shanghai, China), which was dried refluxing with anhydrous $MgSO_4$ overnight and was then distilled under reduced pressure. QIAfilterTM plasmid purification Giga Kit was purchased from Qiagen (Hilden, Germany). GelRed™ was purchased from Biotium (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT), and Dubelcco's phosphate buffered saline (PBS) were purchased from Invitrogen (CA, USA). The Micro BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). All other reagents were analytical grade and used as received. 150 mM NaCl solution was used to mimic the physiological saline environments (34).

Synthesis of Biotinylated PEI

The biotinylated PEI (BP) was prepared according to the literature (30). Biotin (30 mg) was activated with NHS (14 mg) and EDC (23 mg) in dimethyl sulfoxide (3 mL). The activated biotin solution was added to 25 kDa PEI (200 mg) in deionized water (12 mL). The reaction mixture was stirred at 4°C for 24 h, purified dialyzed against water for 2 days (MWCO: 8,000-12,000), lyophilized and defined as BP.

Conjugating of Avidin with Biotinylated PEI

Avidin (0.5 mg) was dissolved in 150 mM NaCl, and then 20-fold weight excess of BP was added. The mixture was vortexed and reacted at room temperature for 30 min to obtain the ABP solution.

^{1}H NMR

^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR) of PEI and BP were recorded on a Varian Unity 300 MHz spectrometer with D_2O as the solvent.

Cell Culture

Human hepatoblastoma cells (HepG2), human cervix carcinoma cells (HeLa) and human embryonic kidney transformed 293 cells (293 T) were incubated in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10,000 U/mL) at 37°C in a fully humidified atmosphere of 5% CO_2.

Amplification and Purification of Plasmid DNA

Luciferase (pGL-3) and expressing green fluorescent protein (pEGFP-C1) plasmids were used in this study. The former one as the luciferase reporter gene was transformed in *E. coli* JM109 and the latter one as the green fluorescent protein gene was transformed in *E. coli* DH5α. Both plasmids were amplified in terrific broth media at 37°C overnight at 250 rpm. The plasmids were purified by an EndoFree QiAfilter™ Plasmid Giga Kit. Then the purified plasmid were dissolved in TE buffer solution and stored at −20°C. The integrity of plasmid was confirmed by agarose gel electrophoresis. The purity and concentration of plasmid were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

Acid-base Titration

The buffer capability of 25 kDa PEI, BP and ABP were determined by acid-base titration assay over the pH from 10 to 2 as described by Benns *et al* (22, 35). Briefly, 0.2 mg/mL of each sample solution was prepared in 30 mL 150 mM NaCl solution. The sample solution was first titrated by 0.1 M NaOH to a pH of 10, then different volume of 0.1 M HCl was added to the solution, and the different pH value was measured using a microprocessor pH meter.

Agarose Gel Retardation Assay

The PEI/pDNA, BP/pDNA and ABP/pDNA complexes at different N/P ratios range from 2 to 20 (the primary amino groups of PEI in the BP to phosphate groups of pDNA) were prepared by adding appropriate volume of BP solution (in 150 mM NaCl solution) to 0.8 μL of pEGFP-C1 pDNA (120 ng/μL in 40 mM Tris-HCl buffer solution). The complexes were diluted by 150 mM NaCl solution to a total volume of 6 μL, and then the complexes were incubated at 37°C for 30 min. After that the complexes were electrophoresed on the 0.7% (W/V) agarose gel containing GelRed™ and with Tris-acetate (TAE) running buffer at 80 V for 80 min. pDNA was visualized with a UV lamp using a Vilber Lourmat imaging system (France).

Particle Size and ζ-potential Measurement

The particle size and ζ-potential were measured by Nano-ZS ZEN3600 (MALVERN Instr.) at 25°C. The complexes at various N/P ratios range from 2 to 60 were prepared by adding 1 μg pGL-3 pDNA (in 40 mM Tris-HCl buffer solution) to appropriate volume of ABP solution or 25 kDa PEI solution (in 150 mM NaCl solution or in deionized water). Then the complexes were incubated at 37°C for

30 min. After that the complexes were diluted by 150 mM NaCl solution or deionized water to 1 mL volume prior to measurement. NaCl solution is unavailable for zeta potential as it will damage the electrodes of zeta potential sample cell. Difference between particle sizes formed in water and formed in NaCl solution at different N/P ratios was studied.

Scanning Electron Microscopy (SEM)

The morphologies of PEI, ABP and their pDNA complexes (at N/P=10 and 40) were observed respectively by SEM (FEI-QUANTA 200). PEI and ABP were dissolved in deionized water and filtrated, respectively. The complexes were prepared by adding 1 μg pDNA (in water) to appropriate volume of PEI solution (in water) and ABP solution (in water), respectively. The complexes were diluted to a total volume of 100 μL by water and then incubated at 37°C for 30 min. The SEM samples were prepared by dropping the polymer solution or polymer/pDNA complexes solution onto the glass slip and then kept in aseptic manipulation cabinet at 30°C for 2 h for drying. Before SEM observation, the samples were coated with gold for 7 min.

In Vitro Cytotoxicity Assay

The *in vitro* cytotoxicities of ABP and ABP/pDNA complexes were examined by MTT assay following literature procedures (36). 25 kDa PEI and PEI/pDNA complexes were used as the control. HepG2, Hela and 293 T cells were used in this assay.

For polymers cytotoxicity assay, cells were seeded in the 96-well plates at a density of 6000 cells/well and cultured 24 h in 200 μL DMEM containing 10% FBS. After that, polymer solutions with different concentrations were added to each well. After the polymers were added for 48 h, the medium was replaced with 200 μL of fresh medium.

For polymer/pDNA complexes cytotoxicity assay, cells were seeded in the 96-well plates at a density of 12000 cells/well and cultured 24 h in 200 μL DMEM containing 10% FBS. Polymers were complexed with 0.2 μg pDNA at different N/P ratios (5–60) for 30 min, which was the same as transfection conditions. After that, the complexes were added to each well. After the complexes were added for 4 h, the medium was replaced with 200 μL of fresh medium and cultured 44 h.

