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Biotinylated transferrin/avidin/biotinylated disulfide containing PEI bioconjugates mediated p53 gene delivery system for tumor targeted transfection

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ABSTRACT

As mutation and dysfunction of p53 gene could induce most of human cancers, the p53 tumor suppressor gene was used to replace them and recover their normal functions in cancer cells. In this paper, biotinylated transferrin/avidin/biotinylated disulfide containing PEI bioconjugates (TABP-SS) mediated p53 gene delivery system was formed attributed to the 'avidin–biotin bridge'. Characteristics of the obtained TABP-SS and its p53 complexes were evaluated in terms of acid–base titration, agarose gel electrophoresis, SEM, particle size and ζ-potential measurements. The acid–base titration results showed that TABP-SS had good buffer capability. The results of gel electrophoresis indicated that TABP-SS could fully condensed DNA and would be degraded by reducing agent inside cells. *In vitro* cell viability and transfection of TABP-SS were investigated in COS7, HepG2, and HeLa cells. Among the three different cell lines, TABP-SS exhibited much lower cytotoxicity and higher transfection efficacy in HepG2 and HeLa cells due to the specific interactions between transferrin ligands and their receptors on tumor cells. Apoptotic morphology was observed using confocal microscopy, and the expression of p53 protein in transfected cells was evaluated by western blotting. All the results indicated that TABP-SS/p53 complex could be considered as a low toxic and high efficient tumor targeted gene delivery system, which has great potential for further clinical application.

1. Introduction

Tumor gene therapy aims at correcting gene defaults and mutations that observed in severe hereditary pathologies or occurred in cancer tissues. The p53 gene is recognized as tumor suppressor and genome guard, which plays a crucial role in diverse cellular activities, such as DNA repair, cell cycle regulation, and apoptosis [1,2]. Recently, researches reported that mutation and dysfunction of p53 gene could induce more than 50% human cancers [3]. Thus, to replace the aberrant p53 gene and recover its normal functions in cancer cells is one of the most efficient and critical approaches for tumor gene therapy. After the successful transfection of normal p53 gene into tumor cells, the apoptosis program will be activated again, and this will prompt cancer cells to 'commit suicide' [4]. Several studies in human tumor models have shown that wild-type p53 gene transfer induces apoptosis and tumor regression, proving that the clinical application of p53 gene therapy is available [5–8].

As the most classical nonviral gene vector, polyethylenimine (PEI) has been utilized to mediate p53 gene, and their gene therapy systems were also reported [9–11]. However, lacking specific targeted capability, those systems exhibited very low transfection efficacy. Hence, in order to let gene vectors recognize the target cells, achieve remarkably enhanced transfection efficacy, and lower the toxicity to the host, cell targeting ligands should be introduced which can specifically interact with their receptors on target cells.

Transferrins are typically monomeric glycoproteins (Mw: 80 kDa) that can transport ferric ions from blood to cells through receptor-mediated endocytosis [12]. It is known that tumor cells with a high rate of proliferation usually over-express transferrin receptors, so they could act as ideal targets in antitumor drug delivery and gene therapy [13,14]. Researchers have coupled transferrin to PEI in order to improve transfection efficiency in tumor cells through the receptor-mediated uptake mechanism [15–17]. According to literature, transferrin-PEI/DNA complexes have shown high activity in a broad variety of applications *in vitro* and promising results in topical application in vivo. Avidin is a 66 kDa highly glycosylated protein that shows extremely high affinity for biotin, and due to this strong affinity, the 'biotin–avidin bridge' has provided useful and versatile intermediates and been employed as a linker for biochemical studies [18–20].

In our previous study, biotinylated disulfide containing PEI (PEI-SS) was prepared as an efficient and low toxic gene vector [21]. In

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this study, biotinylated transferrin was bound with PEI-SS through the 'biotin–avidin bridge' to prepare a novel tumor targeted gene vector, biotinylated transferrin/avidin/biotinylated disulfide containing PEI bioconjugates (TABP-SS). Then, TABP-SS was complexed with the tumor suppressor gene p53 to form a tumor targeted therapeutic gene delivery system. Characteristics of TABP-SS and its DNA complexes were evaluated in terms of acid–base titration, agarose gel electrophoresis, SEM morphology observation, particle size and ζ-potential measurements. The *in vitro* cytotoxicity and transfection efficiency of TABP-SS in different cell lines were studied, the expression level and the antitumor activity of p53 gene were also evaluated.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (PEI) with molecular weight of 800 Da, cystamine dihydrochloride (98%), d-biotin, biotinylated transferrin, *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma–Aldrich and used as received. Acryloyl chloride of analytical grade and 1,4-dithiothreitol (DTT) were purchased from Shanghai Chemical Reagent Co., China and used as received. Avidin was purchased from Pierce. Dimethyl sulphoxide (DMSO) was obtained from Shanghai Chemical Reagent Co., China, which was dried refluxing with anhydrous $MgSO_4$ overnight and was then distilled under reduced pressure. QIAfilterTM plasmid purification Giga Kit (5) was purchased from Qiagen (Hilden, Germany). GelRed™ was purchased from Biotium (CA, USA). Molecular probe (Hoechst 33258) was purchased from Invitrogen (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 Corp. The Micro BCA protein assay kit was purchased from Pierce. All other reagents were analytical grade and used as received. 150 mM NaCl solution was used to mimic the physiological saline environments.