Following the above steps, 20 μL MTT (5 mg/mL) solutions were added for 4 h. Thereafter, the medium was removed and 150 μL DMSO was added. The absorbance of color was measured at 570 nm by a microplate reader (BIO-RAD, Model 550, USA). The relative cell viability was calculated according to the following equation: Cell viability (%)=[OD_{570} (treated cells)-OD_{570} (background)/OD_{570} (untreated cells)-OD_{570} (background)]×100.

In Vitro Transfection

Transfection experiments of ABP were performed with HepG2, Hela and 293 T cells in comparing with 25 kDa PEI. For transfection experiments, including the lucifease assay and the expressing green fluorescent proteins assay, the polymer/DNA complexes were prepared at N/P ratios range from 2 to 60. Both PGL-3 and pEGFP-C1 plasmid DNA were utilized to evaluate the transfection efficiency. First, cells were seeded at a density of 6×10^4 cells/well in the 24-well plate with 1 mL of DMEM containing 10% FBS and incubated at 37°C for 24 h. Then the complexes were prepared by adding appropriate volume of ABP solution to 1 μg plasmid DNA (1 μg DNA per well). Before transfection, cells were washed with PBS (0.1 M, pH 7.4), and then ABP/DNA complexes were added with serum-free DMEM for 4 h at 37°C. The serum-free DMEM was replaced by fresh DMEM containing 10% FBS, and the cells were further incubated for 48 h.

The luciferase assay was performed according to manufacturer's protocols. Relative light units (RLUs) were measured with chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was measured according to a BCA protein assay kit. Luciferase activity was expressed as RLU/mg Protein. The cells expressing green fluorescent proteins were directly observed by an inverted microscope (IX 70, Olympus, Japan).

Preparation of FITC-labeled Polymer

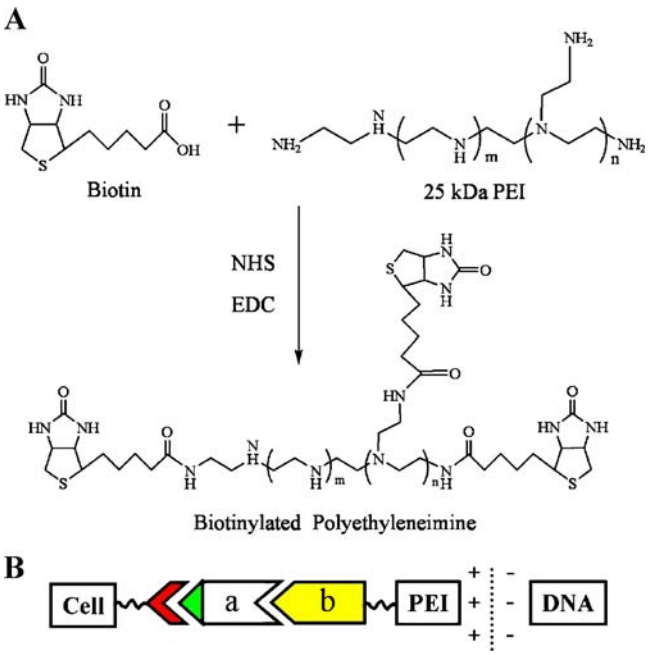
To study the cellular uptake of polymer/pDNA complex during transfection, FITC was labeled on BP and PEI according to the previously reported method (37). Briefly, BP (200 mg) was dissolved in DMSO (8 mL), and then FITC (2 mg) was added. After stirring for 24 h at room temperature, the mixture was purified by dialysis (MWCO: 8,000–12,000) against distilled water for 10 days, and lyophilized to yield the final product designated as FITC-BP. FITC-PEI was prepared by the same method. FITC-BP was conjugated with amount of avidin to form FITC-ABP.

Live Cell Laser Scanning Confocal Microscopy

Live cell confocal microscopy was used to image ABP/pDNA and PEI/pDNA complexes uptake into HepG2, Hela and 293 T cells. Cells were seeded into LabTek chamber slide dishes at a density of 4.0×10^4 cells/well with 1 mL of DMEM containing 10% FBS and incubated at 37°C for 24 h. Then the complexes were prepared at an N/P ratio of 10 by adding appropriate volume of FITC-ABP or FITC-PEI solution to 1 μg pGL-3 plasmid. Culture medium was removed and 1 mL of fresh DMEM containing complexes was added into each well and incubated with cells for 4 h at 37°C. Before observation, medium was removed, cells in each well were washed by 0.5 mL PBS solution twice, and then 1 mL fresh DMEM with FBS was added. The control experiment was also carried out under the same condition by the use of FITC-PEI. The fluorescent images of cells were observed under excitation at 488 nm using confocal laser scanning microscopy (Leica TCS SP2AOBS, Germany). All confocal images were slice images to distinguish PEI or ABP internalized from that adherent to the outside cellular membrane.

Statistical Analysis

The results are representative of replicate experiments performed with a sample size equal to three or four in each



Scheme 1. **A** Synthesis of biotinylated polyethyleneimine. **B** Model of the avidin-mediated gene delivery. ABP is formed via the strong noncovalent interaction between avidin (a) and biotin (b) of BP; positive amines of ABP complex with negative phosphate groups of DNA via electrostatic interactions. The unknown interaction between avidin and HepG2 cells could enhance gene transfection efficiency.

experiment, as indicated on the figure legend. The results are represented by the mean and standard deviation. All statistical analyses were performed using Student's t-test. Probability values less than 0.05 ($P<0.05$) were considered to be indicative of statistical significance.

RESULTS AND DISCUSSION

Synthesis and Characterization of Biotinylate PEI

In this study, biotin was linked to 25 kDa PEI using EDC. The synthesis route for BP is illustrated in Scheme 1 (A). The product was further purified by dialysis in dialysis tube (MWCO: 8000–12000) to remove the residues of solvent and the free biotin. The structure of products was confirmed by ^{1}H NMR spectroscopy, and the spectrum is shown in Fig. 1. It can be seen that biotin signal was very weak due to the low amount of this compound in BP. The peaks of biotin (m, bridgehead CH of biotin) and D_2O solvent overlap between δ 4.6 and 4.4 ppm in ^{1}H NMR spectra. The peaks near δ 2.1 ppm belongs to CH_2 of biotin (Fig. 1a), and the peak between δ 2.4 and 2.6 ppm belong to the CH_2 protons of PEI (Fig. 1d–g). Biotin to PEI ratio was calculated based on integrations with respect to the CHx protons of biotin (Varian 300 MHz, 2.8–3.3 ppm (br. m, CHxS of biotin)) (Fig. 1b,c). According to ^{1}H NMR spectra, it can be calculated that one PEI molecule was coupled with 22.3 biotin molecules.

Gel Retardation Assay

The binding capability of polycation to pDNA is a prerequisite for the gene vector. The ability of ABP and PEI to condense pDNA was assessed through the elimination of electrophoretic mobility using agarose gel electrophoresis at various N/P ratios ranging from 0 to 20. Because some primary aminos of PEI were substituted by biotins and coupled with avidins, the binding capability of BP and ABP became weaker. Obviously, both ABP and BP were able to completely stop the electrophoretic mobility of pDNA at an N/P ratio of 4 (Fig. 2), indicating that the pDNA was fully condensed at this N/P ratio or larger N/P ratios and could be used in the following transfection study.

Buffer Capability

The buffer capability of gene vector is very important for complexes escaping from the endosomes and promoting transfection efficacy (16). In this study, the buffer capabilities of 25 kDa PEI, BP and ABP were evaluated by acid-base titration. The buffer capabilities of BP and ABP were slightly lower than that of 25 kDa PEI shown in Fig. 3 (A). It is reported that 25 kDa PEI contains varying levels of 1°, 2° and 3° amines that can become protonated under a range of pH, giving rise to the so-called "proton sponge" effect that may contribute to the high efficiency of PEI as a gene delivery vehicle (16,17). However, BP and ABP exhibited lower positive charge density than that of PEI due to the introduction of biotin and avidin. Nonetheless, ABP presented very good protonation capability due to the presence of 1° and 2° amino groups on the polymer, which could dramatically improve the endosomal escape capability of complexes during the transfection process.

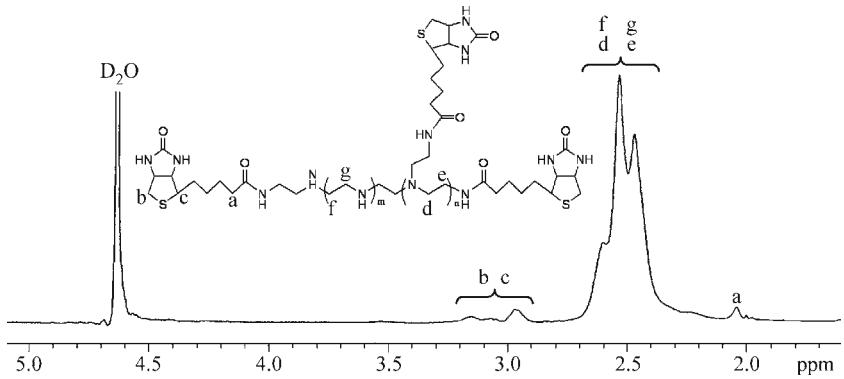


Fig. 1. ^{1}H NMR spectrum of biotinylated polyethyleneimine in D_2O.

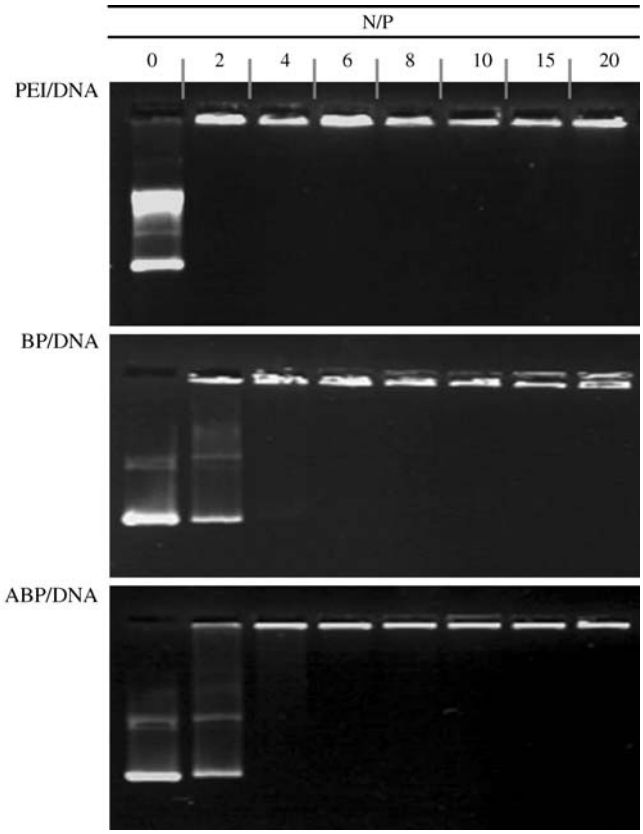


Fig. 2. Agarose gel electrophoresis retardation assay of polymer/DNA complexes was prepared at different N/P ratio: 0, 2, 4, 6, 8, 10, 15, 20; and were separated on a 0.7% agarose gel. The leftmost lane (N/P=0) shows the mobility of plasmid only used as a control.