2.2. Preparation of biotinylated transferrin/avidin/biotinylated disulfide containing PEI bioconjugates (TABP-SS)

First of all, the biotinylated PEI-SS/avidin bioconjugate (ABP-SS) was prepared according to our previous paper [21]. In brief, PEI-SS was synthesized from the Michael addition between cystamine bisacrylamide (CBA) and branched 800 Da PEI, and then biotin was linked to PEI-SS using EDC. The product was purified by dialyzing against water (MWCO: 3500), lyophilized and defined as BP-SS. Appropriate amount of avidin were dissolved in 150 mM NaCl, and then 20-fold weight excess of BP-SS was added. The mixture was vortexed and reacted at room temperature for 30 min to obtain the ABP-SS solution. Then, 40 μL and 80 μL biotinylated transferrin solution (0.5 mg/mL in 150 mM NaCl solution) was respectively added into the ABP-SS solution, and the mixture was reacted at room temperature for 30 min to obtain the T_1ABP-SS and T_2ABP-SS solution.

2.3. Cell culture

Human hepatocellular carcinoma cells (HepG2), African green monkey SV40-transformed kidney fibroblast cells (COS7) and human cervix adenocarcinoma cells (HeLa) 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.

2.4. Amplification and purification of plasmid DNA

Luciferase reporter gene plasmid (pGL-3) and red fluorescent protein-tagged p53 expression plasmid (pDsRed2-N1-p53) were used in this study. The former one was transformed in *E. coli* JM109 (the positive clones were screened by Amp resistance), and the latter one was transformed in *E. coli* DH5α (the positive clones were screened by Kana resistance). Both plasmids were amplified in LB media at 37 °C overnight at 250 rpm and purified by an EndoFree QiAfilter™ Plasmid Giga Kit (5). Then the purified plasmid were dissolved in deionized water 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.

2.5. Acid–base titration

The buffer capability of 25 kDa PEI, 800 Da PEI, PEI-SS and TABP-SS were determined by acid–base titration assay over the pH from 10 to 2 as described by Sun et al. [22]. 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.

2.6. Agrose gel retardation assay

The T_1ABP-SS/DNA and T_2ABP-SS/DNA complexes at different w/w ratios range from 0 to 30 were prepared by adding appropriate volume of TABP-SS solution (in 150 mM NaCl solution) to 1 μL of pGL-3 DNA (100 ng/μL in water) with or without 50 mM DTT. 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. DNA was visualized with a UV lamp using a Vilber Lourmat imaging system (France).

2.7. Particle size and ζ-potential measurement

The particle size and ζ-potential were measured by Nano-ZS ZEN3600 (MALVERN Instr.) at 37 °C. The complexes at various w/w ratios range from 2 to 60 were prepared by adding appropriate volume of polymer solution (in 150 mM NaCl solution) to 1 μg of pGL-3 DNA (in water). After that the complexes were diluted by 150 mM NaCl solution for particle measurement or diluted by distilled water for ζ-potential measurement to 1 mL. Then the complexes were incubated at 37 °C for 30 min and ready to be measured.

2.8. Scanning electron microscopy (SEM)

The morphologies of TABP-SS/DNA, ABP-SS/DNA and PEI-SS/DNA complexes at w/w = 20 were observed respectively by SEM (FEI-QUANTA 200). The complexes were prepared by adding 1 μg of DNA (in water) to appropriate volume of polymer solution (in water). 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/DNA complexes solution onto the glass slip and then kept in aseptic manipulation cabinet at 30 °C for 4 h for drying. Before SEM observation, the samples were coated with gold for 7 min. The micrographs of complexes were obtained at magnification of 20,000×.

2.9. Cytotoxicity assay

The *in vitro* cytotoxicities of TABP and TABP/pGL-3 complexes were examined by MTT assay following literature procedures [21,23]. 25 kDa PEI, PEI-SS and naked DNA were used as the control. HepG2, COS7 and HeLa 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/DNA complexes cytotoxicity assay, cells were seeded in the 96-well plates at a density of 12,000 cells/well and cultured 24 h in 200 μL DMEM containing 10 % FBS. Polymers were complexed with 0.2 μg pGL-3 at different w/w ratios (5–30) 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.

Followed 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 (%) = $[OD_{570\ (\text{treated cells})} - OD_{570\ (\text{background})}/OD_{570\ (\text{untreated cells})} - OD_{570\ (\text{background})}] \times 100$.

2.10. In vitro transfection

Transfection experiments of TABP-SS, ABP-SS, PEI-SS were performed with HepG2, COS7 and HeLa cells, and jetPEI was used as the control. Both pGL-3 and pDsRed2-N1-p53 plasmid DNA were utilized to evaluate the transfection efficiency. 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. The complexes were prepared at w/w ratios range from 2 to 60 by adding appropriate volume of polymer solution to 1 μg plasmid DNA per well, and then incubated at 37 °C for 30 min. Then the polymer/DNA complexes were added into the plate and incubated at 37 °C for 4 h. After that, old medium was replaced with fresh medium and the cells were further incubated for 2 days. For luciferase assay, the medium was removed and cells were washed by PBS, then the cells were lysed using 200 μL reporter lysis buffer (Pierce). The 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 (Pierce) and luciferase activity was expressed as RLU/mg protein.