Particle Size and ζ-potential Measurement

The small and compact complex particle plays an important role in polycation gene delivery. And the particle size would apparently affect the cytotoxicity and transfection efficiency of gene vectors (17). It is known that the particle size depends on many parameters, including pDNA concentration, sequence of addition of polycation or pDNA, and ionic strength of solvent (38). In this study, the particle sizes of ABP/pDNA complexes at various N/P ratios ranging from 2 to 60 were measured by adding ABP solution to 1 μg pDNA at physiological ionic strength condition (in 150 mM NaCl solution) or in deionized water. 25 kDa PEI was used as the control. As presented in Fig. 3 (B_i), the particle sizes of ABP/pDNA complexes formed in 150 mM NaCl solution were less than 220 nm, similar to PEI/pDNA complexes. The particle size ascended before N/P ratio arrived at 30, and then descended gradually, which could be considered that aggregates appeared among those small complexes and induce precipitation when avidin content was added. Moreover, the particle sizes of polymer/pDNA complexes formed in deionized water were a little smaller than that formed in NaCl solution. Hattori *et al.* reported that the presence of NaCl could weaken the association with DNA by neutralizing the cationic charge on PEI and enlarge the particle size of complexes (39). Additionally, larger particle sizes are also a benefit for transfection, as avidin could give pDNA better protection from enzyme inhibition during gene delivery.

The positively charged complex is essential for binding the negatively charged cellular membrane, which facilitates the entering of complex into nucleus by cellular uptake (16,17). The ζ-potential values of polymer/pDNA complexes

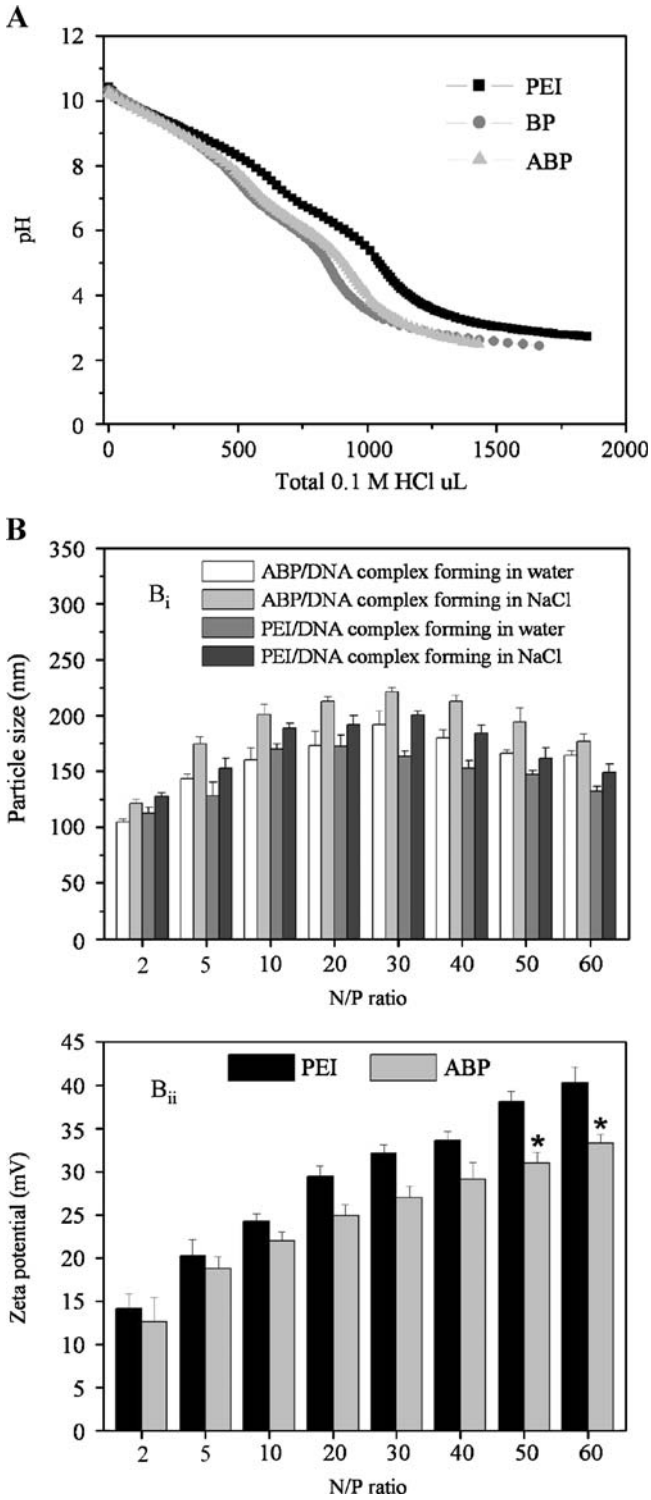


Fig. 3. **A** Acid-base titration profile of 25 kDa PEI, BP and ABP in 150 mM NaCl solution was obtained. **B** Particle size (B_i) and ζ-potential (B_{ii}) of ABP/DNA complexes at N/P ratios range from 2 to 60. PEI (25 kDa) was used as a control. Data were shown as mean± SD (n=3). (*: $p<0.05$ as compared with the data of PEI/DNA complexes).

were also measured at N/P ratios ranging from 2 to 60. Fig. 3 (B_{ii}) reveals that the ζ-potential values of ABP/pDNA and PEI/pDNA complexes have a similar trend with the increasing N/P ratios. The mean ζ-potential of ABP/pDNA complex was about 27 mV, and that of PEI/pDNA complex was about 33 mV, suggesting that avidin and biotin may slightly decrease the positive charge of PEI. It is generally believed that higher surface charge enhances the interaction of the complex with the negatively charged cell membrane (16), and the positive ζ-potentials could stabilize the complexes against aggregation and guarantee a small complex size. However, higher positive charges also lead to the unexpected higher cytotoxicity of polycation (40), which may reduce the transfection efficacy and limit the clinical application. Accordingly, ABP/pDNA complexes with lower positively charged surface may display lower cytotoxicity in comparison with PEI/pDNA complexes.

SEM Morphology Observation

Based on the results of particle size for all the complexes (Fig. 3(B_i)), ABP and 25 kDa PEI complexed with pDNA at two different N/P ratios (10 and 40) were evaluated respectively via SEM morphology. The morphologies of PEI and ABP were observed as the control to compare the effect of plasmid complexation on morphology of formulations. As shown in Fig. 4(A&B), most of PEI formulations without plasmid formed uniform nanospheres less than 20 nm, whereas ABP formed larger particles around 50 nm due to

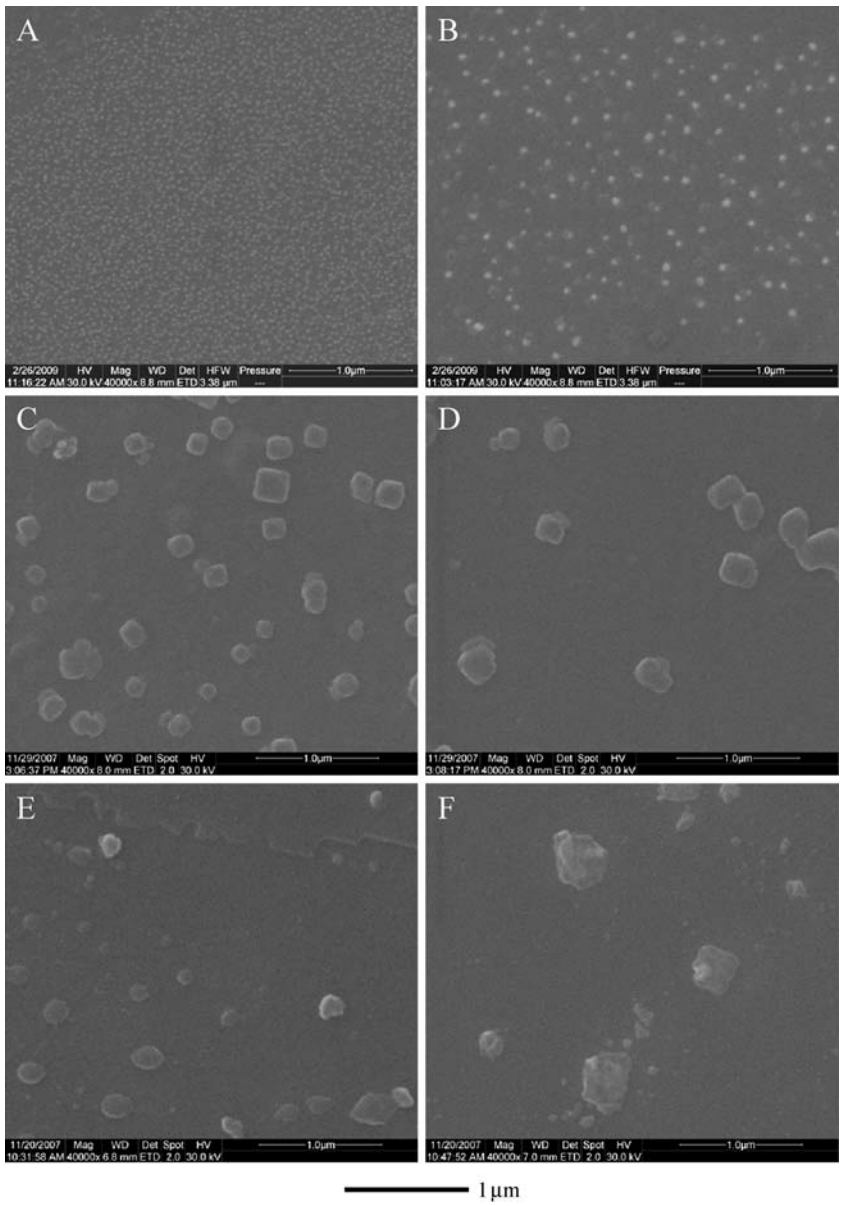


Fig. 4. SEM of PEI (25 kDa), ABP and their pDNA complexes. **A** PEI; **B** ABP; **C** PEI/pDNA complexes at N/P=10; **D** PEI/pDNA complexes at N/P=40; **E** ABP/pDNA complexes at N/P=10; **F** ABP/pDNA complexes at N/P=40. The micrographs are obtained at magnification of 40000×.

the introduction of avidin. Additionally, it was considered that these nanospheres consisted of several PEI or ABP molecules. In contrast, the SEM images demonstrated that the sizes of both polymer/DNA complexes were about 200-400 nm and at least 10 times larger than the pure polymer control, which was similar to the report by Clamme *et al.* (the PEI/DNA complexes are composed on the average by 3.5 (±1) DNA plasmids and about 30 PEI molecules) (41). But

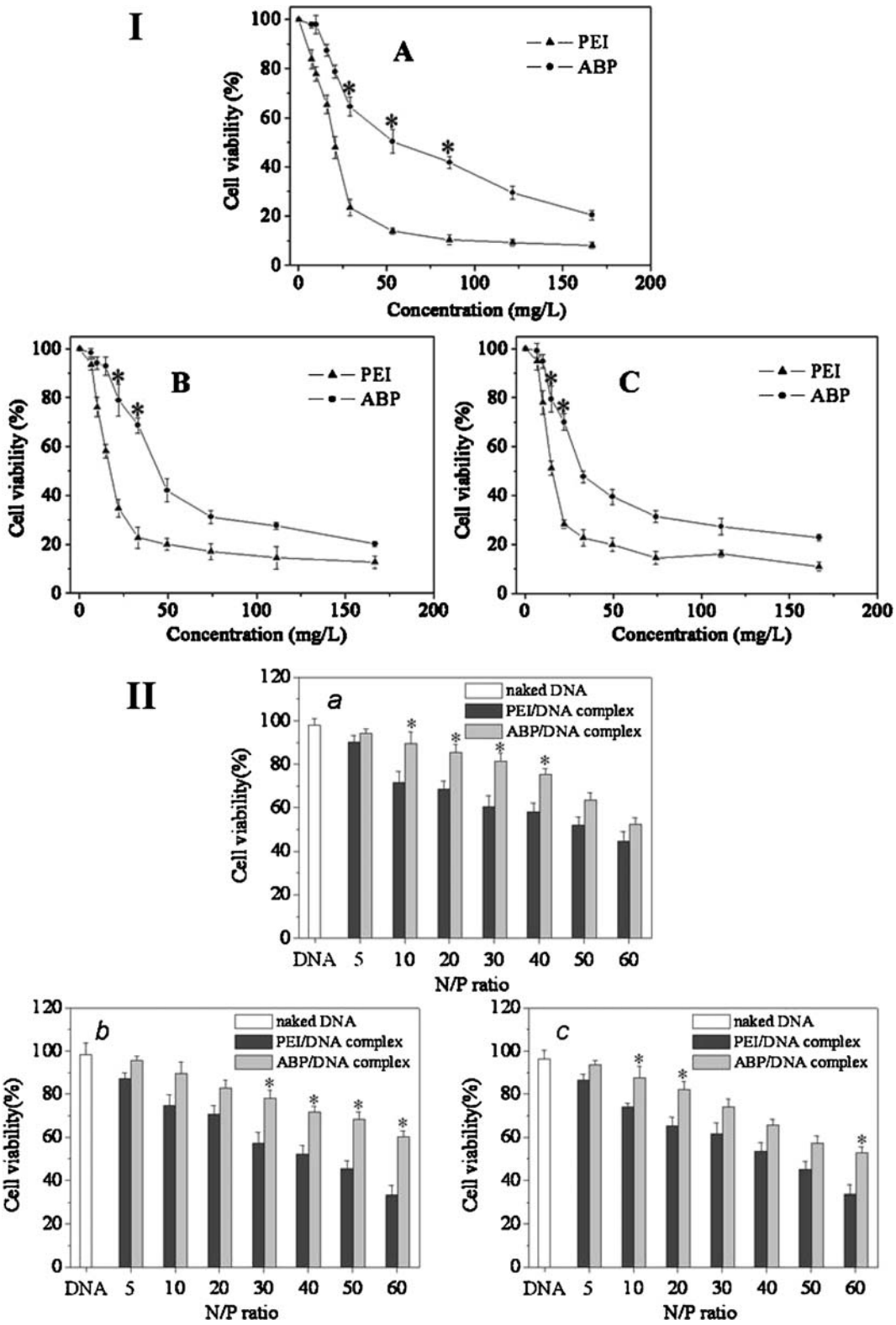


Fig. 5. Cell percentile viabilities of PEI (25 kDa), ABP and their pDNA complexes at different N/P ratios in HepG2 (A & a), Hela (B & b) and 293 T cells (C & c). Naked DNA was used as control. Data were shown as mean±SD (n=3). (*: p<0.05 as compared with the data of PEI or PEI/DNA complex).

these particle sizes were larger than the results measured by Nano-ZS ZEN3600, which may be due to the aggregation during the drying process of sample preparation. Moreover, the typical images showed that the complexes had a spherical shape and a compact structure (Fig. 4(C–F)). It could be inferred that ABP would condense pDNA compactly as well as 25 kDa PEI. Obviously, the morphology of ABP complexes at N/P of 40 was irregular in shape and significantly larger than that of ABP complexes at N/P of 10, indicating that the increasing content of ABP led to the aggregation of small spherical complexes due to the strong interaction between avidin and BP.

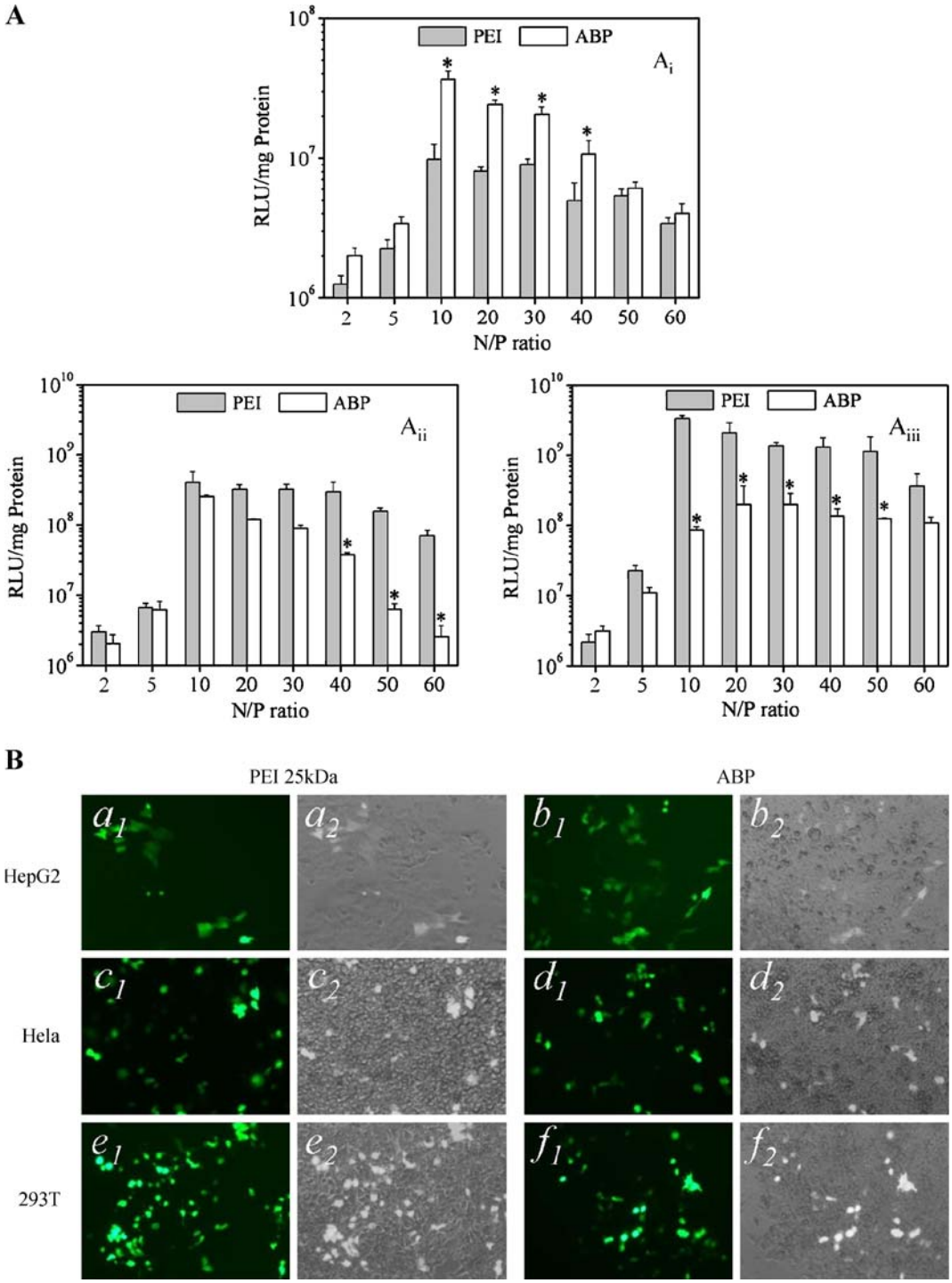


Fig. 6. **A** Transfection efficiency of ABP/DNA complexes at N/P ratios range from 2 to 60 in (A_i) HepG2, (A_{ii}) HeLa and (A_{iii}) 293 T cells. Data were shown as mean±SD (n=3). (*: p<0.05 as compared with the data of PEI/DNA complexes). **B** The fluorescence images of HepG2, HeLa and 293 T cells after transfection of ABP/pEGFP and PEI/pEGFP complexes at N/P=10. HepG2 cells (a, b), Hela cells (c, d), 293 T cells (e, f), green fluorescence image (a_1, b_1, c_1, d_1, e_1, f_1), bright field image (a_2, b_2, c_2, d_2, e_2, f_2). The micrographs are obtained at magnification of 100×.

In Vitro Cytotoxicity

The cytotoxicity of gene vector is definitely essential to the clinical application, which strongly depends on the biocompatibility of material. In order to evaluate the toxicity of ABP and its DNA complexes, the percentage cell viabilities of three different cell lines, HepG2, HeLa and 293 T cells, were tested by MTT assay. 25 kDa PEI and naked DNA were used as the control.

According to the results of polymer cytotoxicity assay, both ABP and PEI exhibited minimal cytotoxicity and maintained high cell viability at lower concentrations, shown in Fig. 5. However, among the cell lines, ABP demonstrated a significantly lower cytotoxicity than those of PEI at higher concentrations. This is considered to be due to the introduction of biotins and avidins, which might reduce the surface charge intensity of polycation and benefit to the bioactivity of cells. In addition, the viability of HepG2 cells was more than 50% in the presence of ABP at the concentration of 50 mg/L, which was much higher than those of HeLa cells and 293 T cell ($p<0.05$). It's suggested that there might exist somewhat unknown interaction between avidin and HepG2 cells which could make the polycation vector more ecological, biocompatible and less toxic.

For polymer/pDNA complexes cytotoxicity assay, nearly 90% cell viabilities were observed in the case of ABP/DNA complexes (N/P=10), whereas PEI/DNA complexes were found to be highly toxic. Moreover, N/P ratios of polymers exceeding 30 presented dramatic reductions of cell viabilities due to the increasing concentrations of polymers. In conclusion, cytotoxicity assay clearly showed that ABP and its complexes were much less toxic than PEI.

In Vitro Transfection

The synergistic effect of avidin, biotin and PEI could greatly benefit the transfection efficacy. Wojda *et al*. (42,43) reported that targeting PEI-avidin-DNA to antibody biotinylated cells increased transfection efficiency several fold over untargeted PEI, and the surface membrane biotinylation could enhance the endocytosis of avidin bioconjugates into nucleated cells. In this paper, the transfection efficiency of ABP/pDNA complexes was compared with PEI/pDNA complexes in three different cell lines: HepG2 cells, 293 T cells and HeLa cells (shown in Fig. 6). In luciferase assay, the pGL-3 was used as the reporter gene. The efficiencies of ABP/pDNA complexes were evaluated at N/P ratio ranging from 2 to 60, and the 25 kDa PEI was used as the control. Obviously, Fig. 6 (A) shows that ABP/pDNA complexes present higher transfection levels than PEI/pDNA complexes in HepG2 cells, but not in HeLa cells and 293 T cells, which indicates that avidin greatly enhances transfection efficacy in HepG2 cells. This suggests that the presence of avidin component facilitates the uptake of the ABP/pDNA complex into the HepG2 cells. ABP/pDNA complexes with N/P ratio ranging from 10 to 40 exhibit a 4-fold enhancement in transfection in HepG2 cells compared with plain PEI (Fig. 6 (A_i)). For example, the transgene expression of ABP/pDNA complexes at N/P ratio of 10 (3.66×10^7 RLU/mg Protein) is statistically significantly higher than that of 25 kDa PEI at the

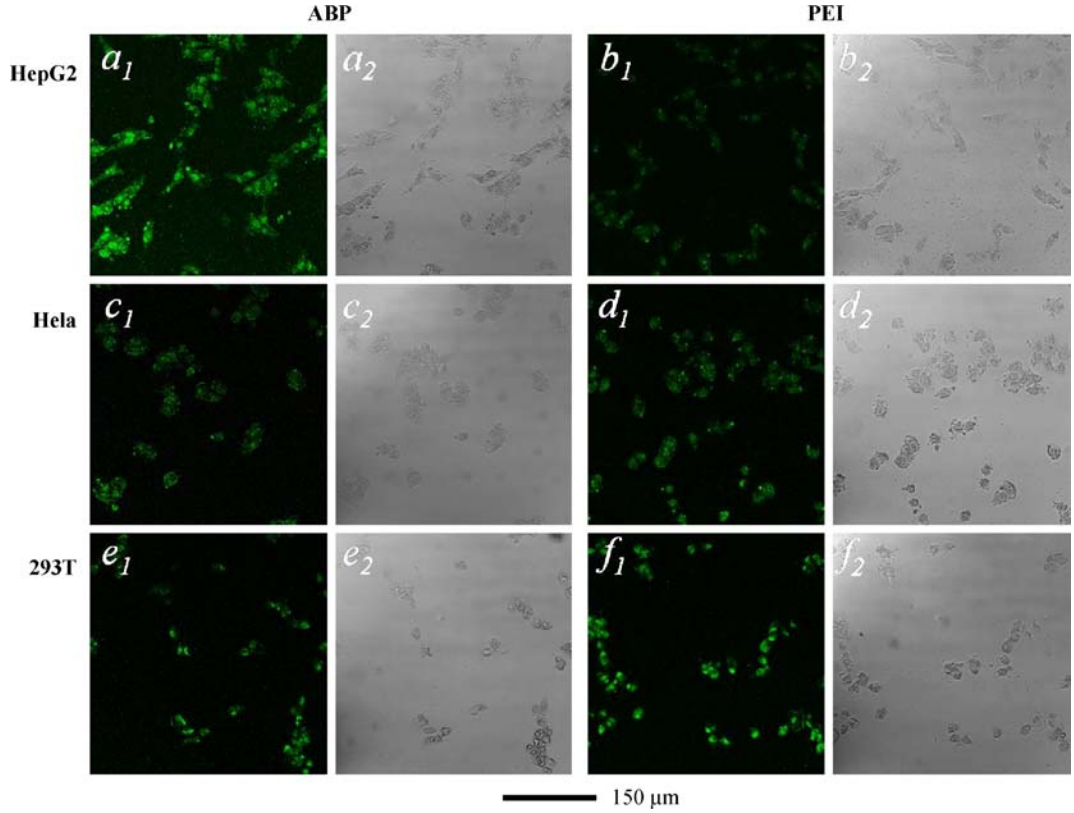


Fig. 7. Confocal images of HepG2, Hela and 293 T cells after incubated with the complexes of pGL-3 and FITC-ABP or FITC-PEI. HepG2 cells (a, b), Hela cells (c, d), 293 T cells (e, f), confocal fluorescence image (a_1, b_1, c_1, d_1, e_1, f_1), bright field image (a_2, b_2, c_2, d_2, e_2, f_2). The micrographs are obtained at magnification of 400×.

same N/P ratio (8.78×10^6 RLU/mg Protein) ($p<0.05$). On the other hand, although the *in vitro* transfection capabilities in Hela cells and 293 T cells are different, the luciferase values in two cell lines are similar. With respect to HeLa cells (Fig. 6 (A_{ii})), the transfection efficiency of ABP/pDNA complexes at N/P ratio of 10 (2.51×10^8 RLU/mg Protein) is slightly lower than that of 25 kDa PEI/pDNA complexes at the same N/P ratio (4.06×10^8 RLU/mg Protein) ($p>0.05$). However, the transfection expression of ABP/pDNA complexes at each N/P ratio is much lower than that of PEI/pDNA complexes in 293 T cells (Fig. 6(A_{iii})). Under the same conditions and at the same N/P ratio, the plain PEI exhibits 5-fold to 10-fold enhancement in luciferase expression compared with ABP ($p<0.05$).

Moreover, pEGFP-C1 was employed as another reporter gene so that the transgene expression level of complexes can be directly visualized by observation of EGFP positive cells using the inverted microscope. The fluorescent images of ABP/pDNA complexes at the optimal N/P ratio of 10 in HepG2 cells, Hela cells and 293 T cells are shown in Fig. 6 (B). For HepG2 cells, the strong fluorescent density can be seen when cells are transfected with ABP/pEGFP-C1 complexes, whereas the weak fluorescent density is displayed when cells are transfected with PEI/pEGFP-C1 complexes (Fig. 6(B(a,b))). However, on the contrary, the fluorescent densities have not shown obvious difference or even been dramatically reduced when Hela cells and 293 T cells are transfected with ABP/pEGFP-C1 complexes (Fig. 6(B(c–f))). These images indicate that the transfection efficiency of ABP/pDNA complexes in HepG2 cells is much higher than that of PEI/pDNA, which further reveals that ABP has remarkable targeting ability.

The probable mechanism for transfection enhancement could be explained as follows (Scheme 1 (B)): pDNA was condensed with ABP, which had better biocompatibility, and it can be speculated that there may exist unknown interaction between avidin and HepG2 cells which could target the liver cancer cells. After binding to the cell surface, ABP/pDNA complexes were internalized, and small fractions of them escaped from the endosome and translocated to the nucleus, where genes within the pDNA were expressed. Enhanced cell interaction leads to more particle uptake and greater gene expression.

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The cellular internalization of complex is essential to gene delivery. Fig. 7 exhibits the images of three different types of cells treated with FITC-ABP/pDNA complexes for 4 h, and FITC-PEI/pDNA complexes were taken as a control. For HepG2 cells, compared with the images of FITC-PEI/pDNA complexes, the green fluorescence of the FITC-ABP/pDNA complexes could be seen evidently, and the images reveal the presence of the fluorescence also in the nuclei. The cellular uptake in HepG2 cells showed that when BP conjugated with avidin, cell uptake of ABP was markedly enhanced. ABP was shown to have a higher uptake to HepG2 cells compared with PEI. Fig. 7 also shows weaker fluorescent expression of FITC-ABP/pDNA complexes can be observed in Hela cells and 293 T cells when compared with FITC-PEI/pDNA complexes, probably attributed to fluid phase pinocytosis. These results demonstrate that ABP easily transfects cells having specific interaction with avidin.

CONCLUSIONS

In conclusion, we synthesized a novel gene vector biotinylated polyethyleneimine/avidin bioconjugates (ABP) by coupling biotin onto the backbone of high molecular weight branched PEI (25 kDa) and bio-conjugating with avidin by the avidin-biotin strong affinity. The resulted gene vector ABP was evaluated in terms of gel retardation assay, particle size, ζ-potential, *in vitro* cell viability and transfection efficiency. Results indicated that ABP demonstrates significant lower cytotoxicity and higher transfection efficacy in HepG2 cells due to the biocompatibility of avidin and the unknown interaction between avidin and HepG2 cells. ABP obtained here will have potential in targeting gene delivery of liver cell and could also be useful for other forms of hepatic disease.

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